Nanomaterials and Regenerative Medicine

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Yunfeng Lin
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PREFACE

Tissue engineering and tissue regeneration is rapidly expanding research area which involves interdisciplinary approaches to the development of regenerative medicine aimed at restoring and improving the functioning of tissue as well as a whole organ. It brings together various disciplines from material engineering, natural science and life science fields with the intention to alleviate present challenges of harvesting and storing tissues for transplantation. It has been demonstrated that adipose derived stem cells possess versatile therapeutic potential in various clinical contexts such as facilitation of wound healing, bone and cartilage regeneration and rehabilitation of cardiac functions among others.

On the other hand material engineering has developed improved procedures for preparation of nano-sized materials which emerged as promising candidates in producing scaffolds able to better mimic the nanostructure in natural extracellular matrix. Overall, nanomaterials exhibit superior performance comparing to microparticulate matter. They exhibit improved biocompatible, mechanical, physico-chemical and magnetic properties which advance tissue growth and regeneration.

The aim of this book is to address recent advances in the field and to review the preparation and functioning of various nano-materials (nanoparticles, nanofibers and the surface modifications of implantable materials) in the biological context. Furthermore, the book summarizes the applications of nano-materials to various tissues, which are classified into four types depending on their functions: protective, mechano-sensitive, electro-active, and shear stress-sensitive tissues.

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WHOLE-ORGAN ENGINEERING WITH NATURAL EXTRACELLULAR MATERIALS

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1.1. INTRODUCTION

Since 1994, when Cao *et al.* showcased their auricular implant grown on a mouse's back and thereby inspired the field of biomedical research with awe [1,2], whole-organ engineering has been proof-of-concept and a growing interest in the field of regenerative medicine. Whole-organ engineering attempts concentrate on creating fully functional organs *in vivo*. Therefore it is essential to deepen our understanding of the composition of individual tissues, and the interfaces between tissue types. Besides offering a three-dimensional physical structure to house the cells within a given tissue, the extracellular matrix (ECM) also allows for signal transductions that direct the morphogenetic process integrated into the tissue. Cell-surface receptor binding sites found on ECM molecules promote cell proliferation, differentiation, adhesion, migration and survival. Natural ECMs consist of proteins and polysaccharides secreted by cells. Many of these molecules are 'multimodal', providing binding sites for both cell and ECM protein [3]. Comprehensive reports on specific natural ECM molecules and their respective functions have been published [4-8]. ECM in organ systems comprises two major components, which include the basal lamina and the stromal matrix. Although ECM composition of various tissues can be dissimilar, the basal lamina, found bordering epithelial sheets, primarily consists of collagen IV and laminins, while stromal or interstitial matrices include diverse compositions such as fibrillar collagen I or hyaluronic acid [3].

Incorporating natural materials into engineered tissues and organs has many benefits. As described above these natural molecules provide functional cell and ECM adhesion sites. In addition natural ECM molecules offer improved biocompatibility over synthetic alternatives, as well as genetic conservation in terms of use with xenogenic products [8,9]. Over the years, a shift has been notable. Biomaterials have advanced from incorporating inert materials into the body, to creating bioactive and bioresponsive material implantations, in the pursuit of repairing organ functionality or regenerating tissue. The use of natural ECM materials integrates all of these efforts.

The main focus of this chapter is to showcase the developments in whole-organ engineering, which reclaims natural tissue derived materials and natural / synthetic blends in innovative ways (Table 1). In this chapter, we use the tooth as an illustrative example of a developing organ, and discuss current efforts and future applications toward whole-organ tooth engineering using natural ECM molecules.

Table 1. Natural extracellular matrix molecules incorporated into engineered organs and tissues

Natural ECM components	Engineered organ/tissue	Ref.
Collagens	Cartilage	[2,42]
	Blood vessels	[44,48,49,117]
	Nerves	[45,125]
	Ligament	[54]
	Bladder	[55]
	Teeth	[82, 94-97, 133]
	Lymphoids	[121]
Gelatins	Skin	[51,52,136]
	Blood vessels	[44]
	Cartilage	[135]
Hyaluronic acid/hyaluronan	Blood vessels	[42,117]
	Nerves	[125]
Laminin	Peripheral nerves	[43]
Genipin	Skin	[53,136]
	Nerves	[73]
	Cartilage	[135]
Silk/fibroin	Skin	[53]
	Ligament	[54]
	Mineralized osteodentin	[92]
Chitin/chitosan	Cardiac muscle	[66]
	Bone	[67]
Fibrin	Cardiac muscle	[66]
	Blood vessels	[71]
Syndecan-1	Breast	[69]
Perlecan	Lymph vessel	[123]

1.2. THE CURRENT STATE OF THE ART FOR NATURAL SCAFFOLDS

Even though in an early stage, current progresses in engineering whole organs with natural materials include decellularizing cadaveric or animal tissue for scaffold use, electrospinning nanofiber scaffolds or functionalizing environmentally responsive matrices (Diagram 1). Whereas in decellularization the 3D tissue architecture is reused, maintained and

characterized, electrospun fibers and responsive materials attempt to recreate functional niches for cells within a 3D-fabricated environment. These technologies are thoroughly tested along a continuum from prototypical *in vitro* models to human clinical studies. However, significant information can be acquired through the incorporation of natural ECM molecules in all of these efforts as we advance towards engineered whole organs.

Diagram 1. Schematic diagram summarizing the different natural scaffolds

1.2.1. Decellularization of tissue

Reconstructing a fully functional ECM remains an elusive task. Therefore, decellularization, a gentle process of cell and associated nucleic acid debris removal, has been used to create acellular scaffolds for tissue and organ engineering. These scaffolds retain the original architecture of organs and tissue interfaces, giving rise to a source of allogenic and xenogenic whole-organ grafts. Decellularization has been carried out on a variety of tissues of various organs such as the urinary bladder, small intestinal submucosa, blood vessels, heart valves, pericardium, tooth buds, trachea and esophagus, as well as musculoskeletal regions such as the temporomandibular joint [9-22]. This method of creating acellular scaffolds has resulted in commercial products and tissue substitutes [23-27].

As bioengineering methods advance towards the formation of more complex geometries, the decellularization process has evolved to meet the strict standards needed for allograft studies. As Gilbert *et al.* suggested, there is variability amongst tissue types in terms of effective decellularization methods [24]. While a certain decellularization treatment may efficiently remove cellular material in a given tissues, it may be less effective in another tissue. Denser, thicker tissues and organs may require more robust decellularization methods to insure penetration throughout the tissue, while less dense tissues may require more gentle techniques. In addition, the effectiveness of cell removal and tissue damage varies based upon the method of decellularization (chemical, enzymatic or physical) [13,14,24,28]. Decellularization methods attempting to optimize decellularization reagent incubation time, temperature, concentration or number of solvent cycles, have been reported with varying degrees of success based upon histology, scanning-electron microscopy and mechanical testing of the resulting decellularized scaffold product [12,19,20,28].

In human allograft studies of tracheal tissue, optimized tissue penetration was achieved by circulating cycles of detergents to remove nucleic materials [19,20]. While verification of cell removal is typically quantified by DNA content post-decellularization by means of spectrophotometry [29-32], further characterization of the remaining major histocompatibility complexes (MHCs) is essential for clinical human allograft studies. For the decellularized human whole-trachea graft, MHC class I and II removal was monitored. To decellularize the tissue, multiple washes in 4 % sodium deoxycholate and 2000 kU deoxyribonuclease I were alternated with water rinses. After 25 cycles of detergent washes, immunohistochemistry demonstrated removal of human leukocyte antigen-A (HLA-A), HLA-B and HLA-C antigens, with minimal expression of MHC II antigens. Likewise, serology screens of HLA recipient antidonor antibody production verified complete absence of foreign proteins for as long as 2 months post-surgery [20].

Most of the commonly known decellularization methods require multiple days of treatment, and require large rinsing volumes in order to insure complete decellularization. Novel decellularization procedures based on sodium chloride show promising results in obtaining intact decellularized tissues while significantly reducing the procedure time. Bühler *et al.* have demonstrated the decellularization of full-sized minipig livers within 24 h [33], while Price *et al.* were able to achieve the same using lungs from 20–30 kg pigs using their own method [34]. In both studies collagen and glycosaminoglycans were preserved. However, using these new rapid protocols may require more attention to the preservation of intact ECM components.

Excessive decellularization can degrade the ECM, resulting in collagen damage, glycosaminoglycan depletion, as well as elastin cleavage [24,35]. Accordingly, protocols should be designed to detect and minimize destruction, while preserving the mechanical integrity of the tissue. Often, retention of ECM

architecture is validated in comparison to untreated cadaveric tissue with microscopy to ensure tissue integrity is not compromised [12,13,17,22,28,32,35]. Using histological methods, the extent of ECM damage in collagen-dominated tissue has been quantified by measuring collagen crimping amplitude and periodicity, which can result from exposure to detergents such as sodium dodecyl sulfate [17,35]. Other imaging techniques such as scanning-electron microscopy have been used to visualize and compare nanostructures (*e.g.*, the weave, coil and strut fibers in the decellularized heart study) in decellularized and non-decellularized cadaveric tissue [32]. Together, these studies show that with optimal treatment, decellularized tissues can maintain ECM antibody epitope expression in such molecules as collagens, laminin and fibronectin in immunohistochemistry and immunofluorescence studies [13,15,18,21,30,36]. Table 2 summarizes the various ECM molecules characterized in select decellularized organ and tissue studies.

Just as the chemical composition of the ECM affects organ functionality, mechanical integrity must also be preserved in decellularized tissues. Mechanical testing can be used to quantitatively evaluate the effects of decellularization. As an example, to accommodate ventricular load and associated cardiac stresses, decellularized rat heart cross-sections were demonstrated to exhibit ventricular tissue anisotropy measurements that were similar to cadaveric tissue, with respect to stiffness and tangential modulus.

Table 2. Extracellular matrix molecules characterized in decellularized organ and tissue studies

Natural ECM components	Engineered organ/tissue	Ref.
Collagens	Bladder	[9]
	Blood vessels	[11]
	Heart valve	[12,13,15,17]
	Pericardium	[18]
	Trachea	[20]
	Esophagus	[21]
	Cornea	[28]
	Pulmonary roots	[29]
	Meniscus	[30]
	Ligament	[31,35,36]
	Hearts	[32]
	Teeth	[103]
Laminin	Bladder	[9]
	Heart valve	[13]
	Esophagus	[21]
	Teeth	[103]

Natural ECM components	Engineered organ/tissue	Ref.
Elastin	Blood vessels	[11]
	Heart valve	[12,13,15,17]
	Pericardium	[18]
	Esophagus	[21]
Cytokeratins	Trachea	[4]
Fibronectin	Esophagus	[21]
	Heart	[32]
	Teeth	[103]
Chondroitin sulfate	Meniscus	[30]
Glycosaminoglycans	Bladder	[9]
	Pericardium	[18]
	Heart valve	[12,13,17]
	Meniscus	[30]
	Ligament	[31,35,36]

Recent developments in decellularization methods focus on retaining whole-organ functionality in animal models [32] and in allogenic clinical studies [20]. As mentioned previously, Ott *et al.* has characterized the mechanical properties and ECM composition in an animal model, whereas Macchiarini *et al.* characterized clinical factors for allograft implantation of tracheal tissue in humans. In both studies, decellularization approaches were followed by functional studies of the decellularized tissues, to confirm recapture of the necessary physiologic functions of the recellularized organ.

Measures of restored function have included quantification by physiological parameters. For example, 24 h after recellularization in a coronary perfusion bioreactor, electrical stimulation was introduced in vitro to an entire rat heart. The decellularized rat heart, which was repopulated with a mixed population of cardiac cells, regained approximately 2 % pump function of an adult rat heart, and 25 % pump function of a 16 week fetal human heart as evaluated by its performance under physiologic preload, afterload, intraventricular pressure and electrical stimulation. Furthermore, doses of phenylephrine administered to the recellularized whole heart were also able to stimulate contractility similar to extrinsic control mechanisms that exist physiologically [32]. Likewise, improved muscle contractility force up to 85 % of pre-injury levels was seen after muscle-derived decellularized ECM was implanted in full-thickness defects in the lateral gastrocnemius of Lewis rats. Seven days post-injury, the implanted decellularized ECM were reseeded with bone-marrow-derived mesenchymal stem cells which led to the formation of new muscle tissue [37].

Similarly, in the decellularized human whole-trachea graft study, the restored physiological function of airway clearance in the allogenic, trachea graft was evaluated. The ratio of forced expiratory volume in 1 s to forced vital capacity (FEV1 : FVC) taken before and 3 months after surgery, determined that the graft resulted in a reversal of airway obstruction [20]. As additional clinical studies are completed, we will continue to learn more about the instructive nature of decellularized grafts, including the preservation of angiogenic cytokines [20], as well as other chemokines preserved from previous niches that may affect morphogenesis [4,14,38,39].

1.2.2. Electrospun nanofibers

Electrospinning is a nanofabrication technique that generates nonwoven fibers of uniform, reproducible composition on a physical scale that is topographically compatible with the cells that reside upon them. The ability to electrospin natural materials offers the benefits of incorporated peptide motifs that synthetic materials lack, although natural materials can be too weak to form reproducible nanofibers, and are therefore often blended with biocompatible, synthetic polymers to create composite fibers with added strength [40]. Furthermore, the addition of natural polymers to synthetic fibers has improved the adhesion, viability and proliferation over synthetic material alone [41,42].

Besides having the ability to form nanoscale fibers, 3D assemblies of natural or natural / synthetic blend fibers can also be used to create composite structures, allowing for a closer replication of natural nanoscale ECM fiber composition within an organ. For instance, the basement membrane bordering epithelial sheets consists primarily of fibrillar proteins such as collagen IV and laminin [3]. In terms of structure, electrospun laminin I meshes have recently been fabricated to resemble basement membrane [43]. Bead-like structures, usually indicative of spinning limitations in terms of thinness, were preserved in the laminin spun fibers due to the replication of 'matrisome' structures found in the natural basement membrane. Laminin meshes, in comparison to laminin films, induced more adherent, elongated morphologies commonly associated with neuronal-like cells, as well as $\beta 3$ tubulin expression in serum-free media, without the use of chemical additives. The addition of exogenous nerve growth factor (NGF) did not increase the frequency of neurite extensions *per cell*, in comparison to the frequency of laminin nanofibers without NGF. Neal *et al.* postulated that the topography and the associated high surface area of electrospun fibers were attributable to the extensions [43].

Layer-by-layer depositions, multilayer mixing, dual-mandrel electrospinning and magnetic electrospinning have also been used to manufacture multidimensional scaffolds [44-47]. Elastomeric vascular graft-fabrication methods have been investigated for over 30 years. Recent efforts have incorporated electrospinning to create a composite graft from poly(ethylene

oxide), segmented polyurethane and UV-crosslinked collagen I by rotating a traversing movable mandrel [44]. The use of cytotoxic crosslinkers was avoided for the bioadhesive, collagen component of the scaffold. Instead, UV crosslinking was used to render the collagen insoluble, but still capable of swelling.

Since the time that Kidoaki *et al.* demonstrated multidimensional construction of an electrospun vascular graft [44], other groups have proceeded to implant and characterize 3D electrospun scaffolds *in vivo*. Using sheep and rabbit models [48], a poly(ϵ -caprolactone) / collagen vascular graft was evaluated based upon functional and physiologic parameters such as patency, platelet adhesion resistance, as based on previous studies that had established upper limits for burst pressure (4912 ± 155 mmHg), tensile strength (4.0 ± 0.4 MPa) and adequate elasticity (2.7 ± 1.2 MPa) [49].

The current challenge is to devise methods that allow for reducing the cytotoxic solvent and / or crosslinker levels currently used to produce natural and natural / synthetic blended fibers [50]. The process of electrospinning often includes the addition of organic solvents to dissolve polymers prior to spinning [51]. Crosslinkers are used to strengthen spun fibers and render natural fibers more water resistant [42]. Some natural-spun fibers have circumscribed or reduced cytotoxic solvent or crosslinker usage by using techniques such as melt electrospinning, or by replacing organic solvents and crosslinkers with less toxic alternatives [40,43,52,53].

Electrospun fibers are increasingly becoming incorporated into many different applications. 3D tissues are being formed with spun nanofibers incorporated into composite designs [48,54-57], and hydrogel 3D composites with stitched fibers have been tested in animal models [54,55]. Thus, as biomimetic studies reduce the amount of cytotoxic crosslinkers and solvents used in electrospun scaffolds, continued efforts to improve biocompatibility may increase the frequency and efficacy of electrospun scaffold use in clinical trials for whole-organ engineering applications.

1.2.3. Responsive materials, remodeling and engineered gradients

The term 'smart material' encompasses many materials that recapitulate the dynamic nature of ECM remodeling in an engineered whole organ. Traditionally, 'smart materials' include materials such as piezoelectric sensors, shape memory alloys and polymer-responsive gels, which can respond to environmental fluctuations through physical or chemical changes. Smart polymers respond to changes in pH, ionic strength, chemical species, enzyme-substrate interactions, magnetic fields, temperature, electric fields, mechanical stimulation, as well as ultrasound irradiation [58,59]. These materials exploit responsiveness for a triggered release. In response to the changing environment, smart polymer hydrogels can swell, thus facilitating an intended deployment of the triggered response, such as drug release.

Various corneal tissue-engineering groups have harvested *ex vivo* cellular sources found in short supply, and expanded and implanted the cells using tissue sheet technologies that employ thermoreversible polymer coated tissue culture plates [60,62]. While poly(*N*-isopropylacrylamide) (PIPAAm) derived implanted cell sheets restored function to the affected corneas, PIPAAm derived cell sheets have most successfully been used in 2D applications to date, and cannot fully replicate cell-matrix remodeling dynamics of large, 3D organs [58]. The combined use of PIPAAm derived cell sheets with other synthetic scaffold materials and natural ECM polymers may be more successful in recapitulating 3D tissue and organ activities.

Natural matrix remodeling has been well documented in organs such as bone. In orthopedics, guided bone regeneration has been pursued using natural materials fabricated into scaffolds, such as demineralized bone, which possesses osteoinductive properties [62]. Recently, demineralized bone has been manufactured as a nanoscale bone matrix (NBM) powder and incorporated within poly(L-lactide) (PLA) electrospun fibers [63]. Human mesenchymal stem cells (MSCs) osteodifferentiated on both PLA and PLA-NBM scaffolds *in vitro*, and accelerated mineralization was observed of human MSC seeded PLA-NBM fibers implanted *in vivo* [64]. PLA-NBM also provided a scaffold exhibiting mechanical properties higher in average tensile strength and Young's modulus than PLA alone, while exhibiting lower values than cortical bone [63]. As a result, PLA-NBM constructs can provide osteoinductive cues and may approximate the mechanical properties of immature bone undergoing remodeling with minimal loading [63]. After 12 weeks *in vivo*, the defect was almost 90 % smaller than its original size, demonstrating the responsive remodeling potential of PLA-NBM [64].

Smart materials are seen as an attractive alternative to decellularized donor tissue, whose supplies will always remain limited. These smart materials preferably contain ECM molecules as well. To address this problem, Shiloh *et al.* developed a method to collect ECM secreted by skeletal muscle myoblasts, and form it into implantable smart scaffolds [65]. In this study, rat skeletal myoblasts were seeded onto foams that served as a scaffold. After 4 weeks in culture, a construction of skeletal myoblasts, ECM and scaffold material was formed. At this time, the scaffold was dissolved using a solvent; leaving behind tissue consisting of accumulated ECM and skeletal myoblasts. After decellularization the ECM was implanted in a rat dorsal subcutaneous site. At 4 weeks post-implantation, the implanted ECM materials were found to be incorporated into the surrounding tissue, and host cells had penetrated the material. By 12 weeks post-implantation almost all of the implanted ECM material was degraded.

Likewise, the fabrication of heart muscle using natural ECM matrices is being claimed as a new type of natural smart material [66]. Blan *et al.* manufactured a composite scaffold of chitosan, a natural biomaterial that was solubilized

with acetic acid, and later pH neutralized after freezing and lyophilization. Fibrinogen was added for scaffold gelation, followed by a thrombin coating, the incorporation of cardio myocytes and an added layer of fibrin. In terms of organ function, this group monitored contractility as well as ECM architecture. To create a responsive scaffold, degradation time was manipulated based upon lysozyme concentrations from 2 h – 2 weeks. One advantage of using a responsive enzyme-substrate scaffold is the ability to match changes in matrix metalloprotease activity to that of cell-secreted matrix remodeling [66,67]. With controlled degradation, cell migration can also be manipulated, although it is important to consider and control for potential unbalanced remodeling and tumorigenic consequences [68-70].

Scaffold degradation processes can also dynamically change cellular cues such as migration. As proteolytic activity degrades the ECM, cryptic binding sites are unveiled, and matrix-bound growth factors become untethered [68,71]. Cell migration occurring along a gradient, such as that which occurs during chemotaxis (chemical gradient), haptotaxis (cellular adhesion site gradients) and durotaxis (rigidity gradients), can therefore be manipulated based upon the design of the ECM [39,72]. Further studies in 3D have been able to demonstrate more physiologically relevant applications by creating matrices with natural materials that take advantage of durotaxis [73,74].

Although advances in whole-organ engineering have origins in 2D *in vitro* models, current methods for bioengineering functional tissues, including tissue decellularization and use of electrospun nanofibers and smart materials, are progressing to 3D scaffolds. It has been shown to be an oversimplification that cells behave the same in 3D as they behave in 2D [74-78]. Cell-matrix adhesions to the ECM are exaggerated in 2D cell cultures—the adhesions are stronger than those of cells in 3D models and of the cell *in vivo* [76]. Likewise, 3D matrix adhesions are not recreated in 2D [76]. Although better than 2D modeling, 3D models mimic static, short-term conditions and may oversimplify parameters such as oxygen diffusion [75,79]. Therefore, it is important and necessary to verify *in vitro* results with parallel *in vivo* model investigations.

1.3. WHOLE-TOOTH ENGINEERING

The tooth is an excellent example of a developing organ directed by the reciprocal interactions of mesenchymal and epithelial tissues over time [80]. Bioengineering strategies have targeted tooth regeneration using various combinations of scaffolds, growth factors and cells [81-84]. Approaches using tissue recombinations, and pelleting cells in a scaffold-free environment, are also popular strategies for whole-tooth engineering [85,86]. In addition, groups have investigated the location of the implantation site for engineered implants as a morphogenetic means of developmental signaling. The diastema, or toothless region within the rodent jaw, has been shown to provide a site for

whole-tooth formation, thus demonstrating the potential for teeth to regenerate and erupt in a native environment [87,88].

While cell pellets alone have been used in other studies to avoid the need for scaffolds [89,90], Nakao *et al.* included a collagen droplet method to contain pelleted E14.5 dental epithelial and mesenchymal cells within a collagen gel matrix [82]. Dissociated cell pellets generated from mouse embryonic molar and incisor dental epithelium and mesenchyme were both able to form predentin, dentin and enamel-like tissue, with specificity confirmed by *in situ* hybridization studies. These E14.5 droplet constructs were able to reassemble into proper tissue layers, whereas older E16.5 cells could not self assemble [82]. As the effects of collagen were not explored in this model, additional studies can be used to improve our understanding of ECM provided spatial and temporal cues for tooth development.

Ongoing studies in the Yelick laboratory have worked towards engineering a whole tooth, as well as hybrid tooth–bone constructs [91-97]. Notably, these studies have focused exclusively on the use of post-natal dental epithelial and mesenchymal tissue derived cells, as opposed to embryonic dental cells. Tooth regeneration studies using adult post-natal dental cells derived from unerupted pig molar teeth and human wisdom teeth are more relevant to human tooth tissue engineering, as human embryonic dental cell populations are generally not available for this purpose. In order for these approaches to achieve clinical status, what remains to be understood and perfected is how best to maintain the spatial organization of the dental epithelial and dental mesenchymal tissues, to guide not only the shape and size of a tooth and surrounding alveolar bone, but also to achieve proper compartmentalization of individual tissue types [92]. For instance, studies have observed ectopic alveolar bone growth within dental tissues [81,96]. Likewise, the ECM components associated with functional tooth-root formation and for functional tooth eruption are critical to our understanding. Histological and immunohistochemical approaches are followed to study the presence of critical ECM components like collagen, fibronectin, and laminin in bioengineered tooth-buds (Figure 1). Similarly, preliminary analyses in a 5.5 month old porcine molar model have demonstrated some ECM fiber organization within the bioengineered tooth-bud and surrounding alveolar bone. Areas of collagen, keratin and fibronectin were shown to co-localize within vascular regions of the dental pulp as well as in enamel epithelium [98]. Future studies will elucidate further ECM organization and composition in these bioengineered teeth.

Figure 1. Immunohistochemical analyses of the extracellular organization of bioengineered porcine molar tooth-buds show positive staining for fibronectin (A), collagen I (B) and laminin (C). Higher magnification images of the boxes in (A) are found in (i) and (ii). Likewise, higher magnification images of the boxes in (B) and (C) are found in (iii), (iv) and (v). Fibronectin expression is observed in blood vessels and near odontoblasts (dental papilla). Collagen I is seen in the dental follicle, dentin and near odontoblasts. Laminin- α 1 was stained brown in the dental papilla, follicle and dentin. Scale bars = 1000 μ m in (A, B and C) and 100 μ m in (i-vi).

With respect to human tooth re-engineering efforts, further characterization of the ECM molecules involved during critical stages of tooth development are imperative. Others have applied the roles of biglycan, and subsequent amelogenin expression, to nanofiber scaffolding to study enamel maturation [99-101]. Mouse knockout models have also elucidated the role of Tbx1 and enamel formation [102]. Still, further exploration of additional ECM molecules and corresponding spatial organization within the tooth are needed for the educated design of effective scaffolds for whole tooth tissue engineering. ECM molecules such as collagen I, osteocalcin, amelogenin, dentin matrix protein, dentin sialoprotein and bone sialoprotein were present in the bioengineered toothbud and tooth-bone hybrid constructs made of silk and poly(glycolic acid) (PGA) / poly(lactide-co-glycolide) (PLGA), respectively [92,95,96]. Some of these ECM molecules, such as collagen I, fibronectin, collagen IV, and laminin can be preserved in decellularized porcine tooth buds [103]. When reseeded with dental progenitor cells, these ECM molecules could contribute to an enhanced organization of the seeded cells. As we continue to characterize the locations and 3D organizations of additional fibrillar proteins and proteoglycans in native tissue and natural ECM scaffolds, we will gain an improved understanding of the spatial organization and function of the natural tissues within the tooth and bone, which guide and maintain final form and functions.

1.3.1. Ongoing challenges in whole-organ engineering

Thus far, the majority of clinically relevant grafts that have been constructed to date are designed for use in hollow organ regeneration and repair [55]. Implicit in the creation of solid organs, including teeth, is the proper integration of functional angiogenesis, lymphangiogenesis and neurogenesis within an organ or organ system [104,105]. An ongoing obstacle in angiogenesis is defining a targeted approach to facilitate invasion of vasculature into the developing organ, to provide sufficient oxygen to tissues with volumes greater than 2–3 mm³. As host-vessel ingrowth requires a finite time to penetrate into the depth of the implanted tissue, necrosis can occur prior to sufficient vascularization, resulting in implant failure. Many studies have explored means to facilitate bioengineered angiogenesis [106,107] and ongoing *in vitro* attempts to prevascularize engineered tissue may have a future in *in vivo* applications [104,108–110]. Notably, one study has already demonstrated evidence of angiogenesis *in vivo* attributed to multi-cell type co-culture [111]. Others have incorporated mesenchymal cells with endothelial cells in 3D scaffold models to successfully create neo-vasculature network formation [112].

Despite these advances, the extent of successful penetration of functional host vessels into bioengineered tissues and organs has yet to be determined. Additional efforts have considered designing vascular network-based physiological models that consider length, diameter, pressure and flow rate, as vessels anastomose into vascular beds as well as recon-verge. These networks are especially applicable in branched, ordered tissues such as the lung or liver [105]. Furthermore, control of vessel architecture is relevant not only in terms of geometric function, but also in terms of maintaining vessel growth [70,105,113,114]. Specific to matrix-induced angiogenic efforts, groups are beginning to identify pro- and anti-angiogenic motifs on natural ECM peptides such as collagen IV [113,115,116]. From these data, gradients to drive angiogenic potential within a construct have been manufactured [117].

Another relatively new subset of angiogenic tissue engineering involves integrating lymphatic system formation in bioengineered tissues and organs. Similar concerns that exist in promoting angiogenesis persist with lymphatogenesis, in terms of controlling tissue growth and preventing metastatic behavior [118,119]. Animal models are currently being explored to investigate balancing pathologies in the lymphatic system [120–122]. However, it was found that lymphatic endothelial cells do not sprout to form a vessel unit, but rather the cells migrate to a site individually and allow unidirectional flow [123]. Perlecan, an ECM molecule involved in vascular homeostasis, was produced in a tissue-engineered model of skin regeneration within a rat model, although interestingly the ECM molecule was only formed after the construction of the lymph vessel and was expressed in the direction of flow [123]. Therefore, integration of a lymphatic system to prevent edema from

developing in an engineered organ may also be affected by changes in the ECM environment. As engineered organs become more prevalent, systemic integration with the lymphatic system will provide an immune response 'watchdog' to maintain homeostasis *via* lymphatic vessels, and to sustain hydrostatic and osmotic pressures at vessel-tissue interfaces [122].

Although tissue-engineering platforms exist to guide axonal growth [124], more cohesive strategies are needed to innervate engineered constructs in a controlled manner. Currently, autologous epineurium [125], or better still an autologous sural graft [126], serve as gold standards for peripheral nerve regeneration. However, autografts have caveats, from supply shortages to the fact that they may not be made-to-order in terms of nerve length, may create wound pain and often have a motor function recovery rate of less than 40 % [125,126]. To innervate an organ, the graft should match the mechanical properties of surrounding areas, allow for diffusion, provide the appropriate degradation when a nerve regenerates and provide migratory guidance cues to recruit nearby axons from a neural growth cone or other means of taxis [124,126]. Current neural-tube materials include, but are not limited to, PGA-collagen, PLGA, poly(L-lactic acid), silicon and hydroxyapatite (HA)-collagen hydrogels [125,126]. While synthetic neural tubes may offer improved stiffness, the same synthetic materials have caused complications such as inflammatory responses and scarring [125,126].

Directing neurogenesis in a precise manner for efficient integration of implanted tissues within the host, and to test for restored function of the nerve, are ongoing struggles. A recent rabbit facial nerve defect model, which tested a HA-collagen hydrogel construct with NT-3 growth factor and xenogenic neural stem cells, found minimal signs of immune response and successful migration of donor cells to affected areas [125]. However, Zhang *et al.* were not able to demonstrate improved neuromuscular function using HA-collagen implants, based on ethology and electromyography results. Functional innervation studies are still difficult to prove in animal models, although various tests have been established for peripheral nerve recovery [126,127]. As Li *et al.* have previously surmised that axonal guidance needs multiple cues for nerve regeneration, which could include integrating physical stimulation [124]. For example, 780 nm laser phototherapy, when applied transcutaneously to peripheral nerve-damaged patients, significantly improved motor function as well as voluntary muscle activity in partially paralyzed limbs [128]. These studies demonstrate that cell growth and specific antibody expression should be coupled to a test of restored function, and may need to include multiple cues for guided regeneration.

1.4. UNMET NEEDS

Efforts to engineer organs, including teeth, are still struggling to identify available and reliable cell sources, and to optimize efficient incorporation of cells into scaffolds. While the multipotency of adult stem cells is being realized as an autologous source for various tissues, limitations remain for re-engineering immature tissue [129]. For instance, since enamel forming tissues have undergone apoptosis prior to tooth eruption, it is necessary to identify a dental epithelial cell source for bioengineered enamel production. Although current studies use murine, rat and porcine tooth germ tissues, animal-derived cells are not suitable for clinical applications in humans. Epithelial Rests of Malassez (REM), present in mature human periodontal tissues, may be a suitable source for human dental epithelium [130]. In addition, current attempts to reprogram cell sources include the development of spontaneously derived immortalized murine dental epithelial cell lines, which when grown in low calcium-supplemented media, can express enamel markers such as amelogenin [131,132], and the use of single-cell suspensions generated from tooth germ tissue [133]. Lessons from these studies should assist efforts to generate suitable human dental epithelial cell lines from available tissues such as Hertwig's epithelial root sheath or epithelial cell REM. Recently, however, Sharpe *et al.* used adult human gingival cells as an epithelial cell source. When combined with mouse embryonic tooth-inducing mesenchyme cells, dental tissue was formed. The adult human gingival cells contributed to the formation of ameloblast-like cells and rests of Malassez [134]. Furthermore, chimeric teeth, generated from autologous dental mesenchyme combined with xenogenic dental epithelium, may eventually be used as a clinically relevant alternative approach [86,90].

Incorporating cells into decellularized and / or fabricated scaffolds is also a parameter in need of improvement. While many studies use bioreactors to facilitate cell seeding, reports of uneven adhesion and proliferation persist [11,32]. Direct injection of cells into scaffolds may create cell clusters rather than even cell distributions, depending on cell migration and cell adhesion receptor binding sites. For example, although Blan *et al.* directly injected cardiomyocytes into smart chitosan material, this approach did not produce the necessary contractile responses owing to low cell retention. However, injection of cells mixed with fibrin gel allowed for cell adhesion to the scaffold [66]. Methods for cell penetration into, retention on and proliferation within scaffolds are anticipated to improve as strategies to promote angiogenesis throughout bioengineered organs continue to advance, and allow for continuous recruitment of host progenitor cells to the implant site. As supporting scaffolds degrade over time, questions remain, such as: Will the regenerating tissue unit be able to grow and remodel? Will engineered organs possess the appropriate ECM signals for proper growth and remodeling, based upon the proposed regression, and pruning models of blood vessel and nerve formation [71,113,126]? Although defined methods to properly manipulate

and monitor angiogenesis, lymphangiogenesis and neurogenesis in bioengineered organs remain unclear, they will undoubtedly improve as additional functional, long-term studies using whole-engineered organs are completed.

1.5. FUTURE PERSPECTIVE

Whole-organ engineering is a new field with plenty of room to grow, and a multitude of multi and inter-disciplinary talents are involved in this pursuit. As we continue to learn more about natural ECM materials, fabrication technologies will also advance. Over the next few years, we will continue to learn more about the composition, degradation and remodeling rates of decellularized cadaveric allografts, as the demand for clinical studies and the number of potential commercial products increases, and as methods for improved removal and detection of MHC antigens continue to improve. As microarray techniques continue to improve high throughput screening, we will be able to better characterize serology screens for foreign antigens in decellularized cadaveric tissues.

Likewise, the generation of thin electrospun fibers with less toxic solvents and crosslinkers will improve current tissue-engineering efforts. Natural crosslinkers, such as genipin, have already shown improved biocompatibility over glutaraldehyde in tissue fixation [135-137]. Other groups have demonstrated incorporation of genipin into natural ECM hydrogels [73,138] and natural electrospun nanofibers [53]. With improved nanofiber production methods, more complex 3D electrospinning techniques can be used to find better ways to integrate a variety of fabricated scaffold layers on the mesoscopic scale.

Smart polymer technologies should increase in clinical relevance as the demonstrated use of composite corneal implants becomes more prevalent. As 3D synthetic scaffolds develop or as natural ECM analogs are better characterized based on remodeling characteristics, implants will become more dynamic, thus reducing the number of surgeries needed for a procedure. Similarly, more long-term studies will assist in our understanding of the biocompatibility and degradation processes of scaffolds over time, along with the associated cascade of matrix metalloprotease activities and soluble cytokines, which redefine and remodel the ECM. Looking beyond the next few years, pluripotent reprogramming technologies such as induced pluripotent stem cells and piggyBac will continue to improve [139-141], and new and useful human embryonic cell lines will be created. Thus, these cells will provide new sources for organ engineering to study ECM synthesis and turnover. As instructive scaffold technologies continue to improve, we anticipate being able to directly control and monitor cell behavior, migration and differentiation throughout the morphogenetic process of any organ

formation. As such, the field of whole-organ tissue engineering has a bright and promising future.

REFERENCES

1. Y.L. Cao, J.P. Vacanti, *39th Annual Meeting of the Plastic Surgery Research Council*, Transplantation of chondrocytes utilizing a polymer-cell construct to produce tissue-engineered cartilage in the shape of a human ear, Ann Arbor, MI, USA. 1994.
2. S.J. Shieh, S. Terada. *Biomaterials* **25** (2004) 1545–1557.
3. B.O. Palsson, S.N. Bhatia, *Tissue Engineering*, Pearson Prentice Hall, NJ, USA, 2004, p. 20–33.
4. J.F. Mano, G.A. Silva. *J. R. Soc. Interface* **4** (2007) 999–1030.
5. I. Capila, R.J. Linhardt. *Angew. Chem. Int. Ed.* **41** (2002) 391–412.
6. J.H. Miner, P.D. Yurchenco. *Ann. Rev. Cell Dev. Biol.* **20** (2004) 255–284.
7. J.H. Miner. *Microsc. Res. Tech.* **71** (2008) 349–356.
8. M. van der Rest, R. Garrone. *FASEB J.* **5** (1991) 2814–2823.
9. F. Bolland, S. Korossis. *Biomaterials* **28** (2007) 1061–1070.
10. S.F. Badylak. *Transpl. Immunol.* **12** (2004) 367–377.
11. A. Ketchedjian, A.L. Jones. *Ann. Thorac. Surg.* **79** (2005) 888–896.
12. K. Schenke-Layland, O. Vasilevski. *J. Struct. Biol.* **143** (2003) 201–208.
13. I. Tudorache, S. Cebotari. *J. Heart Valve Dis.* **16** (2007) 567–573.
14. M.T. Kasimir, E. Rieder. *J. Heart Valve Dis.* **15** (2006) 278–286.
15. E. Rieder, M.T. Kasimir. *J. Thorac. Cardiovasc. Surg.* **127** (2004) 399–405.
16. R.L. Knight, H.E. Wilcox. *Proc. Inst. Mech. Eng. H* **222** (2008) 129–143.
17. J. Liao, E.M. Joyce. *Biomaterials* **29** (2008) 1065–1074.
18. S. Mirsadraee, H.E. Wilcox. *Tissue Eng.* **12** (2006) 763–773.
19. M.T. Conconi, P. De Coppi. *Transpl. Int.* **18** (2005) 727–734.
20. P. Macchiarini, P. Jungebluth. *Lancet* **372** (2008) 2023–2030.
21. A.D. Bhrany, B.L. Beckstead. *Tissue Eng.* **12** (2006) 319–330.
22. S.B. Lumpkins, N. Pierre. *Acta Biomater.* **4** (2008) 808–816.
23. S.F. Badylak. *Biomaterials* **28** (2007) 3587–3593.
24. T.W. Gilbert, T.L. Sellaro. *Biomaterials* **27** (2006) 3675–3683.
25. W. Konertz, P.M. Dohmen. *J. Heart Valve Dis.* **14** (2005) 78–81.
26. C. Harper. *Hosp. Med* **62** (2001) 90–95.
27. P.M. Dohmen, S. Hauptmann. *J. Heart Valve Dis.* **16** (2007) 447–449.
28. S.P. Marquez, V.S. Martinez. *Acta Biomater.* **5** (2009) 1839–1847.
29. E. Rieder, A. Nigisch. *Biomaterials* **27** (2006) 5634–5642.
30. T.W. Stapleton, J. Ingram. *Tissue Eng. Part A* **14** (2008) 505–518.
31. R.D. Harrison, P.F. Gratzer. *J. Biomed. Mater. Res. A* **75A** (2005) 841–854.
32. H.C. Ott, T.S. Matthiesen. *Nat. Med.* **14** (2008) 213–221.
33. N.E. Bühler, K. Schulze-Osthoff. *J. Biosci. Bioeng.* **119** (2015) 609–613.
34. A.P. Price, L.M. Godin. *Tissue Eng. Part C Methods* **21** (2015) 94–103.
35. P.F. Gratzer, R.D. Harrison. *Tissue Eng.* **12** (2006) 2975–2983.
36. J.H. Ingram, S. Korossis. *Tissue Eng.* **13** (2007) 1561–1572.
37. E.K. Merritt, M.V. Cannon. *Tissue Eng. Part A* **16** (2010) 2871–2881.
38. P.A. Jimenez, S.E. Jimenez. *Am. J. Surg.* **187** (2004) S56–S64.
39. J.E. Reing, L. Zhang. *Tissue Eng. Part A* **15** (2009) 605–614.

40. S. Agarwal, J.H. Wendorff. *Polymer* **49** (2008) 5603–5621.
41. J. Yuan, J. Shen. *Polym. Int.* **57** (2008) 1188–1193.
42. T.G. Kim, H.J. Chung. *Acta Biomater.* **4** (2008) 1611–1619.
43. R.A. Neal, S.G. McClugage. *Tissue Eng. Part C* **15** (2009) 11–22.
44. S. Kidoaki, I.K. Kwon. *Biomaterials* **26** (2005) 37–46.
45. K. Klinkhammer, N. Seiler. *Tissue Eng. Part C Methods* **15** (2009) 77–85.
46. P.D. Dalton, D. Klee. *Polymer* **46** (2005) 611–614.
47. D. Yang, B. Lu. *Adv. Mater.* **19** (2007) 3702–3706.
48. B.W. Tillman, S.K. Yazdani. *Biomaterials* **30** (2009) 583–588.
49. S.J. Lee, J. Liu. *Biomaterials* **29** (2008) 2891–2898.
50. J.W. Xie, X.R. Li. *Macromol. Rapid Commun.* **29** (2008) 1775–1792.
51. Z.M. Huang, Y.Z. Zhang. *Polymer* **45** (2004) 5361–5368.
52. H.C. Chen, W.C. Jao. *Polym. Adv. Technol.* **20** (2009) 98–103.
53. S.S. Silva, D. Maniglio. *Macromol. Biosci.* **8** (2008) 766–774.
54. X. Chen, Y.Y. Qi. *Biomaterials* **29** (2008) 3683–3692.
55. D. Eberli, L.F. Filho. *Methods* **47** (2009) 109–115.
56. A.K. Ekaputra, G.D. Prestwich. *Biomacromolecules* **9** (2008) 2097–2103.
57. L. Moroni, R. Schotel. *Adv. Mater.* **18** (2008) 53–60.
58. P.M. Mendes. *Chem. Soc. Rev.* **37** (2008) 2512–2529.
59. J.F. Mano. *Adv. Eng. Mater.* **10** (2008) 515–527.
60. T. Sumide, K. Nishida. *FASEB J.* **20** (2006) 392–394.
61. G. Sitalakshmi, B. Sudha. *Tissue Eng. Part A* **15** (2009) 407–415.
62. J.R. Lieberman, G.E. Friedlaender. *Bone Regeneration and Repair: Biology and Clinical Applications*, Humana Press, NY, USA, 2005.
63. E.K. Ko, S.I. Jeong. *Macromol. Biosci.* **8** (2008) 1098–1107.
64. E.K. Ko, S.I. Jeong. *Tissue Eng. Part A* **14** (2008) 2105–2119.
65. S.A. Hurd, N.M. Bhatti. *Biomaterials* **49** (2015) 9–17.
66. N.R. Blan, R.K. Birla. *J. Biomed. Mater. Res. A* **86A** (2008) 195–208.
67. A.M. Martins, M.I. Santos. *Acta Biomater.* **4** (2008) 1637–1645.
68. W.P. Daley, S.B. Peters. *J. Cell Sci.* **121** (2008) 255–264.
69. V. Nikolova, C.Y. Koo. *Carcinogenesis* **30** (2009) 397–407.
70. C. Chang, Z. Werb. *Trends Cell Biol.* **11** (2001) S37–S43.
71. C.M. Ghajar, S.C. George. *Crit. Rev. Eukaryot. Gene Expr.* **18** (2008) 251–278.
72. M.E. Fleury, K.C. Boardman. *Biophys. J.* **91** (2006) 113–121.
73. H.G. Sundararaghavan, G.A. Monteiro. *Biotechnol. Bioeng.* **102** (2009) 632–643.
74. E. Hadjipanayi, V. Mudera. *Cell Motil. Cytoskeleton* **66** (2009) 121–128.
75. K.M. Yamada, E. Cukierman. *Cell* **130** (2007) 601–610.
76. E. Cukierman, R. Pankov. *Science* **294** (2001) 1708–1712.
77. M. Singh, C. Berkland. *Tissue Eng. Part B Rev.* **14** (2008) 341–366.
78. F. Pampaloni, E.G. Reynaud. *Nat. Rev. Mol. Cell Biol.* **8** (2007) 839–845.
79. C.M. Ghajar, X. Chen. *Biophys. J.* **94** (2008) 1930–1941.
80. I. Thesleff, A.M. Partanen. *J. Craniofac. Genet. Dev. Biol.* **11** (1991) 229–237.
81. Y.C. Li, F. Jin. *Tissue Eng. Part A* **14** (2008) 1731–1742.
82. K. Nakao, R. Morita. *Nat. Methods* **4** (2007) 227–230.
83. E.L. Scheller, P.H. Krebsbach. *J. Oral Rehabil.* **36** (2009) 368–389.
84. M.J. Honda, H. Fong. *Med. Mol. Morphol.* **41** (2008) 183–192.
85. B. Hu, A. Nadiri, 8th International Conference on Tooth Morphogenesis and Differentiation, Tissue engineering of tooth crown, root, and periodontium, York, UK, 2004.

86. J.H. Yu, J.N. Shi. *Tissue Eng. Part B Rev.* **14** (2008) 307–319.
87. A. Ohazama, S.A.C. Modino. *J. Dent. Res.* **83** (2004) 518–522.
88. Y.Q. Song, M.Q. Yan. *Dev. Dyn.* **237** (2008) 411–416.
89. J.H. Yu, J.N. Shi. *Biochem. Biophys. Res. Commun.* **346** (2006) 116–124.
90. C.F. Ferreira, R.S. Magini, 2nd *Journal of Oral Rehabilitation Summer School, Biological tooth replacement and repair*, Bevagna, Italy, 2006.
91. S.E. Duailibi, M.T. Duailibi. *J. Dent. Res.* **87** (2008) 745–750.
92. W.P. Xu, W. Zhang. *Tissue Eng. Part A* **14** (2008) 549–557.
93. M.T. Duailibi, S.E. Duailibi. *J. Dent. Res.* **83** (2004) 523–528.
94. C.S. Young, S. Terada. *J. Dent. Res.* **81** (2002) 695–700.
95. C.S. Young, H. Abukawa. *Tissue Eng.* **11** (2005) 1599–1610.
96. W.B. Zhang, H. Abukawa. *Methods* **47** (2009) 122–128.
97. H. Abukawa, W. Zhang. *J. Oral Maxillofac. Surg.* **67** (2009) 335–347.
98. S.B. Traphagen, P.C. Yelick. *Regen. Med.* **4** (2009) 747–758.
99. X. Wen, Y.M. Zou. *Anat. Rec.* **291** (2008) 1246–1253.
100. D.M.H. Tenorio, M.F. Santos, *International Symposium on Extracellular Matrix (SIMEC 2002)*, Distribution of biglycan and decorin in rat dental tissue, Angra dos Reis, RJ, Brazil. 2002.
101. Z. Huang, T.D. Sargeant. *J. Bone Miner. Res.* **23** (2008) 1995–2006.
102. J. Caton, H.U. Luder. *Dev. Biol.* **328** (2009) 493–505.
103. S.B. Traphagen, P.C. Yelick. *Biomaterials* **33** (2012) 5287–5296.
104. J.J. Moon, J.L. West. *Curr. Top. Med. Chem.* **8** (2008) 300–310.
105. D.M. Hoganson, H.I. Pryor. *Pediatr. Res.* **63** (2008) 520–526.
106. E.M. Brey, S. Uriel. *Tissue Eng.* **11** (2005) 567–584.
107. P. Carmeliet. *J. Intern. Med.* **255** (2004) 538–561.
108. C.K. Griffith, C. Miller. *Tissue Eng.* **11** (2005) 257–266.
109. N.C. Rivron, J. Liu. *Eur. Cell. Mater.* **15** (2008) 27–40.
110. M.I. Santos, K. Tuzlakoglu. *Biomaterials* **29** (2008) 4306–4313.
111. S. Levenberg, J. Rouwkema. *Nat. Biotechnol.* **23** (2005) 879–884.
112. N. Koike, D. Fukumura. *Nature* **428** (2004) 138–139.
113. G.E. Davis, D.R. Senger. *Circ. Res.* **97** (2005) 1093–1107.
114. S.E. Epstein, R. Kornowski. *Circulation* **104** (2001) 115–119.
115. J. Sottile. *Biochim. Biophys. Acta* **1654** (2004) 13–22.
116. E.D. Karagiannis, A.S. Popel. *Biochem. Biophys. Res. Commun.* **354** (2007) 434–439.
117. C. Borselli, O. Oliviero. *J. Biomed. Mater. Res. A* **80A** (2007) 297–305.
118. L.G. Griffith, M.A. Swartz. *Nat. Rev. Mol. Cell Biol.* **7** (2006) 211–224.
119. H.M.E. Hajjami, T.V. Petrova. *Histochem. Cell Biol.* **130** (2008) 1063–1078.
120. W.S. Shin, S.G. Rockson. *Ann. NY Acad. Sci.* **1131** (2008) 50–74.
121. S. Suematsu, T. Watanabe. *Nat. Biotechnol.* **22** (2004) 1539–1545.
122. M.A. Swartz, J.A. Hubbell. *Semin. Immunol.* **20** (2008) 147–156.
123. J.M. Rutkowski, K.C. Boardman. *Am. J. Physiol. Heart Circ. Physiol.* **291** (2006) H1402–H1410.
124. G.N. Li, D. Hoffman-Kim. *Tissue Eng. Part B Rev.* **14** (2008) 33–51.
125. H. Zhang, Y.T. Weing. *J. Transl. Med.* **6** (2008) 67.
126. S. Ichihara, Y. Inada. *Injury* **39** (2008) S29–S39.
127. C.M. Nichols, T.M. Myckatyn. *Behav. Brain Res.* **163** (2005) 143–158.
128. S. Rochkind. *Neurosurg. Focus* **26** (2009) E8.
129. C. Morsczech, G. Schmalz. *Clin. Oral Invest.* **12** (2008) 113–118.

130. H. Nam, J.H. Kim. *Mol. Cells* **37** (2014) 562–567.
131. A. Nakata, T. Kameda. *Biochem. Biophys. Res. Commun.* **308** (2003) 834–839.
132. A. Komine, M. Suenaga. *Biochem. Biophys. Res. Commun.* **355** (2007) 758–763.
133. S. Iwatsuki, M.J. Honda. *Eur. J. Oral Sci.* **114** (2006) 310–317.
134. A. Angelova Volponi, M. Kawasaki. *J. Dent. Res.* **92** (2013) 329–334.
135. A. Bigi, G. Cojazzi. *Biomaterials* **23** (2002) 4827–4832.
136. S.M. Lien, W.T. Li. *Mater. Sci. Eng. Biomim. Mater. Sens. Syst.* **28** (2008) 36–43.
137. W.H. Chang, Y. Chang. *J. Biomater. Sci. Polym. Ed* **14** (2003) 481–495.
138. H.G. Sundararaghavan, G.A. Monteiro. *J. Biomed. Mater. Res. A* **87A** (2008) 308–320.
139. K. Takahashi, S. Yamanaka. *Cell* **126** (2006) 663–676.
140. K. Kaji, K. Norrby. *Nature* **458**(7239) (2009) 771–775.
141. K. Woltjen, I.P. Michael. *Nature* **458**(7239) (2009) 766–770.

**THE PROMISING ROLE FOR
ADIPOSE-DERIVED MESENCHYMAL STEM
CELLS IN TISSUE REGENERATION**

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2.1. INTRODUCTION

Biomaterials and engineered tissue constructs face a significant risk of failure due to the immune response to such foreign bodies. While synthetic materials can be precisely engineered to maximize the desired therapeutic effects, further interventions are needed to mitigate this host response. A new area of research has focused on the immunomodulatory properties of adipose-derived mesenchymal stem cells (ASCs), which have been demonstrated in numerous *in vitro* and *in vivo* studies to significantly modulate immune responses and immune cell activity in a variety of contexts.

Since stem cell-seeded constructs and scaffolds have already been developed, it naturally follows whether ASCs can be incorporated into future iterations of engineered tissues to reduce the negative effects of a host immune response against the therapeutic intervention. This chapter will focus on evidence for the promising role of ASCs in facilitating the successful therapeutic implantation of engineered tissues by means of immunomodulation.

2.2. FOREIGN BODY IMMUNE RESPONSES IN TISSUE ENGINEERING

Surgical insertion of an engineered scaffold for tissue engineering will yield a two-fold reaction: one mediating acute wound healing, and another mediating the foreign body response to the artificial substrate. The former process has been well studied and follows a highly predictable course. Acute wound healing is mediated primarily by immune cells (such as neutrophils and macrophages), associated chemokines / cytokines, platelets, fibroblasts, among other mediators intrinsic to the damaged tissue. The clotting cascade, mediated by platelets and fibrin clots, initiates the healing process, and leads to secretion of platelet-derived growth factor (PDGF) and transforming growth factor- β (TGF- β). These factors, as well as mast cell degranulation, facilitates the chemotactic influx of inflammatory cells such as neutrophils. TGF- β also serves to guide matrix deposition in conjunction with other signaling mediators. Epithelialization and subsequent fibrosis of the wound leads to final closure [1].

The foreign body response to the biomaterial has significant implications on the functionality and success of that device / tissue and this is a significant challenge for engineers, clinicians, and researchers today. Mitigating the complex immune response to a foreign body serves as a critical point of therapeutic intervention to increase the chance of success in tissue engineering applications. Initial interactions between blood components (particularly plasma proteins and complement mediators) [2] and the biomaterial directly

induce subsequent acute and chronic inflammatory processes, resulting in infiltration of the material and the surrounding tissue by neutrophils, lymphocytes, and monocytes. Macrophages, unable to phagocytose the biomaterial, undergo fusion to form the giant cells that characterize the foreign body response. A complex array of signaling molecules and cellular mediators orchestrates this process [3]. An overview of the foreign body response is depicted in Figure 1.

Figure 1. Overview of the foreign body response to biomaterial implantation and current strategies to minimize its effects

The fibrotic response to foreign bodies has significant downstream effects on the functionality of a biomaterial, particularly if the material is bioactive with regards to delivery of drugs or signaling molecules [4]. Fibrosis and inflammatory responses have also been shown to loosen implanted calcium phosphate-containing organic matrices used in bone regeneration [5]. The pre-existing inflammatory milieu in myocardial infarction, in addition to response to the scaffolds used in cardiac patches for assisted tissue regeneration can decrease the survival of cells within the engineered patch [6]. Thus, it becomes evident that control and modulation of the immune response is critical for the success of a therapeutic intervention involving engineered

tissues and biomaterials, and that biomaterial failure due to pathological fibrosis and immune activation is present in a wide array of therapeutic applications.

Initial studies into this immune response and technological limitations urged biomaterial engineers to either account for inevitable fibrosis or minimize antigenicity of the material components [7]. However, achievement of truly 'bioinert' substances remains challenging, and, thus, biomaterials must be designed in such a way so as to adaptively regulate the immune response, rather than hide from it. In order to achieve this, further characterization of the foreign body immune response in terms of intercellular interactions, phenotypic diversity of the cellular mediators, signaling mediators, and qualities of the scaffold material itself can result in proactive interventions to mitigate the negative effects of pathological fibrosis [8-10].

One approach involved the exploration of degradable polymeric biomaterials. Use of more permanent biomaterials can result in a chronic inflammatory process, leading to increased risk of therapeutic failure. The benefit of degradable constructs is that the host immune response will be inherently self-limited. However, it is necessary to ensure that degradation products of these temporary scaffold structures are non-toxic [11]. Specific modulation of macrophage response to biomaterials has also been suggested by utilizing materials with uniform, spherical, and interconnected pores, modulation of nano-scale biomaterial topography, local interleukin-4 (IL-4) administration, and administration of phosphatidylserine-presenting liposomes (to mimic anti-inflammatory characteristics of apoptosis [12]. Biomaterials can also be modified to modulate macrophage adhesion by grafting specific peptide sequences to the material surface [13].

Other techniques have included coating with biocompatible materials, such as natural polymers and synthetic hydrogels. However, these coatings may themselves be immunogenic and prone to degradation over time, in addition to concerns regarding coating adherence to the biomaterial and safety of synthetic coatings [14]. Local reservoirs of steroids or non-steroidal anti-inflammatory drugs (NSAIDs) can also serve to artificially modulate the immune response to the foreign body. Enhancement of vascularization around the biomaterial may prove necessary in certain applications, and can be achieved by addition of vascular endothelial growth factor (VEGF) and limiting use of corticosteroid immunomodulatory agents [14].

While multiple approaches currently exist to limit pathological fibrosis of biomaterials used in tissue engineering, no consistent or comprehensive solution exists to effectively modulate the entire immune response. The best solution would be an adjunct to the existing biomaterial that can modulate the immune system with a response that is itself susceptible to feedback. It should also be readily incorporable into existing scaffolds in a wide variety of therapeutic contexts, and enhance pro-tolerogenic immunologic effects

towards biomaterials in a diverse, multifactorial manner. For this reason, adipose-derived stem cells, which have been demonstrated to have potent immunomodulatory effects, and can readily seed scaffolds used in tissue engineering, can serve a powerful role in enhancing the survival of engineered tissues [15].

2.3. ADIPOSE-DERIVED STEM CELLS (ASCs)

Adipose-derived stem cells (ASCs) are a type of mesenchymal stem cell (MSC), which are defined as a cell population that displays adherence to plastic, a specific array of surface antigen expression, and differentiation potential into osteoblast, adipocyte, and chondrocyte lineages [16]. MSCs are isolated from a wide range of tissues, such as bone marrow, placenta, thymus, Wharton's jelly, umbilical cord blood, and adipose tissue, among a number of other tissue sources [17]. Molecular characterization of ASCs from uncultured stromal vascular fraction includes a surface marker expression panel of CD45-CD235a-CD31-CD34⁺CD13⁺CD73⁺CD90⁺CD105⁺. Since ASCs share molecular characteristics with bone marrow-derived MSCs, ASCs can be uniquely characterized from the latter by expression of CD36 and lack of CD106 expression [18]. ASCs are also capable of differentiating into neuronal [19] and myocyte lineages [20,21].

One of the most appealing qualities of ASCs is the relative ease of isolating such cells from body tissues. Protocols have been developed to allow for collection of 250,000–375,000 ASCs from a single milliliter of human lipoaspirate following 4–6 days of culture in 10 % fetal bovine serum. Since lipoaspirate is readily collected during liposuction, tremendous numbers of ASCs can be relatively easily isolated from patients for use in therapeutic interventions [15,22]. Trypsin-mediated tissue digestion for ASC isolation may also provide an inexpensive method to harvest this cell population following lipoaspiration [23]. Successful culture of ASCs in xeno-free media without serum has also been achieved, further supporting consistent isolation and maintenance of ASCs in economically efficient *in vitro* conditions [24].

ASCs have been shown to have therapeutic potential in a wide range of clinical contexts. Addition of ASCs to skin wounds was shown to result in improved healing dynamics, particularly with enhanced angiogenesis/capillary formation in the wound site, increased epithelialization, and better cosmetic results due to decreased scarring. Wound healing enhancement was attributed to both ASC differentiation and release of paracrine molecules [25]. ASCs were also shown to facilitate resolution of chronic, nonhealing wounds [26].

Other current therapeutic applications of ASCs include acceleration of peripheral nerve regeneration following injury [27], regeneration of bone and cartilage, and to facilitate survival of fat grafts in breast augmentation and facial lipoatrophy [28]. ASCs have also served therapeutic roles in

rehabilitation of cardiac function following myocardial infarction / ischemia as well ischemic injury due to peripheral vascular disease. Preliminary evidence for ASC-derived-insulin-secreting cells in the treatment of Type 1 Diabetes has also demonstrated promising results. The role of ASC in fistula repair has been explored, although currently remains inconclusive [29].

Ultimately, the features of ASCs that allow for such widespread therapeutic benefits in a variety of clinical contexts make it a promising candidate for the demands of enhancing success in the clinical use of engineered tissues.

2.3.1. Bioactive properties of ASCs

ASCs have been shown to have significant immunomodulatory properties, in addition to secretion of growth factors. In promoting immunological tolerance and facilitating growth of an engineered tissue construct, ASCs can serve a significant role in the success of therapeutic interventions involving tissue engineering. Not only can stem cells be used to provide cell types needed for tissue regeneration, but the pleiotropic paracrine-mediated effects of ASCs can also facilitate a rich, pro-healing environment.

What exactly constitutes the secretome of ASCs that make it such a potentially bioactive cell population? Signaling mediators include growth factors and immunomodulatory agents such as TGF- β , hepatocyte growth factor (HGF), VEGF, indoleamine-2,3 dioxygenase (IDO), interleukin-10 (IL-10), prostaglandin E2 (PGE2), nitric oxide (NO), heme oxygenase 1 (HO-1), and human leukocyte antigen (HLA-G) among numerous other mediators [30,31]. Other MSC subpopulations are known to secrete IL-6, IL-8, and IL-12, as well as interferon-gamma (IFN- γ), macrophage colony stimulating factor (M-CSF), and hepatocyte growth factor (HGF) [17,32]. While the secretome of the cell population has been closely studied, one cannot also ignore the many secondary effects of ASC-mediated signaling. By modulating secretory patterns of other cells, local ASC administration can have systemic modulations of various extracellular signals, mediators, and structural proteins [33]. However, the ASC secretome has been shown to have intrinsic immunomodulatory effects without the need for ASCs simultaneously present, as evidenced by an experiment demonstrating variation in the level of various cytokines following infusion of a mouse with the supernatant of an ASC culture [34].

The secretome can be characterized by analyzing various functional consequences of ASC paracrine signaling (Figure 2). Angiogenesis mediated by ASCs is largely due to secretion of TGF- β , VEGF, and basic fibroblast growth factor (bFGF) [31]. Secretion of these pro-vascularizing signals is thought to be induced by a localized state of hypoxia [35,36]. Furthermore, ASCs may display characteristics of pericytes and directly secrete various regulatory proteins that control and organize networks of endothelial cells into functional vasculature [37]. In the context of wound healing, ASCs were shown to potentiate this process through the secretion of some of the various growth

factors and extracellular matrix (ECM) components discussed above, such as collagen I, fibronectin, fibroblast growth factor 2, VEGF, and TGF- β , among other signaling mediators [31,38].

Both of these qualities are critically important for the functionalization of an engineered tissue implant. Enhanced vascularization facilitates oxygenation of the otherwise ischemic tissue, in addition to delivery of various metabolic substrates needed for implanted cells to function properly in their therapeutic environment. Activation of wound healing processes leads to secretion and deposition of ECM components, leading to the integration of the implanted biomaterial into surrounding tissue, enhancing its therapeutic success. Although the immune response poses a significant barrier, ASCs are sufficiently capable of reducing this adverse effect.

Figure 2. Immunomodulatory and angiogenic secretome of ASCs (image from American Research Products) [30,31,39-41]

2.4. ASC-MEDIATED IMMUNOMODULATION

The immunomodulatory capacities of MSCs are widespread, affecting various cellular members of the immune system such as T cells, B cells, dendritic cells (DCs), and natural killer cells [42]. However, such capacities differ among the different sources of MSCs, and various studies have looked into identifying the most potent stem cell population for immunomodulatory effects. ASCs have been demonstrated to be more potent at immunomodulation as compared to MSCs isolated from bone marrow (BMSCs) with respect to suppression of peripheral blood mononuclear cell (PBMC) proliferation, inhibition of monocyte differentiation into DCs, and show a higher level of cytokine secretion [43]. Another study compared ASCs and BMSCs with regards to effects on lymphocytes and found the two cell populations to have similar immunosuppressive capacities, dependent highly on the number of cells while also 'highly variable' between different samples of isolated ASCs [44]. Another group demonstrated that MSCs isolated from placenta can show greater immunomodulatory effects than either ASCs or BMSCs [45].

Interestingly, the immunosuppressive properties of ASCs may continue after subsequent differentiation, as proposed by a study examining such properties following TGF- β 3-induced chondrogenesis of ASCs [46]. Additionally, ASCs genetically modified to overexpress IL-4 were shown to have a stronger anti-inflammatory response in the context of experimental autoimmune demyelination [47]. Ultimately, the potent immunomodulatory properties of ASCs may play a role in a diverse array of therapeutic functions, from mediating tolerance in HLA-mismatched cell-based therapies and potentially restoring immunological balance in autoimmunity.

2.4.1. Effects on antigen-presenting cells

Since the immune system consists of a diverse set of players, it is important to understand how MSCs / ASCs are able to modulate the activity and behavior of each cellular component [17]. DCs and macrophages are efficient antigen-presenting cells (APCs), serving a critical initiating role for inflammation by the immune system [42]. Since APCs serve to deliver antigens from scaffolds and other components in engineered tissues to induce the downstream inflammatory response, effective suppression of APC activity can serve a significant role in maintaining the integrity of the engineered construct. Factors secreted by ASCs have been shown to inhibit differentiation of monocytes and DCs. Furthermore, ASCs are able to affect DC endocytosis, hinder migration of DCs to lymph nodes, induce regulatory phenotypes in both macrophages and DCs, and modulate downstream T cell activation [48-52]. Thus, potent modulation of APC activity by MSCs/ASCs can be utilized effectively to increase success in the context of engineered tissues.

Downstream of APC endocytosis of the antigen is its presentation to effector arms of the immune system, including CD4⁺ T cells that activate other arms of

the immune system. Interestingly, ASCs have potent effects on T lymphocytes as well. ASCs can affect helper T cell differentiation by changing the intracellular milieu of transcription factors and cytokine secretion profile [42,53]. It was previously noted that Th1 differentiation could be affected downstream of DC immunomodulation [50]. Specific suppression of the Th17 [54] and Th2 [55] subsets by ASCs has also been observed. In cross-species interaction of human ASCs with murine T lymphocytes *in vitro*, the mechanism of suppression of T cell proliferation was demonstrated to involve cyclooxygenase-2 (COX-2) [56]. Another group demonstrated a significant role for PGE2 in modulating T lymphocyte transcription factors and cytokines to underlie the immunosuppressive effects. Interestingly, exosomes of ASCs were shown to be sufficient in inducing suppressive effects on T cell activation, differentiation, proliferation, and IFN- γ secretion [57]. With regards to cytotoxic T lymphocytes, MSCs could suppress development of but not actual cell lysis by these cells [58].

2.4.2. Effects on T lymphocyte populations

Direct inhibition of T cell activity by ASCs in a model of rat orthotopic liver transplantation was shown to reduce acute rejection [59]. Similar effects were shown in the ASC-mediated immunosuppression of human T cells from patients with rheumatoid arthritis [60]. The ASC-mediated suppressive effects on T cells, although with great therapeutic benefit, may however come with dangerous consequences. ASCs in breast cancers may modulate T cells in such a way so as to induce a tolerogenic environment, thereby protecting tumor cells from immune attack [61].

In addition to direct immunosuppression of effector T cell populations, MSCs / ASCs are capable of inducing the proliferation and maintenance of both CD4⁺ and CD8⁺ regulatory T cell (Treg) populations [60,62]. The elevated presence of Tregs around an engineered implant can enhance immune tolerance of the artificial material. It has been demonstrated that the Tregs induced by ASCs are functional and capable of suppressing proliferation of effector immune cells [63]. Low oxygen conditions were shown to facilitate Treg induction by ASCs [64], which can be of particular utility in engineered implants, which are not immediately vascularized and thus experience locally hypoxic conditions. Supplementation of ASCs to the post-transplant regimen in a rat model of vascularized composite allotransplantation led to increased numbers of Tregs in circulation, which was implicated in the enhanced post-transplant tolerogenesis in these rats [65].

2.4.3. Effects on B lymphocytes

Immunomodulation by ASCs does not end with APCs and T lymphocyte subsets, but may also include regulation on the level of B lymphocytes and natural killer (NK) cells. BMSCs have been shown to suppress B lymphocyte proliferation via the programmed death ligands (PD-L1 and PD-L2) [66]. Other effects on B cells by MSCs include suppression of terminal differentiation [67], *in vitro* inhibition of antibody production [68], and downregulation of factors necessary for chemotaxis [69]. ASCs, in particular, can induce the development of a regulatory B cell population, further controlling B cell activity [70]. MSCs have been known to suppress activity of NK cells with regards to IFN- γ secretion and proliferation [51,71,72]. ASCs, specifically, are capable of such suppression [73] and are themselves poorly susceptible to lysis by NK cells [74].

2.5. IMPORTANT CONSIDERATIONS IN ENGINEERING

The immunomodulatory properties of ASCs are in turn modulated by a diverse list of variables. Local presence of IFN- γ from T and NK cells is necessary for MSC-mediated immunomodulation, as this resulted in secretion of IDO by the MSCs to suppress the activity of other immune mediators [75,76]. IFN- γ stimulation of MSCs was also associated with MHC-II expression, which weighs heavily in MSC antigen presentation and subsequent allogenicity [77]. It is important to note that ASC-mediated suppression of T cell proliferation is strongly dependent on its passage, with early passage ASCs actually stimulating proliferation, and later passage cells suppressing it [78]. Thus, the immunomodulatory characteristics of the ASCs in an engineered construct will depend on the inflammatory microenvironment around the implant.

Application of ASCs/MSCs in engineered tissues requires culturing and integration of cells *in vitro* within constructs. Subsequent proliferation of MSCs may have effects on their immunomodulatory capacities due to degradation of telomeres. Extension of telomeres through enhanced expression of telomerase was shown to retain immunosuppressive capabilities in the immortalized cell lines [79]. Beneficial to its proposed application in controlling inflammatory responses against engineered tissues, ASCs also demonstrate increased potential for immunosuppression in an inflammatory environment [80]. The age of the donor appears to be inversely proportional to the immunosuppressive capacity of the isolated ASCs as well [81].

Particularly important to engineering processes, the method by which ASCs are cultured can also weigh significantly on their immunomodulatory capacity [82]. This finding was corroborated by a later study, which also demonstrated the ability to isolate and expand ASCs in xeno-free and serum-free media, although this negatively impacts its immunosuppressive properties. Hypoxia

also markedly enhances the functions of ASCs, which is useful in poorly vascularized tissue initially surrounding artificial implants. These functional changes include increased proliferation, anti-apoptotic and angiogenic factor secretion, and altered adhesion dynamics to ECM proteins [25,83].

2.5.1. Differentiation potential of ASCs

In addition to serving an immunoprotective role towards the engineered tissue, ASCs are also capable of differentiating into various soft and connective tissues that facilitate integration of that specific tissue into the body (Figure 3). ASCs are multipotent, being able to undergo adipogenic, osteogenic, myogenic, and chondrogenic differentiation, (differentiation is limited to mesodermic lineages) [84]. Interestingly, according to some, ASC spheroids showed similar chondrogenic potential as BMSCs in a monolayer, but were less effective than the latter in cartilaginous differentiation in high-density 3D culture formed from spheroidal aggregates of cells [85]. Human ASCs have also demonstrated the ability to develop a smooth muscle-like morphology [86]. ASCs can also be induced into a neuronal lineage, serving another exciting role for this cell population in the realm of tissue engineering [87]. Other applications include direct differentiation into corneal epithelial-like cells [88], dentine pulp tissue [89], cell types similar to pancreatic β cells with insulin-secreting capabilities [90], and cardiomyocyte-like cells for post-myocardial infarction repair [89,91,92].

As mentioned above, the pro-angiogenic properties of ASCs provide an exciting platform in the realm of tissue engineering. Since the surgical integration of an engineered tissue implant naturally results in loss of perfusion to that area, a proper return of blood flow through direct endothelial differentiation and VEGF secretion in that area is needed to supply nutrients and oxygen to regenerative cells [93]. ASCs have thus demonstrated potent roles for angiogenic repair in ischemic tissues, such as in myocardial infarction [94]. ASCs have been shown to not only directly differentiate into endothelial cells and incorporate into existing vascular structures [95], but may also have a role in supporting that population. Abluminal localization and subsequent development of pericyte-, smooth muscle-, and mesenchymal-like properties in ASCs allows ASCs to directly interact with endothelial cells to maintain the functional and structural aspects of vasculature [96]. These interactions have already been demonstrated to potentiate angiogenesis around implants, raising the possibility for its clinical use in tissue engineering [97].

Figure 3. Demonstrated differentiation potential of ASCs

2.6. CURRENT THERAPEUTIC APPLICATIONS OF ASCs

The bioactive properties of ASCs have been extensively studied in a wide range of clinical contexts, from effects on tumors to post-transplantation immunosuppression and autoimmune conditions. This extensive array of evidence lends credence to the fascinating potential of these cells in treating and mitigating disease. This, coupled with the relative ease by which they can be isolated, creates an exciting future for ASCs.

2.6.1. Immunomodulation in transplantation and immune-mediated disease

Tolerance to engineered tissues can be likened to that which is required following allogeneic transplant. Current post-transplant immunosuppressive regimens contain chemical suppressors of immune mediator proliferation. New research suggests that supplementation of this regimen with immunomodulatory ASCs can enhance the development of tolerance and reduce the onset of rejection [102]. This combination therapy was demonstrated to have significant results in tolerogenesis in an orthotopic hind-limb vascularized composite allotransplantation model in rats [103].

These successful results have also been demonstrated in large animal models, with enhanced prolongation of heterotopic hind-limb transplantation in swine [104]. Several potential issues must be noted in the use of ASCs/MSCs in post-transplant regimens. These include maintenance of the ASC populations *in vivo* due to natural clearance, specifications regarding dosage and method of delivery, and the potential for drugs in the post-transplant regimen to themselves negatively modulate the immunomodulatory capacity of ASCs [105]. The potential for success in using ASCs in transplant has been demonstrated in a humanized mouse model of skin grafts [106] and even in a patient who underwent kidney transplant [107]. Evidence also exists for the capacity of ASCs to mitigate the severity of graft-versus-host-disease (GVHD) following bone marrow transplant [108].

Immunomodulation by ASCs has proved useful in the context of autoimmune and immune-mediated diseases. Systemic treatment with ASCs has been showed to decrease Th1 response and increase Treg levels (with associated elevation in IL-10 levels) to alleviate the severity of colitis in a murine model of drug-induced colitis [109,110]. Early evidence also points to the utility of MSCs as a therapeutic adjunct in the treatment of multiple sclerosis [111,112]. Through suppression of Th1 and Th17 subsets in combination with upregulation of Tregs, ASCs were demonstrated to improve outcomes in murine experimental autoimmune hearing loss [113]. This immunosuppression of T lymphocytes was also demonstrated to reduce the severity of symptoms in rheumatoid arthritis [60]. ASCs were also shown to decrease the incidence of necrosis following autologous fat grafting [114]. Through the upregulation of VEGF and IL-10, intracerebral implantation of ASCs demonstrated significantly improved functional outcomes in a mouse model of Alzheimer's disease [115].

The vast potential of ASCs is readily apparent. This cell population is readily available and plentiful in lipoaspirates, isolatable by various protocols, and has a rich array of bioactive properties ranging from immunosuppression to localized enhancement of growth and repair. As implanted engineered tissues face hefty challenges from the host immune response and demand localized wound repair for proper integration into the body, the mechanisms by which ASCs have been demonstrated to modulate local environments *in vitro* and *in vivo* can be harnessed through integration into scaffolds and artificial constructs. This is another promising area of study, and is discussed in the following section.

2.6.2. Oncology

Stem cell therapy appears to have a mixed picture when it comes to induction or reduction of cancerous tumors. As was previously stated, the combination of growth factor secretion and immunosuppression by localized presence of ASCs can protect and induce the growth of breast cancer cells [61]. Breast cancer

cells co-cultured with ASCs were shown to have increased markers for malignancy and may lead to worse outcomes [98]. ASCs increased motility and metastasis of cancer cells and may stabilize tumor vasculature in breast cancers as well [99]. Interestingly, stromal tissue isolated from adipose tissue was shown to lead to increased cell death in pancreatic cancers [100]. ASCs also demonstrate the capacity to hone on tumor cells, and this has been used therapeutically to assist in drug delivery to these sites [101].

2.7. INTEGRATION OF ASCs WITH SCAFFOLDS AND ARTIFICIAL CONSTRUCTS

To realize the full potential of ASCs enhancing the successes of engineered tissues, it is first necessary to understand how ASCs can be integrated into scaffolds currently used in these applications. Evidence has existed for over a decade regarding the seeding of scaffolds with ASCs. A 2005 publication examining techniques to facilitate osteochondral engineering determined that ASCs could be successfully integrated into chitosan scaffolds without undue toxicity on those cells [116]. The adhesive properties of the scaffold specifically to encourage ASC attachment and proliferation can be enhanced by the application of specific peptide sequences to its surface [117]. ASCs were also shown to have potential in tissue engineering particularly in vascular generation through seeding of tubular scaffolds [118] and adipogenesis following seeding of gelatin sponges [119]. ASCs were also proposed for the seeding of calcium phosphate scaffolds to facilitate surgical repair in spinal fusion [120].

Considerable research has been conducted to determine specific characteristics of the scaffold that facilitate the use of ASCs in various clinical contexts. Not only does the material of the scaffold weigh heavily on ASC seeding and subsequent proliferation and differentiation, but also the pore size and shape, which has significant effects on vascularization of the biomaterial. Understanding how various scaffold-based technologies have proven useful in laboratory and clinical settings can yield useful information into how ASCs can be integrated into existing tissue engineering applications across the body.

2.7.1. Wound healing

One area where ASCs have proven useful is in the context of wound healing. As previously mentioned, the hypoxic conditions within the wound coupled with the angiogenic properties and differentiation capabilities of ASCs make them potent candidates to accelerate the wound healing process. However, effective use of these cells requires their integration into a matrix that can be easily applied within the wound, which may be irregularly shaped. Acellular scaffolds may facilitate these functions of ASCs, and this has been hypothesized to be an effective solution to enhance wound healing [121]. Cell sheets of ASCs have

been demonstrated to enhance the degree of wound healing in a mouse model [122,123]. One study found that ASCs integrated into an ECM patch were necessary for their survival and subsequent enhancement of skin wound healing in mice [124]. Hydrogels have been shown to maintain ASC viability within the wound, thereby prolonging their healing capacities for a longer period of time [125]. Decay in ASC healing properties may be mitigated by the use of low level laser therapy, which can act to stimulate these cells [126]. In terms of biomaterials, chitosan and PLCP-P123 (nonwoven nanofibrous material with structural similarity to ECM) scaffolds have been shown to be effective in the wound healing process mediated by ASCs [127-129].

2.7.2. Bone / soft tissue repair

Effective scaffold-based ASC therapy can also prove useful in the treatment of defects in bone by optimizing the osteogenic potential of these cells. As in skin wound healing, the architecture and composition of the scaffold plays a critical role in this process. Scaffolds that bear resemblance to bone (calcium phosphate-based scaffold with polycaprolactone and hydroxyapatite components), particularly those that have been co-cultured with osteoblasts, show success in facilitating ASC differentiation into bone within the architecture of the scaffold [130]. As seen previously in skin wound healing, use of low-level laser therapy can enhance bone regeneration as well [131]. In addition to mimicking scaffolds with natural bone, the density of the scaffold structure also weighs on the differentiation potential of ASCs [132]. A recent study demonstrated that a stacked scaffold structure can also enhance the osteogenic potential of ASCs [133].

2.7.3. Angiogenesis

Scaffold material (either naturally derived or synthetically generated) has also been shown to weigh on ASC angiogenic potential [134]. Seeding of ASCs into commercial dermal substitutes can also promote angiogenesis [135,136]. It is interesting to note that the method by which ASCs are integrated into the scaffold (injection vs. onlay seeding) can play a role in the extent of subsequent vascularization of the graft due to differential effects on migration of cells through that scaffold [137]. Additionally, seeding of ASC spheroids into a porous polyurethane scaffold proved more effective at facilitating growth of a higher density of vessels than seeding with individual ASCs [138]. However, the angiogenic potential of ASCs need not require direct contact by those cells. Encapsulation of ASC spheroids with a polytetrafluoroethylene filtration membrane, which allows soluble mediators to diffuse into the local environment, demonstrated increased vascularization in that area [139].

2.7.4. Neuronal regeneration

Scaffold-integrated ASCs can also facilitate neuronal regeneration. The use of decellularized adipose tissue as the ECM scaffold grafted into a mouse model of cavernous nerve damage to simulate post-prostatectomy injury, demonstrated neural regrowth and return of penile erection [140]. Seeding of ASCs into porous gelatin and chitosan scaffolds grafted into mouse model of traumatic brain injury also revealed enhanced repair, attributed to induction of ASC differentiation into neuron-like morphologies [141,142]. Targeted axonal repair following transection and Wallerian degeneration is also enhanced with the use of nerve guidance conduits seeded with ASCs [143].

2.7.5. Other applications of ASC-seeded scaffolds

This platform has been utilized in a variety of other clinical contexts as well (Figure 4). Submucosa-seeded ASCs enhance erectile function following damage to tunica albuginea and application of the graft over the injury [144]. Virally modified ASCs integrated into a 3D PLGA/alginate scaffold demonstrated a method to promote the development of cartilage [145]. Scaffolds of decellularized cadaveric tendon seeded with ASCs also facilitated regeneration of tendon tissue through direct induction of tenocyte differentiation [146,147]. Pelvic floor reconstruction, specifically in the context of fascia regeneration, was also enhanced with ASC-seeded implants [148].

What are some of the factors of the scaffold that may regulate ASC activity? As stated before, the actual composition of the scaffold can weigh heavily on this point. Development of scaffolds of composite material mixed with decellularized adipose tissue demonstrated greater ASC viability and subsequent differentiation into adipogenic lineages [149]. These properties were also encouraged by seeding ASCs in layers of calcium alginate hydrogels [150]. Surface modification of a chitosan-based scaffold allowed for a push towards ASC differentiation into cardiac muscle [151]. Use of polyhydroxybutyrate / poly(hydroxybutyrate-co-hydroxyhexanoate) scaffolds enhanced cartilage development from seeded ASCs [152].

Several treatment conditions can encourage ASC seeding and integration into scaffolds. One demonstrated technique to improve adhesion of ASCs to non-woven poly(L-lactic acid) scaffolds is oxygen plasma treatment prior to seeding [153]. Culture conditions, specifically the media / serum, also can have an effect on initial density of ASC seeding of scaffolds [154]. These studies reveal that the exact material of the scaffold, any modifications to the biomaterial, and architecture will have to be tailored carefully to optimize the desired effects of the seeded ASCs. Therefore in future applications of ASCs to enhance the effectiveness of engineered tissues, one must consider how the scaffold can affect the activity and differentiation potential of ASCs, in addition to effects on vasculogenesis, which is largely affected by pore architecture.

Figure 4. Overview of demonstrated uses and subsequent benefits of ASC-seeded scaffolds in clinical contexts

2.8. FUTURE DIRECTIONS AND CONCLUSIONS

All of this established evidence points to a promising future for ASCs in tissue engineering. This is a highly versatile and readily available cell population that has repeatedly demonstrated characteristics favoring its use in this clinical context. Its multipotent properties, ability to secrete a variety of growth-inducing and immunomodulatory mediators (hence the term immunomodulatory secretome), and ability to integrate into existing scaffold materials point to its increased use in new biomaterials and artificial tissues.

However, much work remains before ASCs can become a feasible treatment modality. Current regulations restrict the breadth of ASC-based therapies such that cells must remain within the operating room with the patient. To date, if cells are brought outside the operating room to undergo culturing or engineering in a laboratory, they may not be replaced into a patient. Patient safety is of paramount concern, and *in vivo* animal studies as well as preliminary human safety trials must be conducted to open the doors to further testing. With strong evidence available regarding its benefits, ASC-based therapies must address these necessary safety concerns to open the

doors to further, but well-regulated, clinical studies. All benefits and challenges of ASCs are listed in Figure 5.

Positive	Negative
<ul style="list-style-type: none">• Found in multiple tissue sources• Easily isolatable in large numbers from lipoaspiration• Established culture protocols• Bioactive properties (immunomodulation, angiogenesis...)• Multipotent differentiation capacity• Incorporable into 3D synthetic scaffolds	<ul style="list-style-type: none">• Restrictive regulations for clinical use• Few <i>in vivo</i> human studies• Need to fully understand how to control ASC proliferation and differentiation

Figure 5. Benefits and challenges of using ASCs in clinical applications

In conclusion, the many challenges currently experienced by bioengineers, clinicians, and translational researchers in the effective *in vivo* application of biomaterials may be mitigated or solved by the effective addition to ASCs. However, further work remains to be done to fully characterize and describe their clinical potential and safety to achieve full clinical use. Although ASCs are largely limited to laboratory use and study at this point, as discoveries emerge and as regulations will change, these cells will offer an exciting new chapter in the field of regenerative medicine.

REFERENCES

1. R.F. Diegelmann, M.C. Evans. *Front. Biosci.* **9** (2004) 283–289.
2. B. Nilsson, K.N. Ekdahl, T.E. Mollnes, J.D. Lambris. *Mol. Immunol.* **44** (2007) 82–94.
3. J.M. Anderson, A. Rodriguez, D.T. Chang. *Semin. Immunol.* **20** (2008) 86–100.
4. K.S. Jones. *Semin. Immunol.* **20** (2008) 130–136.
5. F. Velard, J. Braux, J. Amedee, P. Laquerriere. *Acta Biomater.* **9** (2013) 4956–4963.
6. D.O. Freytes, L. Santambrogio, G. Vunjak-Novakovic. *Cells Tissues Organs* **195** (2012) 171–182.
7. J.M. Morehead, G.R. Holt. *Otolaryngol. Clin. North Am.* **27** (1994) 195–201.
8. P.M. Kou, J.E. Babensee. *J. Biomed. Mater. Res. A* **96** (2011) 239–260.
9. Y. Garcia, A. Breen, K. Burugapalli, P. Dockery, A. Pandit. *Biomaterials* **28** (2007) 175–186.
10. R.J. Love, K.S. Jones. *Crit. Rev. Biomed. Eng.* **37** (2009) 259–281.
11. E.E. Falco, M. Patel, J.P. Fisher. *Pharm. Res.* **25** (2008) 2348–2356.
12. N. Mokarram, R.V. Bellamkonda. *Ann. Biomed. Eng.* **42** (2014) 338–351.
13. W.J. Kao. *Biomaterials* **20** (1999) 2213–2221.
14. J.M. Morais, F. Papadimitrakopoulos, D.J. Burgess. *AAPS J.* **12** (2010) 188–196.
15. J.M. Gimble, B.A. Bunnell, T. Frazier, B. Rowan, F. Shah, C. Thomas-Porch, X. Wu. *Organogenesis* **9** (2013) 3–10.
16. M. Dominici, K. Le Blanc, I. Mueller, I. Slaper-Cortenbach, F. Marini, D. Krause, R. Deans, A. Keating, D.J. Prockop, E. Horwitz. *Cytotherapy* **8** (2006) 315–317.
17. A. Rahimzadeh, F.S. Tabatabaei Mirakabad, A. Movassaghpour, K. Shamsasenjan, S. Karimineko, M. Talebi, A. Shekari, V. Zeighamian, M. Gandomkar Ghalhar, A. Akbarzadeh. *Artif. Cells Nanomed. Biotechnol.* (2014) 1–12.
18. P. Bourin, B.A. Bunnell, L. Casteilla, M. Dominici, A.J. Katz, K.L. March, H. Redl, J.P. Rubin, K. Yoshimura, J.M. Gimble. *Cytotherapy* **15** (2013) 641–648.
19. H. Ning, G. Lin, T.F. Lue, C.S. Lin. *Differentiation* **74** (2006) 510–518.
20. Y.S. Choi, G.J. Disting, S. Stubbs, S. Arunothayaraj, X.L. Han, P. Collas, W.A. Morrison, R.J. Dille. *J. Cell. Mol. Med.* **14** (2010) 878–889.
21. P.A. Zuk, M. Zhu, H. Mizuno, J. Huang, J.W. Futrell, A.J. Katz, P. Benhaim, H.P. Lorenz, M.H. Hedrick. *Tissue Eng.* **7** (2001) 211–228.
22. M. Zhu, S. Heydarkhan-Hagvall, M. Hedrick, P. Benhaim, P. Zuk. *J. Vis. Exp.* (2013) e50585.
23. C.F. Markarian, G.Z. Frey, M.D. Silveira, E.M. Chem, A.R. Milani, P.B. Ely, A.P. Horn, N.B. Nardi, M. Camassola. *Biotechnol. Lett.* **36** (2014) 693–702.
24. B. Lindroos, S. Boucher, L. Chase, H. Kuokkanen, H. Huhtala, R. Haataja, M. Vemuri, R. Suuronen, S. Miettinen. *Cytotherapy* **11** (2009) 958–972.
25. W.U. Hassan, U. Greiser, W. Wang. *Wound Repair Regen.* **22** (2014) 313–325.
26. M. Cherubino, J.P. Rubin, N. Miljkovic, A. Kelmendi-Doko, K.G. Marra. *Ann. Plast. Surg.* **66** (2011) 210–215.
27. S.D. Zack-Williams, P.E. Butler, D.M. Kalaskar. *World J. Stem Cells* **7** (2015) 51–64.
28. D.A. Banyard, A.A. Salibian, A.D. Widgerow, G.R. Evans. *J. Cell. Mol. Med.* **19** (2015) 21–30.
29. M.H. Lim, W.K. Ong, S. Sugii. *Expert Rev. Mol. Med.* **16** (2014) e8.

30. E. Soleymaninejadian, K. Pramanik, E. Samadian. *Am. J. Reprod. Immunol.* **67** (2012) 1–8.
31. S.K. Kapur, A.J. Katz. *Biochimie* **95** (2013) 2222–2228.
32. M. Abumaree, M. Al Jumah, R.A. Pace, B. Kalionis. *Stem Cell Rev.* **8** (2012) 375–392.
33. Y.R. Kuo, C.C. Chen, S. Goto, Y.T. Huang, C.C. Tsai, M.Y. Yang. *Plast. Reconstr. Surg.* **134** (2014) 1213–1223.
34. S.M. Lee, S.C. Lee, S.J. Kim. *J. Surg. Res.* **188** (2014) 280–289.
35. H. Suga, J.P. Glotzbach, M. Sorkin, M.T. Longaker, G.C. Gurtner. *Ann. Plast. Surg.* **72** (2014) 234–241.
36. K. Kondo, S. Shintani, R. Shibata, H. Murakami, R. Murakami, M. Imaizumi, Y. Kitagawa, T. Murohara. *Arterioscler. Thromb. Vasc. Biol.* **29** (2009) 61–66.
37. S. Rohringer, P. Hofbauer, K.H. Schneider, A.-M. Husa, G. Feichtinger, A. Peterbauer-Scherb, H. Redl, W. Holnthoner. *Angiogenesis* **17** (2014) 921–933.
38. B.S. Park, K.A. Jang, J.H. Sung, J.S. Park, Y.H. Kwon, K.J. Kim, W.S. Kim. *Dermatol. Surg.* **34** (2008) 1323–1326.
39. H. Yagi, A. Soto-Gutierrez, B. Parekkadan, Y. Kitagawa, R.G. Tompkins, N. Kobayashi, M.L. Yarmush. *Cell Transplant* **19** (2010) 667–679.
40. S.H. Ranganath, O. Levy, M.S. Inamdar, J.M. Karp. *Cell. Stem Cell* **10** (2012) 244–258.
41. G. Ferrari, B.D. Cook, V. Terushkin, G. Pintucci, P. Mignatti. *J. Cell. Physiol.* **219** (2009) 449–458.
42. V. Machado Cde, P.D. Telles, I.L. Nascimento. *Rev. Bras. Hematol. Hemoter.* **35** (2013) 62–67.
43. S.M. Melief, J.J. Zwaginga, W.E. Fibbe, H. Roelofs. *Stem Cells Transl. Med.* **2** (2013) 455–463.
44. B. Puissant, C. Barreau, P. Bourin, C. Clavel, J. Corre, C. Bousquet, C. Taureau, B. Cousin, M. Abbal, P. Laharrague, L. Penicaud, L. Casteilla, A. Blancher. *Br. J. Haematol.* **129** (2005) 118–129.
45. J.M. Lee, J. Jung, H.J. Lee, K.J. Cho, S.G. Hwang, G.J. Kim. *Int. Immunopharmacol.* **13** (2012) 219–224.
46. A. Technau, K. Froelich, R. Hagen, N. Kleinsasser. *Cytotherapy* **13** (2011) 310–317.
47. N.L. Payne, A. Dantanarayana, G. Sun, L. Moussa, S. Caine, C. McDonald, D. Herszfeld, C.C.A. Bernard, C. Siatskas. *Cell. Adh. Migr.* **6** (2012) 179–189.
48. G.M. Spaggiari, H. Abdelrazik, F. Becchetti, L. Moretta. *Blood* **113** (2009) 6576–6583.
49. W. Zhang, W. Ge, C. Li, S. You, L. Liao, Q. Han, W. Deng, R.C. Zhao. *Stem Cells Dev.* **13** (2004) 263–271.
50. W. Peng, T. Gao, Z.L. Yang, S. Zhang, M.L. Ren, Z.G. Wang. *Cell. Immunol.* **278** (2012) 152–157.
51. S. Aggarwal, M.F. Pittenger. *Blood* **105** (2005) 1815–1822.
52. P. Anderson, L. Souza-Moreira, M. Morell, M. Caro, F. O'Valle, E. Gonzalez-Rey, M. Delgado. *Gut* **62** (2013) 1131–1141.
53. A. Mohammadzadeh, A.A. Pourfathollah, S. Shahrokhi, S.M. Hashemi, S.L. Moradi, M. Soleimani. *Int. Immunopharmacol.* **20** (2014) 316–321.
54. K. Lai, K. Zeng, F. Zeng, J. Wei, G. Tan. *Acta Biochim. Biophys. Sin. (Shanghai)* **43** (2011) 805–812.

55. K.S. Cho, H.J. Roh. *Curr. Stem Cell Res. Ther.* **5** (2010) 111–115.
56. J.H. Kim, Y.T. Lee, J.M. Hong, Y.I. Hwang. *Anat. Cell. Biol.* **46** (2013) 262–271.
57. R. Blazquez, F.M. Sanchez-Margallo, O. de la Rosa, W. Dalemans, V. Alvarez, R. Tarazona, J.G. Casado. *Front. Immunol.* **5** (2014) 556.
58. I. Rasmusson, O. Ringden, B. Sundberg, K. Le Blanc. *Transplantation* **76** (2003) 1208–1213.
59. C.D. Wan, R. Cheng, H.B. Wang, T. Liu. *Hepatobiliary Pancreat. Dis. Int.* **7** (2008) 29–33.
60. E. Gonzalez-Rey, M.A. Gonzalez, N. Varela, F. O'Valle, P. Hernandez-Cortes, L. Rico, D. Büscher, M. Delgado. *Ann. Rheum. Dis.* **69** (2010) 241–248.
61. M. Razmkhah, M. Jaberipour, N. Erfani, M. Habibagahi, A.R. Talei, A. Ghaderi. *Cell. Immunol.* **266** (2011) 116–122.
62. K. Le Blanc, O. Ringden. *J. Intern. Med.* **262** (2007) 509–525.
63. A.U. Engela, M.J. Hoogduijn, K. Boer, N.H.R. Litjens, M.G.H. Betjes, W. Weimar, C.C. Baan. *Clin. Exp. Immunol.* **173** (2013) 343–354.
64. T.P. Frazier, J.B. McLachlan, J.M. Gimble, H.A. Tucker, B.G. Rowan. *Stem Cells Dev.* **23** (2014) 968–977.
65. H.Y. Cheng, N. Ghetu, W.C. Huang, Y.L. Wang, C.G. Wallace, C.J. Wen, H.C. Chen, L.Y. Shih, C.F. Lin, S.M. Hwang, S.K. Liao, F.C. Wei. *Cytotherapy* **16** (2014) 369–380.
66. A. Augello, R. Tasso, S.M. Negrini, A. Amateis, F. Indiveri, R. Cancedda, G. Pennesi. *Eur. J. Immunol.* **35** (2005) 1482–1490.
67. S. Asari, S. Itakura, K. Ferreri, C.P. Liu, Y. Kuroda, F. Kandeel, Y. Mullen. *Exp. Hematol.* **37** (2009) 604–615.
68. P. Comoli, F. Ginevri, R. Maccario, M.A. Avanzini, M. Marconi, A. Groff, A. Cometa, M. Cioni, L. Porretti, W. Barberi, F. Frassoni, F. Locatelli. *Nephrol. Dial. Transplant.* **23** (2008) 1196–1202.
69. A. Corcione, F. Benvenuto, E. Ferretti, D. Giunti, V. Cappiello, F. Cazzanti, M. Risso, F. Gualandi, G.L. Mancardi, V. Pistoia, A. Uccelli. *Blood* **107** (2006) 367–372.
70. M. Franquesa, F.K. Mensah, R.T. Huizinga, T. Strini, L. Boon, E. Lombardo, O. DelaRosa, J.D. Laman, J.M. Grinyó, W. Weimar, M.G.H. Betjes, C.C. Baan, M.J. Hoogduijn. *Stem Cells* **33** (2015) 880–891.
71. G.M. Spaggiari, A. Capobianco, H. Abdelrazik, F. Becchetti, M.C. Mingari, L. Moretta. *Blood* **111** (2008) 1327–1333.
72. J.M. Ryan, F.P. Barry, J.M. Murphy, B.P. Mahon. *J. Inflamm. (Lond.)* **2** (2005) 8.
73. A. Ribeiro, P. Laranjeira, S. Mendes, I. Velada, C. Leite, P. Andrade, F. Santos, A. Henriques, M. Grãos, C.M.P. Cardoso, A. Martinho, M.L. Pais, C. Lobato da Silva, J. Cabral, H. Trindade, A. Paiva. *Stem Cell. Res. Ther.* **4** (2013) 125.
74. O. DelaRosa, B. Sánchez-Correa, S. Morgado, C. Ramírez, B. del Río, R. Menta, E. Lombardo, R. Tarazona, J.G. Casado. *Stem Cells Dev.* **21** (2012) 1333–1343.
75. M. Krampera, L. Cosmi, R. Angeli, A. Pasini, F. Liotta, A. Andreini, V. Santarasci, B. Mazzinghi, G. Pizzolo, F. Vinante, P. Romagnani, E. Maggi, S. Romagnani, F. Annunziato. *Stem Cells* **24** (2006) 386–398.
76. B. Kronsteiner, S. Wolbank, A. Peterbauer, C. Hackl, H. Redl, G.M. van, C. Gabriel. *Stem Cells Dev.* **20** (2011) 2115–2126.
77. J.L. Chan, K.C. Tang, A.P. Patel, L.M. Bonilla, N. Pierobon, N.M. Ponzio, P. Rameshwar. *Blood* **107** (2006) 4817–4824.

78. K. McIntosh, S. Zvonic, S. Garrett, J.B. Mitchell, Z.E. Floyd, L. Hammill, A. Kloster, Y. Di Halvorsen, J.P. Ting, R.W. Storms, B. Goh, G. Kilroy, X.Y. Wu, J. M. Gimble. *Stem Cells* **24** (2006) 1246–1253.
79. S. Wolbank, G. Stadler, A. Peterbauer, A. Gillich, M. Karbiener, B. Streubel, M. Wieser, H. Katinger, M. van Griensven, H. Redl, C. Gabriel, J. Grillari, R. Grillari-Voglauer. *Tissue Eng. Part A* **15** (2009) 1843–1854.
80. M.J. Crop, C.C. Baan, S.S. Korevaar, J.N.M. IJzermans, M. Pescatori, A.P. Stubbs, W.F. J van IJcken, M.H. Dahlke, E. Eggenhofer, W. Weimar, M.J. Hoogduijn. *Clin. Exp. Immunol.* **162** (2010) 474–486.
81. B.A. Scruggs, J.A. Semon, X. Zhang, S. Zhang, A.C. Bowles, A.C. Pandey, K.M. Imhof, A.V. Kalueff, J.M. Gimble, B.A. Bunnell. *Stem Cells Transl. Med.* **2** (2013) 797–807.
82. C. Menard, L. Pacelli, G. Bassi, J. Dulong, F. Bifari, I. Bezier, J. Zanoncello, M. Ricciardi, M. Latour, P. Bourin, H. Schrezenmeier, L. Sensebé, K. Tarte, M. Krampera. *Stem Cells Dev.* **22** (2013) 1789–1801.
83. X. Wang, C. Liu, S. Li, Y. Xu, P. Chen, Y. Liu, Q. Ding, W. Wahafu, B. Hong, M. Yang. *PLoS One* **10** (2015) e0118951.
84. P.A. Zuk, M. Zhu, P. Ashjian, D.A. De Ugarte, J.I. Huang, H. Mizuno, Z.C. Alfonso, J.K. Fraser, P. Benhaim, M.H. Hedrick. *Mol. Biol. Cell.* **13** (2002) 4279–4295.
85. A. Winter, S. Breit, D. Parsch, K. Benz, E. Steck, H. Hauner, R.M. Weber, V. Ewerbeck, W. Richter. *Arthritis Rheum.* **48** (2003) 418–429.
86. J.M. Gimble, A.J. Katz, B.A. Bunnell. *Circ. Res.* **100** (2007) 1249–1260.
87. K.M. Safford, K.C. Hicok, S.D. Safford, Y.D. Halvorsen, W.O. Wilkison, J.M. Gimble, H.E. Rice. *Biochem. Biophys. Res. Commun.* **294** (2002) 371–379.
88. T. Nieto-Miguel, S. Galindo, R. Reinoso, A. Corell, M. Martino, J.A. Pérez-Simón, M. Calonge. *Curr. Eye Res.* **38** (2013) 933–944.
89. R.H. Kim, S. Mehrzarin, M.K. Kang. *Dent. Clin. North Am.* **56** (2012) 651–675.
90. A.V. Vanikar, S.D. Dave, U.G. Thakkar, H.L. Trivedi. *Stem Cells Int.* (2010) 582382.
91. M. Peran, E. Lopez-Ruiz, L. Gonzalez-Herrera, M. Bustamante, A. Valenzuela, J.A. Marchal. *Cytotherapy* **15** (2013) 1541–1548.
92. K. Song, Z. Wang, W. Li, C. Zhang, M. Lim, T. Liu. *Appl. Biochem. Biotechnol.* **170** (2013) 459–470.
93. F. De Francesco, V. Tirino, V. Desiderio, G. Ferraro, F. D'Andrea, M. Giuliano, G. Libondi, G. Pirozzi, A. De Rosa, G. Papaccio. *PLoS One* **4** (2009) e6537.
94. R. Madonna, Y.J. Geng, R. De Caterina. *Arterioscler. Thromb. Vasc. Biol.* **29** (2009) 1723–1729.
95. V. Planat-Benard, J.S. Silvestre, B. Cousin, M. André, M. Nibbelink, R. Tamarat, M. Clergue, C. Manneville, C. Saillan-Barreau, M. Duriez, A. Tedgui, B. Levy, L. Pénicaud, L. Casteilla. *Circulation* **109** (2004) 656–663.
96. D.O. Traktuev, S. Merfeld-Clauss, J. Li, M. Kolonin, W. Arap, R. Pasqualini, B.H. Johnstone, K.L. March. *Circ. Res.* **102** (2008) 77–85.
97. D.O. Traktuev, D.N. Prater, S. Merfeld-Clauss, A.R. Sanjeevaiah, M.R. Saadatzaadeh, M. Murphy, B.H. Johnstone, D.A. Ingram, K.L. March. *Circ. Res.* **104** (2009) 1410–1420.
98. J.W. Kuhbier, V. Bucan, K. Reimers, S. Strauss, A. Lazaridis, S. Jahn, C. Radtke, P.M. Vogt. *Plast. Reconstr. Surg.* **134** (2014) 414–423.

99. F.L. Muehlberg, Y.H. Song, A. Krohn, S.P. Pinilla, L.H. Droll, X. Leng, M. Seidensticker, J. Ricke, A.M. Altman, E. Devarajan, W. Liu, R.B. Arlinghaus, E.U. Alt. *Carcinogenesis* **30** (2009) 589–597.
100. B. Cousin, E. Ravet, S. Poglio, F. De Toni, M. Bertuzzi, H. Lulka, I. Touil, M. André, J.L. Grolleau, J.M. Péron, J.P. Chavoïn, P. Bourin, L. Pénicaud, L. Casteilla, L. Buscail, P. Cordelier. *PLoS One* **4** (2009) e6278.
101. L. Kucerova, V. Altanerova, M. Matuskova, S. Tyciakova, C. Altaner. *Cancer Res.* **67** (2007) 6304–6313.
102. S.S. Tholpady, R.C. Ogle, A.J. Katz. *Curr. Opin. Organ Transplant* **14** (2009) 51–55.
103. Y.R. Kuo, C.C. Chen, S. Goto, I.-T. Lee, C.-W. Huang, C.-C. Tsai, C.-T. Wang, C.-L. Chen. *Plast. Reconstr. Surg.* **128** (2011) 661e–672e.
104. Y.R. Kuo, S. Goto, H.S. Shih, F.S. Wang, C.C. Lin, C.T. Wang, E.Y. Huang, C.L. Chen, F.C. Wei, X.X. Zheng, W.P. Lee. *Transplantation* **87** (2009) 1769–1777.
105. J.A. Plock, J.T. Schnider, M.G. Solari, X.X. Zheng, V.S. Gorantla. *Front. Immunol.* **4** (2013) 175.
106. M. Roemeling-van Rhijn, M. Khairoun, S.S. Korevaar, E. Lievers, D.G. Leuning, J.N.M. IJzermans, M.G.H. Betjes, P.G. Genever, C. van Kooten, H.J.W. de Fijter, T.J. Rabelink, C.C. Baan, W. Weimar, H. Roelofs, M.J. Hoogduijn, M.E. Reinders. *J. Stem Cell Res. Ther. Suppl* **6** (2013) 20780.
107. A.V. Vanikar, H.L. Trivedi, S.C. Gopal, A. Kumar, S.D. Dave. *Ren. Fail.* **36** (2014) 457–460.
108. B. Fang, Y.P. Song, L.M. Liao, Q. Han, R.C. Zhao. *Bone Marrow Transplant* **38** (2006) 389–390.
109. M.A. Gonzalez, E. Gonzalez-Rey, L. Rico, D. Buscher, M. Delgado. *Gastroenterology* **136** (2009) 978–989.
110. E. Gonzalez-Rey, P. Anderson, M.A. Gonzalez, L. Rico, D. Buscher, M. Delgado. *Gut* **58** (2009) 929–939.
111. B. Yamout, R. Hourani, H. Salti, W. Barada, T. El-Hajj, A. Al-Kutoubi, A. Herlopian, E.K. Baz, R. Mahfouz, R. Khalil-Hamdan, N.M. Kreidieh, M. El-Sabban, A. Bazarbachi. *J Neuroimmunol* **227** (2010) 185–189.
112. M. Mohyeddin Bonab, S. Yazdanbakhsh, J. Lotfi, K. Alimoghaddom, F. Talebian, F. Hooshmand, A. Ghavamzadeh, B. Nikbin. *Iran J. Immunol.* **4** (2007) 50–57.
113. Y. Zhou, J. Yuan, B. Zhou, A.J. Lee, A.J. Lee, M. Ghawji Jr, T.J. Yoo. *Immunology* **133** (2011) 133–140.
114. M.S. Piccinno, E. Veronesi, P. Loschi, M. Pignatti, A. Murgia, G. Grisendi, I. Castelli, Da. Bernabei, O. Candini, P. Conte, P. Paolucci, E. M. Horwitz, G. De Santis, L. Iughetti, M. Dominici. *Apoptosis* **18** (2013) 1274–1289.
115. S. Kim, K.A. Chang, J. Kim, H.-G. Park, J.C. Ra, H.-S. Kim, Y.-H. Suh. *PLoS One* **7** (2012) e45757.
116. B.M. PP, A.J. Pedro, A. Peterbauer, C. Gabriel, H. Redl, R.L. Reis. *J. Mater. Sci. Mater. Med.* **16** (2005) 1077–1085.
117. L.Y. Santiago, R.W. Nowak, J. Peter Rubin, K.G. Marra. *Biomaterials* **27** (2006) 2962–2969.
118. P. DiMuzio, L. Fischer, S. McIlhenny, C. DiMatteo, N. Golesorhki, D. Grabo, N. Tarola, A. Mericli, I. Shapiro, T. Tulenko. *Vascular* **14** (2006) 338–342.
119. L. Hong, I.A. Peptan, A. Colpan, J.L. Daw. *Cells Tissues Organs* **183** (2006) 133–140.

120. M.N. Helder, M. Knippenberg, J. Klein-Nulend, P.I. Wuisman, *Tissue Eng.* **13** (2007) 1799–1808.
121. C. Nie, D. Yang, S.F. Morris. *Med. Hypotheses* **72** (2009) 679–682.
122. M.M. McLaughlin, K.G. Marra, *Organogenesis* **9** (2013) 79–81.
123. Y.C. Lin, T. Grahovac, S.J. Oh, M. Ieraci, J.P. Rubin, K.G. Marra. *Acta Biomater.* **9** (2013) 5243–5250.
124. M.T. Lam, A. Nauta, N.P. Meyer, J.C. Wu, M.T. Longaker. *Tissue Eng. Part A* **19** (2013) 738–747.
125. Y. M. Kim, S.H. Oh, J.S. Choi, S. Lee, J.C. Ra, J.H. Lee, J.Y. Lim. *Laryngoscope* **124** (2014) E64–72.
126. H. Kim, K. Choi, O.K. Kweon, W.H. Kim, *J. Dermatol. Sci.* **68** (2012) 149–156.
127. J. Gu, N. Liu, X. Yang, Z. Feng, F. Qi. *Biomed. Mater.* **9** (2014) 035012.
128. S. Gomathysankar, A.S. Halim, N.S. Yaacob. *Arch. Plast. Surg.* **41** (2014) 452–457.
129. S.H. Hsu, P.S. Hsieh. *Wound Repair Regen* **23** (2015) 57–64.
130. Z. Lu, S.I. Roohani-Esfahani, G. Wang, H. Zreiqat. *Nanomedicine* **8** (2012) 507–515.
131. K. Choi, B.J. Kang, H. Kim, S. Lee, S. Bae, O.K. Kweon, W.H. Kim. *J. Biomed. Mater. Res. B Appl. Biomater.* **101** (2013) 919–928.
132. H.A. Declercq, T. Desmet, P. Dubruel, M.J. Cornelissen. *Tissue Eng. Part A* **20** (2014) 434–444.
133. W. Wan, S. Zhang, L. Ge, Q. Li, X. Fang, Q. Yuan, W. Zhong, J. Ouyang, M. Xing. *Int. J. Nanomedicine* **10** (2015) 1273–1290.
134. S. Liu, H. Zhang, X. Zhang, X.J. Zhang, W. Lu, X.H. Huang, H. Xie, J. Zhou, W.H. Wang, Y.J. Zhang, Y. Liu, Z.H. Deng, Y. Jin. *Tissue Eng. Part A* **17** (2011) 725–739.
135. M.A. Meruane, M. Rojas, K. Marcelain. *Plast. Reconstr. Surg.* **130** (2012) 53–63.
136. T.S. Iyyanki, L.W. Dunne, Q. Zhang, J. Hubenak, K.C. Turza, C.E. Butler, *Tissue Eng. Part A* **21** (2015) 475–485.
137. I. Komatsu, J. Yang, Y. Zhang, L.S. Levin, D. Erdmann, B. Klitzman, S.T. Hollenbeck. *J. Biomed. Mater. Res. A* **101** (2013) 2939–2947.
138. M.W. Laschke, T.E. Schank, C. Scheuer, S. Kleer, S. Schuler, W. Metzger, D. Eglin, M. Alini, M.D. Menger. *Acta Biomater.* **9** (2013) 6876–6884.
139. K. Wang, L.Y. Yu, L.Y. Jiang, H.B. Wang, C.Y. Wang, Y. Luo. *Acta Biomater.* **15** (2015) 65–76.
140. G. Lin, M. Albersen, A.M. Harraz, T.M. Fandel, M. Garcia, M.H. McGrath, B.R. Konety, T.F. Lue, C.S. Lin. *Urology* **77** (2011) 1509 e1501–e1508.
141. Y.Y. Hsueh, Y.L. Chiang, C.C. Wu, S.C. Lin. *Cells Tissues Organs* **196** (2012) 117–128.
142. S. Gao, P. Zhao, C. Lin, Y.X. Sun, Y.L. Wang, Z.C. Zhou, D.J. Yang, X.L. Wang, H.Z. Xu, F. Zhou, L.M. Cao, W. Zhou, K. Ning, X. Chen, J. Xu. *Tissue Eng. Part A* **20** (2014) 1271–1284.
143. C.C. Shen, Y.C. Yang, B.S. Liu. *J. Biomed. Mater. Res. A* **100** (2012) 48–63.
144. L. Ma, Y. Yang, S.C. Sikka, P.J. Kadowitz, L.J. Ignarro, A.B. Abdel-Mageed, W.J.G. Hellstrom. *Proc. Natl. Acad. Sci. U S A* **109** (2012) 2090–2095.
145. X.B. Jin, Y.S. Sun, K. Zhang, J. Wang, T.P. Shi, X.D. Ju, S.Q. Lou. *J. Biomed. Mater. Res. A* **86** (2008) 1077–1087.
146. A. Kraus, C. Woon, S. Raghavan, K. Megerle, H. Pham, J. Chang. *Plast. Reconstr. Surg.* **132** (2013) 754e–766e.

147. T. Martinello, I. Bronzini, A. Volpin, V. Vindigni, L. Maccatrozzo, G. Caporale, F. Bassetto, M. Patruno. *J Tissue Eng Regen Med* **8** (2014) 612–619.
148. M.J. Hung, M.C. Wen, Y.T. Huang, G.D. Chen, M.M. Chou, V.C. Yang. *J. Formos. Med. Asso.* **113** (2014) 704–715.
149. H.K. Cheung, T.T. Han, D.M. Marecak, J.F. Watkins, B.G. Amsden, L.E. Flynn. *Biomaterials* **35** (2014) 1914–1923.
150. B. Galateanu, D. Dimonie, E. Vasile, S. Nae, A. Cimpean, M. Costache. *BMC Biotechnol.* **12** (2012) 35.
151. B.H. Liu, H.Y. Yeh, Y.C. Lin, M.H. Wang, D.C. Chen, B.H. Lee, S.H. Hsu. *Biores. Open Access* **2** (2013) 28–39.
152. C. Ye, P. Hu, M.X. Ma, Y. Xiang, R.G. Liu, X.W. Shang. *Biomaterials* **30** (2009) 4401–4406.
153. A.D. Hanson, M.E. Wall, B. Pourdeyhimi, E.G. Lobo. *J. Biomater. Sci. Polym. Ed.* **18** (2007) 1387–1400.
154. P. Lund, L. Pilgaard, M. Duroux, T. Fink, V. Zachar. *Cytotherapy* **11** (2009) 189–197.

BIODEGRADABLE POLYMERIC NANOMATERIALS

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3.1. INTRODUCTION

Polymeric nanomaterials are materials ranging from 1–1000 nm in size in at least one dimension and are associated with having a larger surface area than their larger-scale material cousins. Their peculiar nanosized structure confers different properties to the nanomaterials in comparison to bulk polymers. The main advantages of polymeric nanomaterials are that they can be designed with a variety of compositions, sizes and possible morphologies, surface chemistries, and surface topographies [1]. In recent years, polymeric nanomaterials have attracted increased attention as they can be developed to exhibit a variety of unique properties for a wide range of applications to meet market needs. Indeed, if recent trends continue, polymeric nanomaterials will emerge as the fastest growing domain in the near future [2].

Biodegradable polymers contain functional groups that may be cleaved *in vivo* leading to the fragmentation of the polymeric chains and therefore to their solubilisation. Biodegradable polymeric materials are used when it is necessary for a medical device to degrade over the time and these materials must fulfil some prerequisites to be considered useful for such purposes: do not impart inflammatory or toxic responses upon implantation, have an acceptable shelf life, possess a degradation rate in physiological conditions in accordance with the intended application, have appropriate initial mechanical properties with pertinent variations over the material degradation period, generate non-toxic degradation products that may be metabolised and cleared from the body, and have appropriate processability and sterisability for the intended application [3].

Biodegradable nanomaterials can be divided into four categories: spheres, hydrogels, micelles and fibres. They have been employed for a wide variety of applications in the biomedical field such as drug or biomolecular delivery systems, vaccines, scaffolds for tissue engineering and even wound dressings [4,5]. As biodegradable nanoparticles are digested internally and cleared from the body, they are often chosen over their non-biodegradable counterparts. Moreover, their application for the delivery of drugs and bioactive molecules has been shown to improve the bioavailability, the solubility and the retention time of the encapsulated systems. Indeed, with non-biodegradable drug carriers, the release of therapeutic agents may be poor due to their high molecular weights which can impede diffusion through the polymer matrix. Furthermore, when using biodegradable systems, the interaction with the biological environment is also increased leading to better tissue absorption and intracellular penetration. Therefore, the comparative therapeutic index is increased with enhanced tolerability and low toxicity risks [6]. In tissue engineering applications, scaffolds based on biodegradable nanofibres offer several advantages, such as a large surface area-to-volume ratio, high and

tunable porosity, malleability to facilitate a wide variety of shapes, superior mechanical properties compared to regular fibres, and finally high axial strength combined with high flexibility. Moreover, their morphologies closely match the architecture of natural tissue extracellular matrix, since nanofibres possess the same dimensions as collagen or cellular cytoskeleton fibres. Therefore, nanofibre-based scaffolds enhance biological activity resulting in better cell attachment, proliferation and maintenance of phenotypic expression [7].

The first section of this chapter presents a background of the biodegradable polymers used in medical applications. Then, the development of nanofibres and nanofibrous scaffolds as well as nanoparticles, with a view to medical applications, is explored. Finally, the surface modifications applied to such nanomaterials are covered in more detail.

3.2. BIODEGRADABLE POLYMERS

Biodegradable polymers are divided into two categories. Synthetic degradable polymers have the advantage of not presenting immunogenicity, being synthesised with reliable sources, and being manufactured to obtain predictable properties. They mostly degrade hydrolytically since they contain hydrolytically labile chemical bonds in their backbone. Their degradation rates are influenced by the polymer molecular weight, morphology, crystallinity, as well as the device size and shape [8]. Natural polymers share similar traits with extracellular matrix polysaccharides or proteins. They can be recognised by the biological environment, may avoid toxicity issues, and may aid in the attachment, proliferation and differentiation of cells since they contain biofunctional molecules. However, natural polymers are complex and are difficult to purify and characterise, which in-turn makes them difficult to obtain as uniform raw materials. Moreover, natural polymers are enzymatically degraded and their degradation rates, ranging from a few hours to 6 months, are not easily controlled since enzymatic activity can differ between hosts [9]. The chemical structures of some biodegradable polymers, both natural and synthetic, that are widely used to develop nanomaterials are given in Figure 1.

Figure 1. Chemical structures of synthetic and natural biodegradable polymers that are commonly used to develop nanomaterials

3.2.1. Aliphatic polyesters

Aliphatic polyesters, such as polyglycolide (PGA), polylactide (PLA), their copolymers poly(D,L-lactide-*co*-glycolide) (PLGA), and poly(ϵ -caprolactone) (PCL), *etc.*, have been widely used for biomedical applications due to their diversity, synthetic versatility, and ease of degradation [3]. They are generally synthesised at high temperatures by ring-opening polymerisation of the corresponding cyclic monomers. They degrade by hydrolysis of the ester linkages along the backbone producing biodegradable metabolite monomers that can be resorbed *via* metabolic pathways. Their degradation kinetics depend on various factors such as the polymer nature, the monomeric composition, molecular weight, conformation, and physicochemical characteristics [10].

PGA is a highly crystalline polymer that exhibits high melting and glass transition temperatures ($T_m = 225\text{--}233\text{ }^\circ\text{C}$, $T_g = 35\text{--}45\text{ }^\circ\text{C}$). It degrades fairly rapidly *in vivo* (1–12 months) and its degradation results in the production of glycolic acid that, at high concentrations, lowers the pH of the surrounding tissue and may cause inflammation [9]. PLA is a relatively hard semi-crystalline polymer poly(L-lactide) (PLLA) with a high melting temperature ($T_m = 170\text{--}200\text{ }^\circ\text{C}$, $T_g = 55\text{--}65\text{ }^\circ\text{C}$) when synthesised with L-lactide, or an amorphous polymer poly(D,L-lactide) (PDLLA) when obtained with D,L-lactide. PDLLA has only a glass transition temperature of around $55\text{ }^\circ\text{C}$ and therefore has a lower tensile strength in comparison to PLLA. Moreover, PLLA degrades very slowly (5 months–5 years), while PDLLA has an intermediate degradation rate (12–16 months) [11]. Consequently, PLGA made of various compositions of D,L-lactide and glycolide monomers, is one of the most successfully used biodegradable polymers in the development of nanomaterials [6]. Indeed, the mechanical properties and the degradation times can be modulated with the lactide/glycolide ratio. Moreover, PLGA degrades into lactic acid and glycolic acid that are metabolised by the body through the Krebs cycle. As a consequence, the use of PLGA nanosystems is associated with minimal systemic toxicity [12]. The US Food and Drug Administration (USFDA) have approved the use of PLGA for human use and nanomedicines. PLGA is soluble in a wide range of solvents and various therapeutic agents have been successfully encapsulated into or adsorbed onto PLGA nanoparticles [6].

PCL is a semi-crystalline polyester with a low glass transition temperature that makes it semi-rigid at room temperature ($T_m = 58\text{--}65\text{ }^\circ\text{C}$, $T_g = -65\text{ to } -60\text{ }^\circ\text{C}$). Therefore, PCL may be processed to obtain various shapes, such as spheres, fibres, porous materials, *etc.*, as PCL shows good organic solvent solubility and an innate malleability. Moreover, PCL may be compatibly blended with a wide range of other polymers and is also used as a soft block in polyurethane formulations [13]. PCL degrades slowly (2–3 years) in comparison with the other polyesters and is especially interesting for the preparation of long-term drug delivery systems, such as one-year implantable contraceptives [14]. PCL

degrades by hydrolysis leading to low-concentrations of caproic acid that does not cause significant negative reactions in the surrounding tissues, and is completely metabolised since caproic acid enters the citric acid cycle. PCL also degrades through enzymatic attack. The bioactivity of PCL is well documented and it is approved by the USFDA for the formulation of nanoparticles [15,16]. Various molecules have been successfully incorporated into PCL nanoparticles/nanofibres to increase their therapeutic value [6]. PCL-based nanofibres have also widely been developed as scaffolds for tissue engineering [16,17].

Poly(3-hydroxybutyrate) (PHB) is the simplest member of the poly(3-hydroxyalkanoate) family. This natural polyester can be produced biotechnologically leading to a high semi-crystalline polymer ($T_m = 168-182\text{ }^{\circ}\text{C}$, $T_g = 1-15\text{ }^{\circ}\text{C}$). PHB could be used for various applications in the biomedical field due to its biocompatibility and biodegradability, as well as the non-cytotoxicity of its metabolic products [18]. Indeed, PHB degradation leads to D-(-)-3-hydroxy-butyric acid, which is a normal constituent of blood. Although PHB possesses a chemical structure very similar to the synthetic biodegradable polyesters, it degrades very slowly due to its high crystallinity. Therefore, PHB nanoparticles present great potential as depot devices [19]. To reduce its crystallinity and result in materials with better processability, as well as to increase its degradation rate, PHB is often copolymerised with 3-hydroxyvaleric acid (HV). PHB and P(HB-HV) may find applications as tissue engineering scaffolds and drug delivery nanoparticles [20].

3.2.2. Other synthetic biodegradable polymers

Polyanhydrides are the most widely investigated biodegradable polymers. Indeed, anhydride bonds (CO-O-CO) are highly sensitive and degrade through hydrolysis into corresponding dicarboxylic acids. The degradation occurs by a surface erosion mechanism, which allows for the release of encapsulated drugs at constant and slow rates. Furthermore, the chemical composition of polyanhydrides can be customised to develop materials with a wide range of degradation kinetics. Aliphatic linear polyanhydrides degrade within a few days, while polyanhydrides containing aromatic groups take longer to degrade, as much as a year [21]. As a consequence, it is possible to control the drug release rate as well as the release pattern. Moreover, polyanhydrides possess a hydrophobic backbone that prevents water penetration which protects the encapsulated drugs from hydrolysis [22].

Aliphatic polycarbonates, such as poly(trimethylene carbonate) (PTMC), are elastomeric polymers with excellent flexibility and softness that make them easy to process. Moreover, sensitive drugs can be encapsulated into polycarbonate-based nanoparticles under mild conditions. PTMC degrades slowly (> 1 year) by surface erosion and advantageously does not produce acidic degradation products. Indeed, PTMC degradation leads to

1,3-propanediol and carbonic acid [13]. PTMC is generally copolymerised with other cyclic lactones and some of the copolymers have been commercialised and are approved by the USFDA for clinical applications [23].

Polyphosphazenes are hybrid organic-inorganic polymers that contain a phosphorus-nitrogen backbone and organic or organometallic side groups that are attached to the phosphorus atoms, which dictate the polymer properties. A huge selection of substituents may be easily introduced by common organic chemistry and the inorganic backbone can degrade by hydrolysis with rates ranging from a few hours to years, depending on the side groups. The degradation leads to neutral products and may have a pH buffering effect when combined with polyesters [24]. Polyphosphazenes are flexible polymers with good processability and they may participate in noncovalent bonding which in-turn may create new interfacial interactions with biological systems. Consequently, the unique structural diversity of biodegradable polyphosphazenes associated with their potential multifunctionality should allow for the design of nanomaterials with superior biological characteristics [25]. Polyphosphazenes-based nanofibres and nanoparticles show significant promise in drug delivery and tissue engineering applications. Interestingly, organometallic derivatives of polyphosphazenes combine the electronic properties associated with the transition metal anchored to the side groups and the processing advantages of organic polymers. Polyphosphazenes may also be used as coatings on hydrophilic superparamagnetic nanoparticles in order to enhance their water dispersibility and colloidal and chemical stability [26].

Other synthetic biodegradable polymers are also susceptible to hydrolysis. Poly(ortho esters), poly(*p*-dioxanone), poly(propylene fumarate), and poly(ester urethanes) have been used on their own and as copolymers in biomedical applications [27].

3.2.3. Polysaccharides

Polysaccharides are found in numerous organisms, such as in mammals, insects, marine organisms and plants. They are the homopolymers or copolymers of monosaccharides, and are very diverse in their chemical structure and composition, molecular weight, and ionic character, leading to various biological activities. Polysaccharides have been widely used in biomedical applications since they are non-toxic, possess a high content of functional groups that can be easily chemically modified, and have excellent properties. Polysaccharide-based biomaterials can be designed as self-assembled micelles, cross-linked nanogels, and fibrous meshes. Accordingly, they are employed for various nanomedicine applications such as in drug delivery carriers, biosensors and tissue engineering scaffolds [28].

Chitosan is a modified polymer prepared by the partial *N*-deacetylation of chitin, which is one of the most abundant natural polymers and found in the

exoskeletons of insects and arthropods. Its chemical and physical properties depend on its molecular weight, degree of deacetylation, and the distribution of the acetyl groups in the backbone. Chitosan is easier to develop as a biomaterial than chitin due to its better solubility in water and organic solvents. Chitosan is a cationic polymer that possesses polyelectrolyte behaviour and may complex with various negatively-charged biomolecules which enhances its biological activity and makes it a very effective mucoadhesive. Furthermore, it is capable of chelating various metal ions [7]. The structure of chitosan is similar to glycosaminoglycans present in the human body, and it has been shown to elicit a minimal foreign body response and to have a stimulatory influence on immune cells, which may stimulate wound healing processes [3]. Moreover, the cationic structure of chitosan also confers antimicrobial properties, cellular binding capabilities, hemostatic properties, as well as anti-bacterial and anti-fungal properties [29]. Lysozyme and chitosanase degrade chitosan into glucosamine and the *in vivo* degradation rates range from between weeks to 6 months, depending on the degree of deacetylation [30].

Alginates are unbranched copolymers of β -D-mannuronic acid (M) and α -L-guluronic acid (G), existing as mixed salts of calcium, magnesium, sodium and potassium. They are found in brown algae and their physicochemical properties depend on the proportion and the distribution of M and G monomers which are intrinsically linked to the source origin [31]. Alginates do not enzymatically degrade *in vivo* since alginate lyases are found in *algae* and marine microorganisms, and they do not possess bioactive sequences which may be recognised by cells [30]. However, alginates are abundant and are relatively low in cost. They show excellent biocompatibility, low toxicity, and non-immunogenicity. They also possess high functionality and they may easily form hydrogels through simple gelation with divalent cations [29]. Moreover, alginates can electrostatically interact with oppositely charged chitosan and poly(L-lysine) to develop hydrophilic nanocarriers with high potential as vectors in biomedical and pharmaceutical applications [32,33].

Dextran is produced from sucrose through *bacteria* or yeast fermentation. It consists essentially of α -1,6 linked glucopyranoside residues with a small percentage of α -1,3 linked residues. The length and arrangement of branches differ depending on the enzyme's bacterial source. Dextran is cleaved by microbial dextranases and slowly degrades in comparison with other polysaccharides. Due to its high content of hydroxylic groups, dextran may be easily functionalised [34]. Additionally, it is a natural analogue of poly(ethylene glycol) (PEG) and therefore dextran derivatives are used in a wide range of biomedical applications because of their excellent solubility in aqueous solutions, biocompatibility and nonfouling properties. Dextran-based hydrogels can also be formed by physical or chemical crosslinking. Recently, dextran-based nanogels have been developed to act as cell tracking probes for advanced *in vivo* imaging techniques [35].

3.2.4. Glycosaminoglycans

Glycosaminoglycans are linear, anionic, naturally occurring polysaccharides primarily composed of glucuronic acid, iduronic acid and *N*-acetylgalactosamine with variable sulfation patterns, which is critical for biological activities. Indeed, the sulfation content plays a major role in glycosaminoglycan-protein binding. As the synthetic development of glycopolymers with high reproducibility and yield has yet to be achieved, the use of natural glycosaminoglycans is of interest for the design of bioactive biomaterials. Heparin is one of the most studied glycosaminoglycans due to its high negative charge density which can be attributed to the presence of approximately 2.7 sulfate groups per disaccharide unit. Such high negative charge density enables interactions with several proteins. Heparin has mainly been used as a surface coating to functionalise a variety of nanomaterials [36].

Hyaluronic acid (HA) is the most commonly used carbohydrate-based natural polymer in tissue engineering. It is composed of D-glucuronic acid and *N*-acetyl-D-glucosamine. HA is the only non-sulfated glycosaminoglycan and in contrast to other glycosaminoglycans, it does not covalently bond to proteins. HA is synthesised at the inner wall of the plasma membrane and is present in the extracellular matrix, connective tissues and synovial fluids. It is the most abundant natural polymer present in the human body and more than 50 % is found in the skin, lung and intestine. Commercial HA is produced from animal tissues and microbial fermentations [37]. HA can be obtained in a wide range of molecular weights, up to 10^7 g mol⁻¹, which allows HA to assume a variety of roles within the body. Indeed, high-molecular-weight HA is considered anti-angiogenic and non-immunogenic and plays a role in maintaining cell integrity, while low-molecular-weight HA is considered inflammatory, immuno-stimulatory and angiogenic and induces receptor-mediated intracellular signalling. *In vivo*, HA is enzymatically degraded by hyaluronidase, β -D-glucuronidase, and β -*N*-acetyl-hexosaminidase at a high degradation rate (from hours to 1 month). HA degradation leads to glucuronic acid and *N*-acetylglucosamine, which are finally metabolised to carbon dioxide, water and urea [30,38]. Because of its excellent biocompatibility and physicochemical properties, HA has been widely used in wound dressings, drug delivery applications and for tissue engineering including cartilage, liver, vascular, dermal, ophthalmic and nerve repair or regeneration. However, the commercially available HA homopolymer is too mechanically weak to be used as a supportive scaffold and its half-life is too short for long-term clinical applications. As a consequence, HA is often chemically modified or cross-linked to allow for the development of nanofibres, nanogels or self-assembled nanoparticles [29,37,39]. Moreover, it was demonstrated that when sulfur-modified, HA shows a higher binding affinity to growth factors than heparin [36].

3.2.5. Proteins

Proteins are essentially high-molecular-weight amino acid polymers arranged in a three-dimensional folded structure that are naturally degraded by a wide range of proteases. Proteins display a wide range of biological functions in nature which has motivated a significant amount of interest in the development of protein-based biomaterials. Collagen accounts for about a fourth of the total protein content of the human body. It is a fibrous protein that maintains the structural integrity of the extracellular matrix in tissues. Its primary structure is a polypeptide chain composed of repeating triplets of glycine-X-Y, where X and Y are typically proline and hydroxyproline. There are 28 types of collagen molecules and collagen type I (present in the skin, tendons and bones), represents 90 % of all collagen types [40]. Collagen-based nanofibres have been applied for wound dressings and preliminary vascular tissue engineering. However, commercial sources of collagen type I are generally derived from rat tail, bovine dermis, or human placenta. Therefore, recombinant systems have been developed due to the risks associated with infectious disease transmission from allogenic or xenogenic materials, the potential for immunogenicity, as well as the high cost of purification, quality concerns, and product homogeneity for mass production [30].

Gelatin is obtained by the denaturation and physicochemical degradation of collagen and its properties depend on the production process. Gelatin consists of 19 amino acids and is arranged in single-stranded molecules. It possesses similar hemostatic properties to its collagen precursor and is enzymatically degraded by collagenases [41]. Gelatin is soluble in aqueous solutions; however it possesses cationic and anionic groups coupled with strong hydrogen binding ability, which can make fibre-forming a challenge. Many molecules have been successfully encapsulated into gelatin-based nanoparticles, and the mechanical stability of nanofibre-based scaffolds may be enhanced by chemical crosslinking with various agents [6,40].

Silk is a natural protein mainly produced by silkworms and spiders. Silk fibre possesses a crystallised and compact structure consisting of hydrophobic fibroin as the core protein coated by hydrophilic sericin, which maintains the physical structure of fibroin. The presence of sericin is the main concern in terms of usefulness for biomedical applications, since it is associated with hypersensitivity reactions and poor biocompatibility. When sericin is removed, or absent, like in silk fibres from spiders, the immune response is similar to that of other biomaterials [30]. Fibroin is highly biocompatible, biodegradable, induces a limited inflammatory response, and possesses excellent mechanical properties. Moreover, silk-based biomaterials have high thermal stability over a wide range of temperatures (up to 250 °C) and therefore they can be sterilised by autoclave. Silk degradation occurs through enzymatic proteolysis from enzymes such as chymotrypsin, actinase, and carboxylase. The degradation rate *in vivo* can be tailored from months to years based on many

factors, such as the silk processing conditions and the physical characteristics of the material. Silk-based biomaterials have been widely processed as nanofibre-based scaffolds for tissue engineering applications and wound dressings [40,42].

3.3. POLYMERIC NANOFIBRES AND NANOFIBROUS SCAFFOLDS

Polymeric nanofibres have been developed for a variety of applications. In the medical field, the market of nanofibre-based materials is very nascent and therefore research in this area is rapidly expanding [43]. The potential medical applications are in the development of scaffolds for tissue engineering, carriers of bioactive compounds and cells, and in wound dressings. Additionally, in-terms of positively promoting cell-polymeric matrix interactions, the high surface area of the nanostructured, nanofibrous scaffolds allows for oxygen permeability and provides sufficient space for nutrient and waste exchange [44]. Moreover, in wound dressings, fluid accumulation at the wound site is limited and the material pore size prevents bacterial penetration. When used as carriers, nanofibrous materials may offer site-specific delivery of multiple drugs, genes and growth factors. Furthermore, their morphology and porosity can be modulated to control the molecule release profile [45]. Finally, the nanofibre-based scaffold surface can easily be functionalised by chemical or physical methods in order to carry various functionalities.

3.3.1. Fabrication

As illustrated in Figure 2, three main techniques are used to fabricate nanofibres based on biodegradable polymers: electrospinning, thermally-induced phase separation and self-assembly.

Figure 2. Main techniques used for the development of polymeric nanofibres

Among the three, electrospinning is the most widely studied technique. It is a simple, cost-effective, robust, and versatile process capable of producing polymeric fibres from a variety of polymer melts and solutions, with nanoscale diameters ranging from 50–1000 nm or greater. The electrospinning setup includes a high voltage power supply between a polymer solution or melt reservoir and a grounded collector (Figure 2). Due to the electric field, the polymer solution surface at the end of the spinneret is electrically charged, with even charge distribution over the surface. Under the influence of electrostatic interactions, an increase in the electrical potential leads to a drop distortion into a so-called “Taylor cone”. When the applied electric field reaches a critical value, the repulsive electrical forces overcome the surface tension forces and a jet is ejected continuously from the tip of the Taylor cone. Eventually, the electrified jet is attracted by the grounded collectors of opposite polarity placed under the spinneret. The space between the spinneret tip and the collector allows for solvent evaporation and therefore a solid polymeric non-woven fibrous matrix is deposited on the surface of the collector [46]. Conventional electrospinning produces randomly oriented nanofibres; however, aligned electrospun fibres (uniaxially aligned, radially aligned or in a wavy form) can also be obtained when using rotating and dual collectors or by manipulating the electrical field. Finally, the flat matrix may be stacked, folded, wound and twisted to fabricate three dimensional scaffolds with various shapes such as sheets, tubes or threads [47]. The morphology of the fibrous matrix is influenced and controlled by the polymeric solution parameters (viscosity, conductivity, surface tension), processing parameters (electric field, distance between the spinneret and the collector, flow rate and spinneret diameter) as well as ambient parameters (temperature, humidity). Indeed, the polymer nature determines the rate of degradation, while the solution and processing parameters determine the nanofibre diameter and the amount of polymeric beads that may be formed along the fibre [5].

Thermally-induced phase separation (TIPS) is a relatively simple procedure with very minimal requirements in terms of equipment. The TIPS approach allows for great processing flexibility with overall shape and pore structure control, as well as allowing for the design of extracellular matrix-like nanofibres ranging from 50–500 nm. Moreover, TIPS can be combined with other techniques to create simultaneous nano- and macro- architectures through the formation of macroporosity in the nanofibrous matrix, and to fabricate nanofibrous hollow microspheres without the need for a template [48-50]. The TIPS method involves five basic steps: polymer dissolution, liquid-liquid phase separation process, polymer gelation, solvent extraction, and eventually freezing and freeze-drying under vacuum (Figure 2). TIPS is based on the thermodynamic instability of a homogenous solution of polymer in solvent which, by cooling the solution below the polymer glass transition temperature, will spontaneously separate into two phases: polymer-rich and polymer-lean phases. Upon extraction and freeze drying, the polymer-rich

phase solidifies to form the polymer skeleton and eventually leaves behind a solid polymeric scaffold whose morphology is affected by processing variables such as the polymer nature, concentration, solvent, and temperature. Therefore under the right conditions, nanofibrous scaffolds may be developed. Actually, gelation is the step that controls the porous morphology since low gelation temperature leads to nanoscale-fibre scaffolds, whereas high gelation temperature leads to a platelet-like structure due to crystal nucleation and growth. However, the fibre average diameter is not significantly affected by gelation conditions or polymer concentration [5].

Self-assembly involves the spontaneous organisation of individual molecules into an ordered structure or pattern through non-covalent interactions such as hydrogen bonding, van der Waals forces, electrostatic forces, or hydrophobic forces (Figure 2). The goal of self-assembly is the formation of thermally stable protein-like molecular architectures, through the creation of nanofibres from synthesised small molecules and oligopeptides with a well-defined chemistry. For instance, oligopeptides consist of alternating hydrophilic and hydrophobic amino acids forming stable β -sheet structures. When an aqueous peptide solution is added to a physiological salt-containing solution, β -sheets pack together to form double-layered β -sheet nanofibres, without the need for temperature changes [51]. Self-assembly is a simple process for nanofibre fabrication. It can also be used to easily encapsulate cells in a hydrogel and can be used in an injectable form for *in situ* scaffold formation. Control of self-assembly in the design of nanofibres is carried out by switching the pH, through the introduction of divalent ions, and by varying the temperature and the concentration. However, self-assembly is limited by the choice of molecules whose synthesis is time-consuming and quite expensive. Self-assembly also leads to nanofibres in a gel-form, while electrospinning and TIPS give nanofibres in a dehydrated-form. Moreover, the self-assembly process does not easily allow for the control of pore size and shape within the hydrogel, and leads to small fibre diameters in comparison to those fabricated by other techniques. Indeed, individual fibre diameters are around 10–20 nm, and fibre lengths can only reach several micrometers [49].

3.3.2. Properties

The process and the processing parameters have a significant effect on the geometric properties of the nanofibres obtained, which determine the physical and mechanical properties of the polymeric materials. Some examples of polymers and solvents used for designing nanofibres by electrospinning and TIPS, associated to the fibre diameters, are given in Table 1.

Table 1. Examples of nanofibre diameters in correlation with solvent, polymer nature and concentration, obtained by electrospinning and TIPS

Polymer	Solvent ^a	Conc. ^b (w/v%)	FD ^c (nm)	FL/FD ratio ^d	Ref.
<i>Electrospinning</i>					
PCL	CHCl ₃	10	4173	–	[55]
	FA/CHCl ₃	10	222		
	FA/AA	10	266		
PDLLA	TFE	20.8	750	–	[70]
PCL/PLLA	CHCl ₃ /MeOH	8–11	347–1340	–	[54]
PCL/PTMC	DCM/DMF	8	203–302	–	[66]
PHB	CHCl ₃ /DMF	14	950	–	[18]
P(HB-HV)	CHCl ₃	18	571	–	[71]
Polyphosphazene	CHCl ₃	7	1200	–	[72]
Chitin	HFIP	6	110	–	[73]
Chitosan	H ₂ O/AA	7	40–130	–	[73]
Alginate/PEO	H ₂ O	10.6	80–300	–	[74]
Dextran	H ₂ O	75	186	–	[75]
HA	HCl	1.3	49–74	–	[76]
Collagen	HFIP	8	100–1200	–	[63]
Gelatin	H ₂ O/AA	10	110	–	[77]
Silk	HFA	3–7	250–500	–	[42]
<i>TIPS</i>					
PLLA	THF	1–7.5	164–169	6.1–12.5	[60]
PCL/PLLA	THF	5	95–175	–	[78]
PLGA	THF	5–12	259–569	3.1–11.7	[79]
PHB	CHCl ₃ /DIOX	1–5	161–170	4.8–8.0	[61]
Gelatin	MeOH/H ₂ O	5–10	157–177	3.2–6.7	[50]

^a Solvent abbreviations: AA: acetic acid, CHCl₃: chloroform, DCM: dichloromethane, DMF: *N,N*-dimethylformamide, DIOX: dioxane, FA: formic acid, H₂O: water, HCl: hydrochloric acid, HFA: hexafluoroacetone, HFIP: 1,1,1,3,3,3-hexafluoro-2-propanol, MeOH: methanol, TFE: 2,2,2-trifluoroethanol, THF: tetrahydrofuran

^b Conc.: polymer concentration

^c FD: fibre diameter

^d FL/FD ratio: fibre length/fibre diameter ratio

Electrospun nanofibre diameters may be increased by increasing the flow rate, the spinneret tip diameter and its distance from the collector, and decreasing the voltage and the solution charge density. Moreover, the viscosity of the solution impacts on the uniformity of the bead-free nanofibres [52]. Indeed, there is an optimal feed solution viscosity for electrospinning since no continuous fibres may form at very low viscosity, whereas the ejection of jets from highly viscous polymer solutions is very difficult. In the optimal range, a more viscous feed solution, due to the higher polymer concentration and molecular weight, leads to larger and more uniform fibre diameters with a reduction in the number of beads along the fibres [53]. For instance, PCL/PLLA nanofibres electrospun from a chloroform/methanol solution had dense bead structures with a smaller diameter for low concentrations of PCL/PLLA, in comparison with a concentration of 11 w/v % which resulted in fibres almost four times larger with bead-free structures (Table 1) [54]. Van der Schueren *et al.* have demonstrated the importance of the solvent during the electrospinning process. Indeed, different solvents lead to different polymer solution surface tensions, and a high surface tension tends to inhibit the electrospinning process. For instance, using chloroform as the solvent led to uniform PCL fibres in the microscale range, while the binary system of formic acid / chloroform resulted in smaller fibres in the nanoscale range with noticeable beads. When using the formic acid / acetic acid system, the number of beads drastically decreased (Table 1). The composition of the binary system also impacted on the eventual fibre diameter. By increasing the amount of acetic acid from 10 v% to 80 v%, the average fibre diameter showed a minor increase (from 545 to 662 nm), though this was associated with a large increase in the standard deviation (from 80 to 420 nm). This trend was attributed to changes in the solution conductivity [55]. Porous nanofibres can also be fabricated by inducing phase separation between the polymer and the solvent, which can be induced through temperature reduction or by using a highly volatile solvent [56,57]. Finally, the composition of fibres can be easily tailored by using different polymers, composite materials and encapsulations during electrospinning, which may also lead to changes in the nanofibre diameters. Khatri *et al.* have demonstrated that the fibre diameter decreased from 860 to 715 nm with increasing PLLA ratio in PCL/PLLA blends [58]. When magnetite nanoparticles were encapsulated into the PCL nanofibres during the electrospinning process, the nanofibre diameter decreased from 864 nm to 202 nm through the inclusion of up to 15 % of magnetite nanoparticles, which can be attributed to changes in the solution electrical conductivity and viscosity. Nevertheless, the addition of 20 % of magnetite nanoparticles increased abruptly the fibre diameter to 664 nm. This phenomenon was explained by the agglomeration of magnetite nanoparticles resulting in a too high solution viscosity and a low electrospinnability [59].

The nanofibrous structure is also affected by the processing parameters when using the TIPS method for nanofibrous scaffold preparation (Table 1). The

solvent and the gelation temperature have a huge impact on the morphology of the foam. Indeed, if the polymer / solvent mixture is cooled fast to a low temperature that allows the solvent to freeze into a solid state, a solid-liquid phase separation will take place instead of the liquid-liquid phase separation. As a consequence, channel and ladder-like features are observed instead of nanofibrous networks. However, when the polymer / solvent mixture is appropriate, nanofibrous structures are obtained even at low gelation temperatures. Ma *et al.* have demonstrated the peculiarity of the PLLA/THF system, where nanofibrous scaffolds were obtained only with temperatures of below 15 °C [60]. Li *et al.* reported that THF and DMF could not produce PHB nanofibrous structures, while nanofibrous scaffolds were obtained by using a chloroform / dioxane mixture and gelation temperature of below 4 °C [61]. Moreover, the authors have found that the average fibre diameter was not affected by the gelation temperature; however the interfibre spacing, which can be related to the fibre length / fibre diameter ratio, decreased and became more uniform at lower gelation temperatures. The same trend is observed when the polymer concentration was increased [60,61]. In this way, the porosity of the scaffold may be controlled.

Mechanical characterisation of nanofibres and nanofibrous scaffolds is crucial for tissue engineering applications because the scaffold must provide a mechanically stable support for cell development, and must be able to withstand the forces exerted by growing tissues and physiological activities. Some mechanical properties of polymeric nanofibres and nanofibrous scaffolds obtained by electrospinning and TIPS are given in Table 2. The values are different from those obtained in equivalent bulk materials [27]. Indeed for fibre-based materials, the mechanical properties are affected by the polymer molecular weight, morphology, crystallinity, as well as the material size and shape such as porosity, pore area and fibre size, density and orientation [44]. For instance, when electrospun fibre-based materials are developed with aligned fibres, the modulus and the tensile strength increase and the mechanical behaviour becomes anisotropic in comparison to randomly arranged fibres [62,63]. The fibre size also affects the mechanical properties as a reduction in the size improves the orientation and decreases the quantity of defects in the structure. As a consequence, a higher modulus and strength are generally obtained. The mechanical behaviour of electrospun fibre-based materials may also be tailored by: applying thermal post-treatments, using polymer blends, crosslinking the fibres, modifying the fibre surface, as well as encapsulating nanoparticles [44]. Ramier *et al.* have demonstrated that the incorporation of hydroxyapatite nanoparticles within PHB nanofibres increased the elastic modulus and the tensile strength by 67 % and 51 %, respectively. In contrast, when the hydroxyapatite nanoparticles were sprayed over the PHB nanofibres during the electrospinning process, the mechanical properties drastically decreased. This can be attributed to the higher porosity of the scaffold due to weaker interactions between the fibres and the

hydroxyapatite nanoparticles [18]. Nanofibrous scaffolds obtained by TIPS exhibit significantly better mechanical behaviour than solid-walled porous scaffolds [50]. Moreover, the mechanical properties can be tailored by using polymer blends and by varying the polymer concentration in the gelling solution [60,61]. Indeed, an increase in the polymer concentration leads to a higher network density associated with lower porosity. As a consequence, the Young's modulus and the tensile strength both increase [60].

Table 2. Examples of mechanical properties of some polymeric nanofibres and nanofibrous scaffolds obtained by electrospinning and TIPS

Polymer	Young's Modulus (MPa)	Tensile Strength (MPa)	Strain at Break (%)	Ref.
<i>Electrospinning</i>				
PCL	6–60	0.6–40	0.6–7.7	[46,59,65,80]
PDLLA	13.9–70	1.1	1.8	[65,70]
PLLA	10–66	0.1–7.8	1.8–127	[40,65]
PLGA	30–140	1.8–45	2	[62,65]
PHB	238	10.7	7.3	[18]
Chitosan	155	4.07	0.12	[81]
Collagen	80–262	3–12	–	[40,46,63]
Gelatin	105–499	2.3–12	10.6–96	[40,46]
<i>TIPS</i>				
PLLA	4–20	0.15–0.6	6–9	[60]
PHB	40	0.7	6	[61]

The physical properties of the nanofibrous scaffolds are crucial as they influence the degradation behaviour of the material as well as the biological properties. Indeed, it was found that the fibre diameter influences the cell spreading proliferation, migration and differentiation [40,64]. Cells migrate poorly into small-diameter fibre scaffolds and readily penetrate into large-diameter fibre scaffolds. However, cells are restricted to spreading along single fibres for large-diameter fibres, whereas cells are guided by the underlying fibrous matrix for small-diameter fibres. As a consequence, cell differentiation is more extensive on fibres with smaller diameters. Moreover, cells are also affected by the fibre alignment. Since cells adhere and elongate along the fibre axis, a more organised deposition of extracellular matrix is obtained in aligned fibre-based scaffolds. As a consequence, the resulting tissues possess higher stiffness and modulus in comparison with tissues formed by cells seeded onto random fibre-based scaffolds [64]. Finally, cells have a higher proliferation rate on scaffolds that are more stable because

scaffolds lose their porosity and structural integrity when degrading, thus preventing cell adhesion and ingrowth [65]. The degradation behaviour of macroscale degradable polymers has been comprehensively studied and was found to depend on a wide range of parameters, such as the polymer composition, molecular weight, crystallinity, porosity, material size and shape. However, it is important to note that the degradation rate of polymers is different between the bulk material and the nanofibre-based scaffolds. For some polymers, the degradation rate of larger structures is faster than that of nanofibres due to autocatalysis in larger structures [66]. Nevertheless, the hydrolytic degradation was found to be much more rapid for nanofibrous scaffolds obtained by TIPS in comparison to solid-walled scaffolds, even if the wettability in the nanofibrous scaffold is smaller because of small interfibre spacing and the large amount of relatively-hydrophobic surface area. Indeed, the high amount of surface area offers more available sites for polymer hydrolysis. Besides, fibre aggregation occurs during the degradation leading to a decrease in the surface area of the nanofibrous scaffolds, which consequently induces a reduction in the degradation rate with time [67]. Fibre aggregation during degradation is also observed for electrospun nanofibres that are prepared from polymers with glass transition temperatures lower than the degradation temperature. For some polymers, the degradation results in fibre swelling. The fibres may also change from smooth and straight to coil and wavy [65]. For rigid and crystalline polymers associated with slow degradation rates, the fibres tend to break along the fibre axis and the broken ends are more susceptible to hydrolytic attacks [68]. Some attempts to reduce the degradation rate have led to the design of nanofibres with various polymer blends as well as composite nanofibres with encapsulated apatitic nanoparticles. Indeed, Ji *et al.* demonstrated that the incorporation of apatite nanoparticles within PLGA/PLC electrospun fibres had a buffering effect as the nanoparticles neutralised the acidic degradation products that are generated upon polymer degradation, hence slowing down the scaffold degradation [69].

3.3.3. Applications of nanofibrous materials

The ultimate goal of tissue engineering approaches is to successfully repair and restore the function of damaged or diseased tissues. An important feature of tissue engineering is the design of polymeric scaffolds that may be used as carriers for therapeutic cell delivery to the defect region, or may act as space fillers that would recruit surrounding cells and allow tissue development. As a consequence, compelling requirements have been identified for scaffold design: (1) biocompatibility and biodegradability with an appropriate degradation rate; (2) adequate morphology; (3) multi-scale interconnected porous structure; (4) optimal mechanical strength; (5) surface properties that could regulate appropriate cell activities [82]. Various scaffolds have been prepared using many different methods including particulate leaching, textile technologies, and phase separation [83]. Although these scaffolds exhibit

certain advantages, their pore sizes and fibre diameters are often micron-scale, far from the natural nano-scale of extracellular matrix. As a consequence, cells attach and spread in a flatten pattern and still exhibit a two dimensional topography, similar to if they had been cultured on 2D flat surfaces [84]. In addition to the architectural similarity with natural extracellular matrix, nanofibrous and nanofibre-based scaffolds can absorb more proteins due to their high surface area and porosity, and therefore present more binding sites to cell membrane receptors. As a consequence, they may enhance cell adhesion and provide an excellent micro/nano environment for cell proliferation and activity [85]. Since a number of natural and synthetic biodegradable polymers have been successfully fabricated as nanofibres, nanofibrous scaffolds have been explored for many tissue engineering applications. Thus, they have been investigated for use in bone regeneration as well as soft tissue regeneration, such as for the regeneration of cardiovascular tissue, cardiac grafts, nerve, cartilage, ligament and skin [5,42,86]. Therefore, numerous studies have investigated the behaviour of various cells, including cardiomyocytes, chondrocytes, keratinocytes and stem cells seeded over nanofibrous materials [61,65,85,87]. In wound healing applications, nanofibre-based materials possess many advantages. Indeed, they help generate dressings with good oxygen permeation, sufficient drainage of wound exudates, and facilitate the protection of the wound from infection and dehydration. Moreover, they may accelerate the healing process by promoting the migration of cells on the wound surface, and may also reduce wound contraction leading to a decrease in patient morbidity [77,85]. Finally, due to the ease of nanoparticle incorporation within the nanofibres, antimicrobial wound dressings may be further developed [70].

Polymeric nanofibres may also be used to encapsulate and deliver bioactive hydrophilic and hydrophobic molecules for therapeutic applications. The solubility and compatibility of the drug in a drug/polymer/solvent system are decisive factors for the elaboration of nanofibrous carriers, since the system is aimed to deliver a sufficient amount of a drug for an adequate period of time and has to avoid the degradation of drugs during the fabrication process [88]. A wide range of therapeutic molecules, including drugs, proteins, genes and growth factors, have been successfully encapsulated within fibre structures, physically coated or chemically attached on their surfaces [89,90]. For instance in the electrospinning process, drug loading can be achieved through various techniques such as; post-spinning modifications that prevent the drug from exposure to the electrospinning process; direct electrospinning of drug/polymer blends that boasts the advantage of being a single-step method; and co-axial or emulsion electrospinning which can be used to develop core-shell morphologies that contribute to prolonged release [47]. Compared with other formulations, electrospinning also offers a high loading capacity. As a matter of fact, the high surface area of nanofibres allows for fast and efficient solvent evaporation limiting drug crystallisation, and therefore leading to the

formation of amorphous dispersions. Moreover, due to the flexibility of the nanofibre processing, a variety of fibre compositions, porosities and structural architectures may be developed in order to control the drug eluting profiles and to meet the needs of the targeted physiological environment. Indeed, the release mechanism is highly dependent on the distribution of the drug molecules in the fibres as well as the fibre morphology. In this way, the drug release pattern can be tailored by varying the drug travelling distance and diffusion pathway, which are directly related to the polymer degradation mechanism and the drug/polymeric matrix affinity [90]. The principal advantage of nanofibrous carriers over cast-films is the increased drug release, prolonged availability and site-specific delivery into the body, thus achieving high local bioactivity and low systemic side effects. Furthermore, simultaneous administration of multiple drugs may be achieved by encapsulating various therapeutic agents within the same nanofibrous carrier. To ensure the independent controlled release of each drug, sequential electrospinning has been developed to obtain multilayered membranes consisting of various drugs and basement nanofibres [88]. It has also been demonstrated that the encapsulation of drugs into nanospheres which have been subsequently incorporated within nanofibrous scaffolds, reduces the burst release and allows for drug delivery over a prolonged duration [45]. Biodegradable polymeric nanofibre-based carriers can be applied for the prevention of post-surgical adhesions and infections, local chemotherapy, transdermal drug delivery, and tissue engineering [45,47].

3.4. POLYMERIC NANOPARTICLES

Polymeric nanoparticles (PNPs) are defined as particulate dispersions or solid particles of 10–1000 nm in size. Within these PNPs, therapeutic agents (such as drugs, DNA, proteins, *etc.*) as well as fluorescent labels can be dissolved, entrapped, encapsulated or can be attached to the nanoparticle matrix. Two conformations of polymeric nanoparticles are known to exist (Figure 3). The term “nanocapsule” is used when the polymer forms a core, in which a molecule of interest can be entrapped. The other common structure is called nanosphere and refers to a nanoparticle made of entangled polymer chains in which the molecule of interest is present. It is important to note that the molecule can also be absorbed or covalently attached onto the surface of the nanoparticles.

Figure 3. Schematic representation of a nanosphere (left) and a nanocapsule (right)

3.4.1. Advantages and applications of polymeric nanoparticles

There has been considerable research interest in using particulate delivery systems as carriers for small and large molecules in drug delivery. Particulate systems, such as nanoparticles, have been used as a physical approach to alter and improve the pharmacokinetic and pharmacodynamic properties of various types of drug molecules. Indeed, the nanometre-size promotes effective permeation through cell membranes and stability in the blood stream. Polymeric nanoparticles have been extensively studied as particulate carriers in the pharmaceutical and medical fields, because they show promise as drug delivery systems. This potential can be attributed to a number of factors including: their controlled and sustained release properties [91,92], subcellular size, which allows for relatively higher intracellular uptake compared to other particulate systems [93], possible improvement of the active substance stability [94], and biocompatibility with tissues and cells when synthesised from materials that are either biocompatible or biodegradable [95]. Polymers are very convenient materials for the manufacture of countless and varied molecular designs that can be integrated into unique nanoparticle constructs with many potential medical applications [12]. Other advantages of nanoencapsulated systems as active substance carriers include: high drug encapsulation efficiency due to optimised drug solubility in the core, low polymer content compared to other nanoparticulated systems such as nanospheres, drug polymeric shell protection against degradation factors such as pH and light, and the reduction of tissue irritation due to the polymeric shell (Scheme 1) [96,97]. As a consequence, PNPs have been extensively studied as drug carriers in the pharmaceutical field [98,99] and different research teams have published reviews about the nanoparticle formation mechanisms [91-101], the classification of nanoparticulated systems [98], and the techniques employed for the preparation of nanocapsules [96,102].

Scheme 1. Advantages of polymeric nanoparticles (PNPs)

3.4.2. Methods for nanoparticle preparation

The properties of PNPs have to be optimised in accordance with the particular application. In order to achieve the properties of interest, the mode of preparation plays a vital role. Thus, it is highly advantageous to have appropriate preparation techniques at hand in order to obtain PNPs with the desired properties for a particular application. Several methods have been developed with nanoparticle sizes generally ranging in the scale of 100–500 nm [100]. These techniques are classified according to whether the particle formation involves a polymerisation reaction or a direct arrangement of pre-formed polymers and a desolvation of macromolecules [102-104]. The polymerisation methods can be further classified into emulsion and interfacial polymerisation, and there are two types of emulsion polymerisation – organic and aqueous – depending on the continuous phase. The availability of different synthetic approaches allows for considerable flexibility in the preparation and functionalisation strategy of multifunctional PNPs.

3.4.2.1. Self-assembly

Due to their unique chemical structures, amphiphilic (co-)polymers tend to self-assemble into nano-aggregates in aqueous solution [105,106]. To obtain PNPs containing active organic substances, a mixture of amphiphilic (co-)polymers and organic active substances is firstly dissolved in a “good” solvent and then quickly added to an excess amount of a “poor” solvent. Thus,

the hydrophobic segments of the polymer tend to aggregate and encapsulate the organic substance in the core, while the hydrophilic polymer segments act as a shell to stabilise the PNPs. Moreover, upon conjugating the active organic substance to the hydrophobic ends or side chains of the amphiphilic (co-)polymer, the polymer-substance conjugates form nanoparticles with the organic active substance embedded and anchored in the polymeric matrix. Additionally, the polymer hydrophilic segments could be decorated with functional groups for further conjugation with specific targeting moieties to cater to versatile biological tasks.

3.4.2.2. Polymerisation

In a typical polymerisation method, the organic solvent, containing monomers and organic active substances, is uniformly dispersed into stable and small oil droplets in the presence of an emulsifier in an aqueous solution through ultrasonification. The polymerisation of monomers in oil droplets starts with the addition of initiators into the emulsion to yield organic nanoparticle dispersions (Figure 4). Further solvent evaporation results in well-dispersed PNPs. In this method, the organic substance can be either reactive or non-reactive to the monomers during polymerisation [107,108].

Figure 4. Schematic illustration of organic active substance-encapsulated PNPs prepared from *in situ* polymerisation: (1) Addition of monomers and organic substances in water containing an emulsifier; (2) dispersion with sonication; (3) addition of an initiator to initiate the monomer polymerisation to yield organic substance-loaded PNPs

3.4.2.3. Emulsification / solvent evaporation

Emulsification-solvent evaporation involves two steps (Figure 5). The first step requires emulsification of the polymer solution into an aqueous phase. To do so, the polymer organic solution containing the dissolved drug is dispersed into nanodroplets, using a dispersing agent and high-energy homogenisation, in a non-solvent or suspension medium such as ethyl acetate. During the second step, polymer solvent is evaporated by increasing the temperature under pressure or by continuous stirring, inducing polymer precipitation in

the form of nanospheres [109]. When the solvent is added into the emulsifier-containing aqueous solution under ultrasonication or vigorous stirring, small organic droplets are stabilised by the emulsifier to generate a homogeneous oil-in-water emulsion. After organic solvent evaporation, a stable suspension of PNPs in water is obtained and the nanoparticles surfaces can be used for further functionalisation. However, this method can only be applied to liposoluble drugs, and limitations are imposed by scale-up of the high energy requirements in homogenisation. Frequently used polymers in this method are PLA, PLGA, PCL, and PHB. Drugs or model drugs encapsulated in this way include: albumin, texanus taxoid, loperamide, pranziquantel, cyclosporin A, nucleic acid and indomethacin.

Figure 5. Schematic representation of the emulsification-evaporation technique

3.4.2.4. Nanoprecipitation

Nanoprecipitation is also known as the solvent displacement method. It differs from emulsion in organic solvents since it involves the precipitation of a pre-formed polymer from an organic solution and the diffusion of the organic solvent in an aqueous medium, in the presence or absence of a surfactant [109]. Using PLA as an example, PLA is dissolved in a water-miscible solvent of intermediate polarity and this phase is subsequently injected into a stirred aqueous solution containing a stabiliser surfactant. Polymer deposition on the interface between the water and the organic solvent, caused by fast diffusion of the solvent, leads to the instantaneous formation of a colloidal suspension [110]. To facilitate the formation of colloidal polymer particles during the first step of the procedure, phase separation is performed with a totally water-miscible solvent that is also a non-solvent of the polymer [111]. The solvent displacement technique allows for the preparation of nanocapsules when a small volume of non-toxic oil is incorporated into the organic phase. Considering the oil-based central cavities of the nanocapsules, high loading efficiencies are generally reported for lipophilic drugs when nanocapsules are

prepared. Furthermore, hydrophilic segments of the polymeric matrix orient into the aqueous phase and facilitate further functionalisation of the PNPs. However, the usefulness of this simple technique is limited to water-miscible solvents, in which the diffusion rate is enough to produce spontaneous emulsification [110]. Moreover, even though some water-miscible solvents produce certain instability when mixed in water, spontaneous emulsification is not observed if the coalescence rate of the droplets is sufficiently high. Finally, this method is only practically applicable to lipophilic drugs because of the miscibility of the solvent with the aqueous phase, therefore it is not an efficient means to encapsulate water-soluble drugs. This method has been applied using various polymeric materials such as PLGA, PLA, PCL, and poly(methyl vinyl ether-comaleic anhydride) [112-115]. It has been well adapted for the incorporation of cyclosporin A, with entrapment efficiencies as high as 98 % reported [116]. It can also be used for the preparation of highly loaded nanoparticulate systems based on amphiphilic h-cyclodextrins to facilitate the parenteral administration of the poorly soluble antifungal drugs Bifonazole and Clotrimazole.

3.4.2.5. Salting-out

Salting-out is based on the separation of a water miscible solvent from aqueous solution *via* a salting-out effect. The salting-out procedure can be considered as a modification of the nanoprecipitation process. The polymer and drug are initially dissolved in a solvent such as acetone, which is subsequently emulsified into an aqueous gel containing the salting-out agent (electrolytes such as magnesium chloride, calcium chloride, and magnesium acetate, or non-electrolytes such as sucrose) and a colloidal stabiliser such as poly(vinylpyrrolidone) (PVP) or hydroxyethylcellulose. The selection of the salting-out agent is important because it can play an important role in the encapsulation efficiency of the drug (Table 3). Stirring causes the dispersion of the solvent as irregular sized globules in equilibrium with the continuous phase, and the stabiliser is absorbed on the larger interface; further homogenisation results in smaller globules. The oil-in-water emulsion is diluted with a sufficient volume of water or aqueous solution to enhance the diffusion of acetone into the aqueous phase, thus inducing the formation of nanospheres. The addition of water and the heating step destabilises the equilibrium and causes the diffusion of the organic solvent to the external surface. During the transport of the solute, PNPs are produced with sizes ranging from 100–200 nm. A heating step also encourages the production of a final suspension free of organic solvent which is more uniform in size. Both the solvent and the salting-out agent are then eliminated by cross-flow filtration [96]. To remove the non-encapsulated drug, the PNP suspension is generally filtered and ultracentrifuged followed by re-suspension in an adequate volume of water. The salting-out process has been used in the preparation of biodegradable PNPs with high efficiency and is easily scaled up. The main

advantage of salting-out is that it minimises stress to the protein encapsulants [57]. It does not require an increase of temperature and therefore may be useful when heat sensitive substances have to be processed [117]. Moreover, the amount of drug encapsulated into the PNPs can be modulated by varying various parameters such as drug concentration, rate, temperature, and the nature of the polymer ligands. Some studies have suggested that drug loading can also be increased by varying the pH value of the solution [118,119]. The greatest disadvantages are exclusive application to lipophilic drugs and the extensive nanoparticle washing steps [120].

Table 3. Properties, advantages and disadvantages of some non-electrolyte salting-out agents and colloidal stabilisers

Salting-out agents and colloidal stabilisers	Properties	Advantages/disadvantages
Chitosan	Natural, cationic, hydrophilic, linear, biodegradable	Can be used in non-viral gene delivery
Dextran	Natural, branched, hydrophilic, biocompatible	Permits the anchoring of biovectors and drugs when functionalised with amino groups
Sucrose	Natural	Improves the stability of proteins during lyophilisation
PEG	Synthetic, neutral, hydrophilic, linear, biocompatible	Remains stable in high ionic strength solutions with varying pH values Enhances blood circulation time (a few hours) Permits functionalisation
PVP	Synthetic, branched, hydrophilic	Forms covalent bonds with drugs containing nucleophilic functional groups

3.4.3. Examples of nanoparticles obtained from biodegradable synthetic and natural polymers

Chitosan-based nanoparticles have been widely developed to encapsulate proteins such as bovine serum albumin, tetanus and diphtheria toxoid vaccines, anticancer agents, insulin, and nucleic acids [121]. Chitosan enhances the absorption of peptides such as insulin and calcitonin across the nasal epithelium. The methods proposed to prepare chitosan-based nanoparticles are based on the spontaneous formation of complexes between chitosan and polyanions or the gelation of a chitosan solution dispersed in an oil emulsion. The nanoparticles obtained by formation of the spontaneous complex have smaller diameters (200–500 nm).

Collagen has been widely used as a biomaterial for years due to its promising biocompatibility, low antigenicity and biodegradability [122]. Although collagen forms hydrogels without the use of chemical crosslinking, nanoparticle preparation needs additional chemical treatments due to weak mechanical strength. For instance, collagen nanoparticles are often prepared by electrostatic interactions with sodium sulfate employed as a desolvating agent [123]. A recent study reported on the preparation of collagen-based nanoparticles (340 nm) with methods using lipid vesicle cages, which allow for the control of both the particle dimensions and the gelling environment during the collagen polymerisation [123]. Due to the ease of particle size control, their large surface area, high adsorption capacity and dispersion ability in water, collagen-based nanoparticles can be applied for the sustained release of various drugs.

Gelatin solutions undergo a coil-triple helix transition followed by aggregation of the helices, enabling the formation of nanoparticles. Moreover, the high number of functional groups on the polymer backbone can be used for chemical modification such as for crosslinking and the addition of ligands. Thus, gelatin is a much used biopolymer in the production of nanoparticles to be used as delivery carriers. A number of methods have been reported to prepare gelatin-based nanoparticles including desolvation (a thermodynamically driven self-assembly process), emulsion and crosslinking with poly(ethylenimine) and glutaraldehyde, nanoprecipitation, coacervation, and the grafting of hydrophobic anhydrides to the amino groups of primitive gelatin to form self-assembled micelles [109,124–126]. Emulsified gelatin droplets can also be hardened by cooling the emulsion below the gelation point in an ice bath, resulting in gelatin-based gelled nanodroplets which can be subsequently cross-linked with formaldehyde [127]. The particle sizes range from between 100–600 nm with a mean of 280 nm, and crosslinking can significantly increase the particle size. This technique is useful for heat sensitive drugs; however, a number of drugs can be undesirably covalently bound to the gelatin during the formaldehyde treatment. Furthermore, a

significant disadvantage of the crosslinking agent relates to its toxicity, and this point must be carefully considered.

The PLGA-based nanoparticulate system is one of the most successful and interesting colloidal systems. Indeed, PLGA nanoparticles protect the therapeutic agents, increase their stability and can be used for the controlled delivery of therapeutic molecules with improved pharmacokinetic and pharmacodynamic profiles. However, PLGA nanoparticles suffer from a significant limitation due to their high level of opsonization by the reticuloendothelial system (RES) [128]. To address this negative aspect, several methods and procedures have been utilised for the surface modification of PLGA nanoparticles in order to produce PLGA-based nanoparticulate systems which are not readily recognised by RES. This goal has been achieved by coating the surface of the nanoparticles with more hydrophilic agents to cover the hydrophobic surface and to provide stealth nanoparticles. Moreover, in some applications such as bone tissue engineering, there is a need for a resorbable polymeric systems with sufficient strength and toughness. It is most likely that a single phase system would not possess all the necessary material characteristics, and hence modifications of the polymer matrix may be needed to modulate the properties of the composite and generate the required bioactive material. However, separation at the composite filler-polymer interface suggests that bonding between all of the components of a multi-phase system is required to obtain a mechanically and thermally stable material. In this context, the development of PLGA-hydroxyapatite (nHAP) nanoparticles functionalised by collagen is a promising approach [129]. This synthesis is carried out in several steps. In the first step, the ring-opening polymerisation of D,L-lactide and glycolide monomers is initiated by hydroxyapatite nanoparticles. Thereafter, the polymerisation product is activated for collagen attachment (Figure 6).

Figure 6. Schematic representation of the synthesis of collagen-PLGA-nHAP nanoparticles: formation of PLGA-nHAP system followed by activation and attachment of collagen [129]

3.5. SURFACE MODIFICATION

Polymeric nanoparticles, nanofibres and nanofibrous scaffolds have been developed for a variety of applications in the medical field, such as for tissue engineering and for the delivery of bioactive molecules. However, while the biodegradability and bulk properties may match the needs of the intended application and generally motivates the polymer choice, the inert nature of synthetic biodegradable polymers may not facilitate the attachment of bioactive compounds and can lead to a lack of biological recognition. For

instance, the surface chemical nature plays a vital role in tissue engineering because it impacts on the biological response [130]. To develop nanoparticles, nanofibres and nanofibrous scaffolds as useful nanobiomaterials, it is essential to tailor their surface properties and therefore a variety of approaches leading to high functionalities have been developed as post-treatments [49,89,131,132]. Consequently, surface-functionalised nanomaterials allow for coupling with a wide range of biological and therapeutic molecules that may be released in a timely and proper manner or that may provide bio-modulating or biomimetic microenvironments for cells and tissues [89].

3.5.1. Surface modification of nanofibrous scaffolds

In the biomaterial field, a variety of approaches have been proposed to change the polymer surface: introduction of polar groups by surface treatment, adsorption of biomolecules, and the covalent immobilisation of bioactive compounds [89,133-138]. In theory, any techniques used for the surface modification of polymeric biomaterials may be applied to polymeric nanofibres and nanofibrous scaffolds, as long as the process does not degrade the polymer nor changes its nano-features.

Simple physical adsorption is the most straightforward and convenient method to immobilise bioactive agents, such as enzymes, proteins, growth factors and drugs, onto a polymeric surface. In this technique, electrostatic interactions, hydrogen bonding, hydrophobic interactions, or van der Waals interactions are the driving forces leading to the surface adsorption. The efficiency of the adsorption may be enhanced by surface treatments that modify the hydrophilicity of the materials, such as plasma treatment or wet chemical methods [131]. Although it is the simplest approach to functionalisation, the control over the bioactive agent retention is limited, and conformational change and loss of bioactivity may take place when immobilising proteins [49,133]. However, non-covalent adsorption is sometimes desirable in drug delivery applications. For instance, postsurgical anti-adhesion barriers are biomaterials that physically separate the wound site from an adjacent organ or tissue, and concomitantly deliver infection preventing antibiotics. Consequently, a rapid drug release profile is highly desirable [89]. Another way to physically immobilise charged therapeutic molecules is the use of layer-by-layer assembly. In this method, polyanions and polycations are alternately deposited on a charged polymer surface, resulting in a multilayer coating in which the drug is encapsulated. This functionalisation technique is easy to implement and can be used with a wide compositional range of the coating layer. However, it is not applicable to uncharged drugs and the release profile is influenced by the thickness of the coating [89].

In contrast with physical adsorption, covalent immobilisation provides a stable bond between the bioactive compound and the polymer surface, and therefore

provides a more efficient coating, extends the bioactive agent retention, and may prevent its metabolism [133]. Prior to the biomolecule covalent immobilisation, the generation of reactive functional groups on the polymer surface is necessary. This may be achieved by various wet chemical or plasma treatments. For instance, carboxylic acid and amine groups can be generated with oxygen, ammonia, or air plasma treatments [132]. They also may be generated on biodegradable polyesters through hydrolysis with sodium hydroxide and aminolysis with a diamine, respectively [136,137,139,140]. However, strong reaction conditions should be avoided to ensure maintenance of nano-feature and polymer. Subsequently, after activation of the functionalised polymer surface, the biomolecule is tethered directly or through the use of a linker molecule. A variety of conjugation techniques have been developed [133]. Covalent bonding and physical adsorption may also be combined. For instance, various methods have been considered to covalently attach heparin onto polymeric scaffolds for tissue engineering applications. Afterwards, growth factors can be bound to heparin with preservation of their stability and biological activity through the retention of their native conformation [137,141]. In another example, hydroxyapatite was coated onto nanofibrous biodegradable polyester (PLA or PCL) surfaces [131,139]. In a first step, the nanofibres surface was activated in an alkaline solution in order to generate carboxylic acid groups. In a second step, the nanofibrous material was dipped in Ca and P-rich solutions and finally, immersed in simulated body fluid. The mineralization occurs through the carboxylic acid groups that chelate calcium ions and initiate the mineral nucleation. The coating can be tailored by varying the incubation time and the simulated body fluid composition. In the end, the mineralised polymer exhibited higher osteoblastic responses (cells adhesion and growth) with better expression of the bone extracellular matrix genes than those on unmodified nanofibres. These new nanofibrous materials have potential as bone regeneration membrane [139].

The cost and the control for the covalent immobilisation of biomolecules remains a critical step for controlling the cell response. Moreover, the covalent bonding may partially inactivate the bioactive compound. Therefore, the introduction of multi-functional groups is an easier way to change the charge or the chemical composition surface of a polymer, and therefore to tune the rearrangements of proteins that adsorb from the cell culture serum onto the polymer surface and to modulate the ultimate cell response [142,143]. With this in mind, surface graft polymerisation is a simple, effective, and versatile approach with a wide range of possibilities in terms of functionalisation and composition due to the vast choice of monomers available. Moreover, multi-functional groups can be introduced with a high density without modifying the polymer bulk properties. Surface graft polymerisation can be initiated by various techniques, such as plasma discharge, ultraviolet light, ozone oxidation, γ -rays, electron beams, and Cerium IV treatment [144]. Again, the processing conditions should be carefully controlled to ensure the polymer and

nano-feature integrity is maintained. Furthermore, to confer surface hydrophilicity and provide functional groups for the subsequent immobilisation of bioactive compounds, surface graft polymerisation can also be used to covalently attach bioactive polymers, such as poly(sodium styrene sulfonate), which is known to influence protein adsorption and cell response [138,145].

3.5.2. Coupling strategies for the biofunctionalisation of nanoparticles

The association of one or more biologically relevant molecules at the interface of nanoparticles defines a nanoparticle-bioconjugate with biological activity such as selective binding. Biomolecules of interest may include one or more of the following:

- Peptides, proteins, and antibodies
- Enzymes and ribozymes
- Oligonucleotides and aptamers
- Carbohydrates
- Lipids
- Drugs or other biologically active small molecules

The interest in these bioconjugate materials arises from the combination of nanoscale size with the nearly infinite diversity of physical properties and chemical functionality that can be obtained through organic chemistry. Indeed, PNPs can be designed to:

- Carry molecular cargo externally or internally
- Carry hydrophilic or hydrophobic cargo
- Release cargo gradually
- Exhibit “smart” physicochemical responses to environmental stimuli (*e.g.*, pH, thermal response)
- Evade the reticuloendothelial system and other immune responses
- Biodegrade
- Target different tissues or cell types

These different properties are tailored through the selection of the chemical composition of the PNPs. Bioconjugates of polymer and amphiphile nanoparticles are typically prepared to assist targeting, with antibody conjugates being particularly common. While there is no characteristic surface chemistry due to the diversity of materials, the introduction of carboxylic acid or amine groups into the polymer / amphiphile composition for bioconjugation is routine. Overall, the bioconjugation chemistry of PNPs is generally dictated by the functional groups associated with the material.

3.5.2.1. Methods of coupling

Various coupling methods have been widely studied (Figure 7). Carbodiimide coupling is used to covalently link carboxylic acid to amine groups *via* formation of a “zero length” amide bond [146]. The key advantage of this procedure is that it involves no lengthy linker species, so that the hydrodynamic radius of the PNPs is minimised. The most common carbodiimide coupling strategy uses 1-ethyl-3-(dimethylaminopropyl) carbodiimide hydrochloride (EDC or EDAC) as the coupling agent, which has been applied for enzyme-to-PNP coupling with retention of up to 50–80 % of the enzymatic activity, depending on the enzyme [147,148] (Figure 7-A). The efficiency of the coupling reaction can be increased by stabilising the *O*-acylisourea intermediate by formation of the succinimide ester using *N*-hydroxysuccinimide (NHS) or sulfo-NHS. Bis(*N*-hydroxysuccinimide) can also be used without a carbodiimide activation agent, allowing for the conjugation of two amine moieties thanks to the two NHS ester groups [149]. When PNPs bear hydroxyl groups on the surface, the activated species may be directly coupled *via* a net dehydration reaction leading to ester linkages [150]. Maleimide may be used to conjugate primary amines to thiol groups as illustrated in Figure 7-B [151]. The most commonly used maleimide-derived coupling reagent is sulfosuccinimidyl-4-(maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC). Maleimide coupling has been widely used to conjugate biomolecules such as DNA, herceptin and proteins [152-154].

Another common route for bioconjugation is the Cu(I)-catalysed alkyne-azide cycloaddition reaction, also known as “CuAAC” or “click chemistry”, which involves the coupling of an alkyne group to an azide moiety resulting in a 1,2,3-triazole ring as the strong covalent bond between the PNPs and the biofunctional agents (Figure 7-C). This process has been demonstrated to be highly versatile since either alkyne or azide moieties can be expressed on the biofunctional agent, suitable for conjugation of a variety of species including small molecules. Combined with the variety of ligand head groups available for nanoparticle-ligand bond formation, this procedure has a lot of potential as a coupling approach for bioconjugation. Furthermore, the one-step click process has been shown to give the possibility of introducing multiple functionalities onto PNPs [155].

Finally, charged PNPs may be coupled either with oppositely charged biological and polymeric species, or indeed to different oppositely charged nanoparticles (Figure 7-D) [156,157]. Some obvious examples of biological application are the coupling of negatively charged DNA or liposomes to positively charged PNPs [158,159]. Moreover with careful tuning of the pH, it is possible to couple a variety of proteins, which can be cationic, anionic or neutral [160].

Figure 7. Schematic representation of various coupling methods: A) carbodiimide coupling used to covalently link PNP bearing carboxylic acid groups to amine moieties-bearing antibodies, B) Maleimide coupling of amines with thiol groups using sulfo-SMMC as the linker, C) Schematic of Cu-mediated alkyne-azide cycloaddition (“click reaction”), D) Ionic coupling between antibodies and PNPs

3.5.2.2. Polymers as conjugating agents on PNPs

Small molecules can be used as ligands to act as a sort of physical barrier preventing the nanoparticle cores from coming into contact with each other. This approach is associated with a very small increase in the nanoparticle hydrodynamic radius. However, care must be taken not to make the molecular shell too thin as this leads to an insufficient steric barrier, resulting in reduced nanoparticle stability and aggregation [112]. By contrast, polymers also make excellent ligands to surround nanoparticles and act as a substantial physical barrier, which leads to a higher hydrodynamic radius in comparison to using small ligands [128]. This effect is desirable for *in vivo* applications requiring a long circulation time, but disadvantageous if rapid diffusion to the extravascular space is required; essentially, size is a very important factor in the biodistribution of PNPs [161]. Indeed like small-molecule stabilising agents, the concentration of polymeric stabilisers may be used to control the nanoparticle core morphology [112,159].

There are many suitable polymeric ligands that enable water solubility, most of which are based on PEG and common carbohydrates such as starch, dextran and chitosan [102,103,121]. PEG is especially suitable for nanosystems

requiring long circulation times in blood, as it reduces the degree of opsonisation and provides excellent long-term stability in high salt concentrations and pH extremes [162]. Moreover, PEG end chains can be modified to provide chemical functionality or ionic stabilisation, and allow for the selective attachment to nanoparticle surfaces and subsequent biofunctionalisation (Table 4) [156]. Therefore, PNPs decorated with different ligands could be used for therapeutical purposes. For instance, appropriate functionalisation of PNPs with targeting moieties could enable selective recognition and interaction with specific cancer cells. Indeed, the proper orientation of the targeting moiety can promote the antigen binding and the subsequent spontaneous cell internalisation.

Finally, nanoparticle surface charge can also have an important influence on their interaction with cells and on their uptake. Positively charged nanoparticles seem to allow for a higher extent of internalisation, due to the ionic interactions established between the positively charged nanoparticles and the negatively charged cell membranes [159]. Moreover, positively charged nanoparticles seem to be able to escape from lysosomes after being internalised and exhibit perinuclear localisation, whereas the negatively and neutrally charged nanoparticles prefer to colocalise with lysosomes. Nanoparticle surface charges can be modulated through functionalisation with polymeric ligands to facilitate a higher degree of internalisation. For instance, PLGA nanoparticles have negative charges which can be shifted to neutral or positive charges by surface modification with PEG or chitosan, respectively [12,121].

Table 4. Examples of PEG derivative ligands used for chemical functionalisation of PNPs

PEG derivatives used as ligands	Chemical structure	Functionalisation
PEG bis(carboxymethyl)ether		Carbodiimide coupling Ionic PNPs
Thiol-PEG-carboxylic ether		Carbodiimide coupling Ionic PNPs
Cholesterol PEG		Carbodiimide coupling Ionic PNPs
PEG bis(amine)		Sulfo-SMCC coupling Ionic PNPs
PEG bis(azide)		Click chemistry

3.6. CONCLUSION

After a presentation of the biodegradable polymers used in medical applications, this chapter gave an overview of the principal approaches that can generate nanofibres and nanofibrous scaffolds as well as nanoparticles. The choice of the method and the processing parameters are crucial for the control of the nanofibrous and nanoparticle architecture and size as well as their properties. Post-treatments and conjugations are often applied to modify the surface chemistry leading to functionalised materials with biological cues. Due to the annual growing of the global nanomaterial market, research and development at the material scale is still challenging for the production of new nanomaterials that could be used as biomimicking scaffolds and bioactive nanocarriers.

REFERENCES

1. E. Campos, R. Cordeiro, A.C. Santos, C. Matos, M.H. Gil. *Colloids Surf. B Biointerfaces* **88** (2011) 477–482.
2. Transparency Market Research, *Nanomaterials Market – Global Industry Analysis, Size, Share, Growth, Trends and Forecast 2014 – 2020*, 2014.
3. L.S. Nair, C.T. Laurencin. *Prog. Polym. Sci.* **32** (2007) 762–798.
4. S. Naahidi, M. Jafari, F. Edalat, K. Raymond, A. Khademhosseini, P. Chen. *J. Control Release* **166** (2013) 182–194.
5. R. Vasita, D. S. Katti. *Int. J. Nanomedicine* **1** (2006) 15–30.
6. A. Kumari, S.K. Yadav, S.C. Yadav. *Colloids Surf. B Biointerfaces* **75** (2010) 1–18.
7. A. Shukla, R. Tiwari, *Intelligent Medical Technologies and Biomedical Engineering: Tools and Applications*, IGI Global, Hershey, USA, 2010, p. 376.
8. S. Deb, in *Biomaterials Fabrication and Processing Handbook*, P.K. Chu, X. Liu, (Eds.), CRC Press, Boca-Raton, USA, 2008, p. 457–481.
9. D.M. Yoon, J.P. Fisher, in *Biomedical Materials*, R. Narayan (Ed.), Springer, New York, USA, 2009, p. 415–441.
10. S. Dumitriu, *Polymeric Biomaterials, 2nd Edition, Revised and Expanded*, Marcel Dekker, New York, USA, 2002, p. 1168.
11. Q.-Z. Chen, O. Bretcanu, A.R. Boccaccini, in *Biomaterials Fabrication and Processing Handbook*, P.K. Chu, X. Liu (Eds.), CRC Press, Boca-Raton, USA, 2008, p. 3–43.
12. F. Danhier, E. Ansorena, J.M. Silva, R. Coco, A. Le Breton, V. Préat. *J. Control Release* **161** (2012) 505–522.
13. U. Edlund, A.-C. Albertsson, in *Degradable Aliphatic Polyesters, Advances in Polymer Science, vol. 157*, A.C. Albertsson (Ed.), Springer-Verlag, Berlin-Heildeberg, Germany, 2002, p. 67–112.
14. B.D. Ulery, L.S. Nair, C.T. Laurencin. *J. Polym. Sci. Part B: Polym. Phys.* **49** (2011) 832–864.
15. N. Duzgunes, *Methods in Enzymology. Vol. 509. Nanomedicine: Infectious Diseases, Immunotherapy, Diagnostics, Antifibrotics, Toxicology and Gene Medicine*, Academic Press, San Diego, USA, 2012, p. 470.

16. M.J. McClure, S.A. Sell, D.G. Simpson, B.H. Walpoth, G.L. Bowlin. *Acta Biomater.* **6** (2010) 2422–2433.
17. W.-J. Li, R. Tuli, C. Okafor, A. Derfoul, K.G. Danielson, D.J. Hall, R.S. Tuan, *Biomaterials* **26** (2005) 599–609.
18. J. Ramier, T. Boudierlique, O. Stoilova, N. Manolova, I. Rashkov, V. Langlois, E. Renard, P. Albanese, D. Grande. *Mater. Sci. Eng. C Mater. Biol. Appl.* **38** (2014) 161–169.
19. F.S. Poletto, L.A. Fiel, B. Donida, M.I. Ré, S.S. Guterres, A.R. Pohlmann. *Colloids Surf. A Physicochem. Eng. Aspects* **324** (2008) 105–112.
20. C. Peña, T. Castillo, A. García, M. Millán, D. Segura. *Microbial Biotech.* **7** (2014) 278–293.
21. M.C. Hacker, A.G. Mikos, in *Principles of Regenerative Medicine*, A. Atala, R. Lanza, J.A. Thomson, A. Nerem (Eds.), Elsevier, Burlington, USA, 2008, p. 604–635.
22. W.-C. Lee, I.-M. Chu. *J. Biomed. Mater. Res. Part B: Appl. Biomater.* **84B** (2008) 138–146.
23. X. Jiang, H. Xin, J. Gu, X. Xu, W. Xia, S. Chen, Y. Xie, L. Chen, Y. Chen, X. Sha, X. Fang. *Biomaterials* **34** (2013) 1739–1746.
24. R. James, M. Deng, S.G. Kumbar, C.T. Laurencin, in *Natural and Synthetic Biomedical Polymers*, S.G. Kumbar, C.T. Laurencin, M. Deng (Eds.), Elsevier, Burlington, USA, 2014, p. 193–205.
25. A.K. Andrianov, R. Langer, in *Polyphosphazenes for Biomedical Applications*, A.K. Andrianov (Ed.), John Wiley & Sons, Hoboken, USA, 2009, p. 3–14.
26. C. Diaz, M.L. Valenzuela, in *Polyphosphazenes for Biomedical Applications*, A.K. Andrianov (Ed.), John Wiley & Sons, Hoboken, USA, 2009, p. 297–324.
27. G. Rohman, in *Biomaterials*, V. Migonney (Ed.), John Wiley & Sons, Hoboken, USA, 2014, p. 27–81.
28. Y. Wen, J.K. Oh. *Macromol. Rapid Commun.* **35** (2014) 1819–1832.
29. K.Y. Lee, L. Jeong, Y.O. Kang, S.J. Lee, W.H. Park. *Adv. Drug. Deliv. Rev.* **61** (2009) 1020–1032.
30. E.L. Monzack, K. J. Rodriguez, C.M. McCoy, X. Gu, K.S. Masters, in *Biomaterials for Tissue Engineering Applications – A Review of the Past and Future Trends*, J.A. Burdick, R.L. Mauck (Eds.), Springer-Verlag, Wien, Germany, 2011, p. 209–241.
31. W. Paul, C.P. Sharma, in *Degradation Rate of Bioresorbable Materials – Prediction and Evaluation*, F. Buchanan (Ed.), CRC Press, Boca-Raton, USA, 2008, p. 67–94.
32. C.A. Schütz, L. Juillerat-Jeanneret, P. Käuper, C. Wandrey, *Biomacromolecules* **12** (2011) 4153–4161.
33. H.A. Patel, J.K. Patel. *Int. J. Pharm. Sci. Rev. Res.* **4** (2010) 37–41.
34. J. Maia, M.B. Evangelista, H. Gil, L. Ferreira, in *Carbohydrates Applications in Medicine*, M.H. Gil (Ed.), Research Signpost, Kerala, India, 2014, p. 31–53.
35. S. Zhou, H. Dou, Z. Zhang, K. Sun, Y. Jin, T. Dai, G. Zhou, Z. Shen. *Polym. Chem.* **4** (2013) 4103–4112.
36. R.A. Scott, A. Panitch. *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol.* **5** (2013) 388–398.
37. E.J. Oh, K. Park, K.S. Kim, J. Kim, J.-A. Yang, J.-H. Kong, M.Y. Lee, A.S. Hoffman, S.K. Hahn. *J. Control. Release* **141** (2010) 2–12.
38. E.M. Bueno, J. Glowacki. *Nat. Rev. Rheumatol.* **5** (2009) 685–697.

39. K.Y. Choi, H. Chung, K.H. Min, H.Y. Yoon, K. Kim, J.H. Park, I.C. Kwon, S.Y. Jeong. *Biomaterials* **31** (2010) 106–114.
40. D.B. Khadka, D.T. Haynie. *Nanomedicine: NBM* **8** (2012) 1242–1262.
41. I. Vroman, L. Tighzert. *Materials* **2** (2009) 307–344.
42. X. Zhang, M.R. Reagan, D.L. Kaplan. *Adv. Drug. Deliv. Rev.* **61** (2009) 988–1006.
43. Transparency Market Research, *Nano-Fiber Cell Scaffold Market – Global Industry Analysis, Size, Share, Growth, Trends and Forecast, 2012 – 2018, 2014.*
44. B. Ma, J. Xie, J. Jiang, F.D. Shuler, D.E. Bartlett. *Nanomedicine (Lond)* **8** (2013) 33.
45. M. Prabakaran, R. Jayakumar, S.V. Nair, in *Biomedical Applications of Polymeric Nanofibers, Advances in Polymer Science, vol. 246*, R. Jayakumar, S.V. Nair (Eds.), Springer-Verlag, Berlin-Heildeberg, Germany, 2012, p. 241–262.
46. N. Bhardwaj, S.C. Kundu. *Biotechnol. Adv.* **28** (2010) 325–347.
47. A.J. Meinel, O. Germershaus, T. Luhmann, H.P. Merkle, L. Meinel. *Eur. J. Pharm. Biopharm.* **81** (2012) 1–13.
48. J.M. Holzwarth, P.X. Ma. *Biomaterials* **32** (2011) 9622–9629.
49. Z. Zhang, J. Hu, P.X. Ma. *Adv. Drug. Deliv. Rev.* **64** (2012) 1129–1141.
50. X. Liu, P.X. Ma. *Biomaterials* **30** (2009) 4094–4103.
51. J. Mey, G. Brook, D. Hodde, A. Kriebel, in *Biomedical Applications of Polymeric Nanofibers, Advances in Polymer Science, vol. 246*, R. Jayakumar, S.V. Nair (Eds.), Springer-Verlag, Berlin-Heildeberg, Germany, 2012, p. 131–170.
52. S.J. Lee, J.J. Yoo, in *Handbook of Intelligent Scaffold for Tissue Engineering and Regenerative Medicine*, G. Khang (Ed.), Pan Stanford Publishing, Singapore, 2012, p. 201–218.
53. N. Lavielle, A.-M. Popa, M. de Geus, A. Hébraud, G. Schlatter, L. Thöny-Meyer, R.M. Rossi. *Eur. Polym. J.* **49** (2013) 1331–1336,
54. P.S. Gungor-Ozkerim, T. Balkan, G.T. Kose, A. Sezai Sarac, F.N. Kok. *J. Biomed. Mater. Res. Part A* **102A** (2014) 1897–1908.
55. L. Van der Schueren, B. De Schoenmaker, Ö.I. Kalaoglu, K. De Clerck. *Eur. Polym. J.* **47** (2011) 1256–1263.
56. X. Qin, D. Wu. *J. Therm. Anal. Calorim.* **107** (2012) 1007–1013.
57. J. Xie, X. Li, Y. Xia. *Macromol. Rapid. Commun.* **29** (2008) 1775–1792.
58. Z. Khatri, R. Nakashima, G. Mayakrishnan, K.-H. Lee, Y.-H. Park, K. Wei, I.-S. Kim. *J. Mater. Sci.* **48** (2013) 3659–3664.
59. R.K. Singh, K.D. Patel, J. Ho Lee, E.-J. Lee, J.-H. Kim, T.-H. Kim, H.-W. Kim. *PloS One* **9** (2014) e91584.
60. P.X. Ma, R. Zhang. *J. Biomed. Mater. Res.* **46** (1999) 60–72.
61. X.-T. Li, Y. Zhang, G.-Q. Chen. *Biomaterials* **29** (2008) 3720–3728.
62. J. Xie, X. Li, J. Lipner, C.N. Manning, A.G. Schwartz, S. Thomopoulos, Y. Xia, *Nanoscale* **2** (2010) 923–926.
63. A.O. Gee, B.M. Baker, A.M. Silverstein, G. Montero, J.L. Esterhai, R.L. Mauck. *Cell Tissue Res.* **347** (2012) 803–813.
64. S.H. Lim, H.-Q. Mao. *Adv. Drug. Deliver. Rev.* **61** (2009) 1084–1096.
65. W.-J. Li, J.A. Cooper Jr, R.L. Mauck, R.S. Tuan. *Acta Biomater.* **2** (2006) 377–385.
66. D. Han, K.C. Cheung. *Polymers* **3** (2011) 1684–1733.
67. V.J. Chen, P.X. Ma. *Biomaterials* **27** (2006) 3708–3715.
68. K. Zhang, A. Yin, C. Huang, C. Wang, X. Mo, S.S. Al-Deyab, M. El-Newehy. *Polym. Degrad. Stabil.* **96** (2011) 2266–2275.

69. W. Ji, F. Yang, H. Seyednejad, Z. Chen, W.E. Hennink, J.M. Anderson, J.J.J.P. van den Beucken, J.A. Jansen. *Biomaterials* **33** (2012) 6604–6614.
70. H. Rodriguez-Tobias, G. Morales, A. Ledezma, J. Romero, D. Grande. *J. Mater. Sci.* **49** (2014) 8373–8385.
71. A. Suslu, A.Z. Albayrak, A.S. Urkmez, E. Bayir, U. Cocen, *J. Mater. Sci.: Mater. Med.* **25** (2014) 2677–2689.
72. L.S. Nair, S. Bhattacharyya, J.D. Bender, Y.E. Greish, P.W. Brown, H.R. Allcock, C.T. Laurencin. *Biomacromolecules* **5** (2004) 2212–2220.
73. M.Z. Elsabee, H.F. Naguib, R. Elsayed Morsi. *Mater. Sci. Eng. C Mater. Biol. Appl.* **32** (2012) 1711–1726.
74. C.A. Bonino, K. Efimenko, S. In Jeong, M.D. Krebs, E. Alsberg, S.A. Khan. *Small* **8** (2012) 1928–1936.
75. F. Cengiz-Çalloğlu. *e-Polymers* **14**(2014) 5–13.
76. I. Chul Um, D. Fang, B.S. Hsiao, A. Okamoto, B. Chu. *Biomacromolecules* **5** (2004) 1428–1436.
77. M. Dubský, S.A. Kubinová, J. Sirc, L. Voska, R. Zajíček, A. Zajícová, P. Lesný, A. Jirkovská, J. Michalek, M. Munzarová, V. Holan, E. Syková. *J. Mater. Sci.: Mater. Med.* **23** (2012) 931–941.
78. L. He, B. Liu, G. Xipeng, G. Xie, S. Liao, D. Quan, D. Cai, J. Lu, S. Ramakrishna. *Eur. Cell Mater.* **18** (2009) 63–74.
79. J. Mao, S. Duan, A. Song, Q. Cai, X. Deng, X. Yang. *Mater. Sci. Eng. C Mater. Biol. Appl.* **32** (2012) 1407–1414.
80. E. Guadalupe, D. Ramos, N.B. Shelke, R. James, C. Gibney, S.G. Kumbar. *J. Appl. Polym. Sci.* **132** (2015) 41879.
81. J.D. Schiffman, C.L. Schauer. *Biomacromolecules* **8** (2007) 594–601.
82. E. Sachlos, J.T. Czernuszka. *Eur. Cell Mater.* **5** (2003) 29–40.
83. P.X. Ma. *Mater. Today* **7** (2004) 30–40.
84. F. Gelain, A. Horii, S. Zhang. *Macromol. Biosci.* **7** (2007) 544–551.
85. S. Agarwal, J.H. Wendorff, A. Greiner. *Polymer* **49** (2008) 5603–5621.
86. S. Srinivasan, R. Jayakumar, K.P. Chennazhi, E.J. Levorson, A.G. Mikos, S.V. Nair, in *Biomedical Applications of Polymeric Nanofibers, Advances in Polymer Science, vol. 246*, R. Jayakumar, S.V. Nair (Eds.), Springer-Verlag, Berlin-Heildeberg, Germany, 2012, p. 1–20.
87. O. Ishii, M. Shin, T. Sueda, J.P. Vacanti. *J. Thorac. Cardiovasc. Surg.* **130** (2005) 1358–1363.
88. M. Zamani, M.P. Prabhakaran, S. Ramakrishna. *Int. J. Nanomedicine* **8** (2013) 2997–3017.
89. H.S. Yoo, T.G. Kim, T.G. Park. *Adv. Drug Deliver. Rev.* **61** (2009) 1033–1042.
90. X. Hu, S. Liu, G. Zhou, Y. Huang, Z. Xie, X. Jing. *J. Control Release* **185** (2014) 12–21.
91. L. Cruz, L.U. Soares Costa, T.D. Mezzalira, G.N.P. da Silveira, S.S. Guterres, A.R. Pohlmann. *Int. J. Pharm.* **313** (2006) 198–205.
92. E. Amaral, A. Grabe-Guimarães, H. Nogueira, G.L. Machado, G. Barratt, V. Mosqueira. *Life Sci.* **80** (2007) 1327–1334.
93. V.C. Furtado, P. Legrand, J.L. Morgat, M. Vert, E. Mysiakine, R. Gref, J.P. Devissaguet, G. Barratt. *Pharm. Res.* **18** (2001) 1411–1419.
94. A.F. Ourique, A.R. Pohlmann, S.S. Guterres, R.C.R. Beck. *Int. J. Pharm.* **352** (2008) 1–4.

95. S. Guinebretière, S. Brianc, H. Fessi, V.S. Teodorescu, M.G. Blanchin. *Mater. Sci. Eng. C* **21** (2002) 137–142.
96. C. Pinto, R.J. Neufeld, A.J. Ribeiro, F. Veiga. *Nanomedicine NBM* **2** (2006) 8–21.
97. N. Anton, J.P. Benoit, P. Saulnier. *J. Control Release* **128** (2008) 185–199.
98. M.V. Chaubal. *Drug Discover. Today* **9** (2004) 603–609.
99. K. Letchford, H. Burt. *Eur. J. Pharm. Biopharm.* **65** (2007) 259–269.
100. D. Quintanar, E. Allémann, H. Fessi, E. Doelker. *Drug Dev. Ind. Pharm.* **24** (1998) 1113–1128.
101. D. Moinard-Checot, Y. Chevalier, S. Briançon, H. Fessi, S. Guinebretière. *J. Nanosci. Nanotechnol.* **6** (2006) 2664–2681.
102. C. Vauthier, K. Bouchemal. *Pharm. Res.* **26** (2008) 1025–1058.
103. E. Fattal, C. Vauthier, in *Encyclopedia of Pharmaceutical Technology*, J. Swarbrick (Ed.), Marcel Dekker, New York, USA, 2002, p. 1864–1882.
104. L. Kai, L. Bin. *Chem. Soc. Rev.* **43** (2014) 6570–6597.
105. M.J. Joralemon, S. Mcrae, T. Emrick. *Chem. Commun.* **46** (2010) 1377–1393.
106. G. Gaucher, R.H. Marchessault, J.C. Leroux. *J. Control Release* **143** (2010) 2–12.
107. J. Chen, F. Zeng, S. Wu, J. Su, Z. Tong. *Small* **5** (2009) 970–978.
108. S. Wang, G. Kim, Y.-E.K. Lee, H.J. Hah, M. Ethirajan, R.K. Pandey, R. Kopelman. *ACS Nano* **6** (2012) 6843–6851.
109. S. Hornig, T. Heinze, C.R. Becer, U.S. Schubert. *J. Mater. Chem.* **19** (2009) 3838–3840.
110. T. Govender, S. Stolnik, M.C. Garnett, L. Illum, S.S. Davis. *J. Control Release* **57** (1999) 171–185.
111. C. Vauthier, C. Dubernet, E. Fattal, P. Alphandary, P. Couvreur. *Adv. Drug Deliv. Rev.* **55** (2003) 519–548.
112. J.M. Barichello, M. Morishita, K. Takayama, T. Nagai. *Drug Dev. Ind. Pharm.* **6** (1999) 425–471.
113. F. Nemati, C. Dubernet, H. Fessi, A.C. Verdier, M.F. Poupon, F. Puisieux. *Int. J. Pharm.* **38** (1996) 237–246.
114. J. Molpeceres, M. Guzman, M.R. Aberturas, M. Chacon, L. Berges. *J. Pharm. Sci.* **85** (1996) 206–213.
115. J.M. Irache, M. Huici, M. Konecny, S. Espuelas, M.A. Campanero, P. Arbos, *Molecules* **10** (2005) 126–145.
116. E. Allemann, J.C. Leroux, R. Gurny. *Adv. Drug Deliv. Rev.* **34** (1998) 171–189.
117. G. Lambert, E. Fattal, P. Couvreur. *Adv. Drug Deliv. Rev.* **47** (2001) 99–112.
118. M. Gajendiran, S. Mohammed, J. Yousuf, V. Elangovan, S. Balasubramanian. *J. Mater. Chem. B* **2** (2014) 418–427.
119. F. Danhier, O. Feron, V. Préat. *J. Control Release* **148** (2010) 135–146.
120. P. Couvreur, C. Dubernet, F. Puisieux, *Eur J Pharm Biopharm* **41** (1995) 2–13.
121. A. Rampino, M. Borgogna, P. Blasi, B. Bellich, A. Cesàro. *Int. J. Pharm.* **455** (2013) 219–228.
122. J.W. Stone, P.N. Sisco, E.C. Goldsmith, S.C. Baxter, C.J. Murphy. *Nano Lett.* **7** (2007) 116–119.
123. M. Papi, V. Palmieri, G. Maulucci, G. Arcovito, E. Greco, G. Quintiliani, M. Fraziano, M. De Spirito. *J. Nanopart. Res.* **13** (2011) 6141–6147.
124. S. Azarmi, Y. Huang, H. Chen, S. McQuarrie, D. Abrams, W. Roa, W.H. Finlay, G.G. Miller, R. Löbenberg. *J. Pharm. Pharm. Sci.* **9** (2006) 124–132.
125. W.-T. Kuo, H.-Y. Huang, M.-J. Chou, M.-C. Wu, Y.-Y. Huang. *J. Nanomater.* **2011** (2011) 646538.

126. E. Leo, A. Vandelli, M.R. Cameroni, F. Forni. *Int. J. Pharm.* **155** (1997) 75–82.
127. A.K. Sailaja, P. Amareshwar. *J. Pharm. Res.* **5** (2012) 1854–1856.
128. L. Shydar, L. Huang. *BBA-Biomembranes* **10** (2009) 2259–2266.
129. D. Bhuiyan, M.J. Jablonsky, I. Kolesov, J. Middleton, T.M. Wick, R. Tannenbaum. *Acta. Biomater.* **15** (2015) 181–190.
130. D.G. Castner, B.D. Ratner. *Surf. Sci.* **500** (2002) 28–60.
131. V. Beachley, X. Wen. *Prog. Polym. Sci.* **35** (2010) 868–892.
132. H. Chen, R. Truckenmüller, C. Van Blitterswijk, L. Moroni, in *Nanomaterials in Tissue Engineering: Fabrication and Applications*, A.K. Gaharwar, S. Sant, M.J. Hancock, S.A. Hacking (Eds.), Woodhead Publishing Limited, Cambridge, United Kingdom, 2013, p. 158–183.
133. J.M. Goddard, J.H. Hotchkiss. *Prog. Polym. Sci.* **32** (2007) 698–725.
134. G. Chan, D.J. Mooney. *Trends Biotechnol.* **59** (2008) 382–392.
135. J.K. Tessmar, A.M. Göpferich. *Adv. Drug Deliver. Rev.* **59** (2007) 274–291.
136. M.-C. Serrano, R. Pagani, M. Vallet-Regí, J. Peña, J.-V. Comas, M.T. Portolés. *Acta Biomater.* **5** (2009) 2045–2053.
137. G. Rohman, S.C. Baker, N.R. Cameron, J.J. Southgate. *Mater. Chem.* **19** (2009) 9265–9273.
138. S. Huot, G. Rohman, M. Riffault, A. Pinzano, L. Grossin, V. Migonney. *Bio-Med. Mater. Eng.* **23** (2013) 281–288.
139. J.-H. Jang, O. Castano, H.-W. Kim. *Adv. Drug Deliver. Rev.* **61** (2009) 1065–1083.
140. D.R. Nisbet, L.M. Yu, T. Zahir, J.S. Forsythe, M.S. Shoichet. *J. Biomater. Sci. Polym. Ed.* **19** (2008) 623–634.
141. C.L. Casper, W. Yang, M.C. Farach-Carson, J.F. Rabolt. *Biomacromolecules* **8** (2007) 1116–1123.
142. S.G. Eskin, T.A. Horbett, L.V. McIntire, R.N. Mitchell, B.D. Ratner, F.J. Schoen, A. Yee, in *Biomaterials Science: An introduction to materials in medicine*, B.D. Ratner, A.S. Hoffman, F.J. Schoen, J.E. Lemons (Eds.), Elsevier Academic Press, San Diego, USA, 2004, p. 237–291.
143. K. Park, Y.M. Ju, J.S. Son, K.D. Ahn, D.K. Han. *J. Biomater. Sci. Polym. Ed.* **18** (2007) 369–382.
144. Z. Ma, Z. Mao, C. Gao. *Colloids Surf. B Biointerfaces* **60** (2007) 137–157.
145. N. Djaker, S. Brustlein, G. Rohman, S. Huot, M. Lamy de la Chapelle, V. Migonney. *Biomed. Opt. Express* **5** (2014) 149–157.
146. X.H. Gao, Y.Y. Cui, R.M. Levenson, L.W.K. Chung, S.M. Nie. *Nat. Biotechnol.* **22** (2004) 969–976.
147. M. Koneracka, P. Kopcansky, M. Antalík, M. Timko, C.N. Ramchand, D. Lobo, R.V. Mehta, R.V. Upadhyay. *J. Magn. Magn. Mater.* **201** (1999) 427–430.
148. L.D. Chen, J. Liu, X.F. Yu, M. He, X.F. Pei, Z.Y. Tang. *Biomaterials* **29** (2008) 4170–4176.
149. P.C. Lin, P.H. Chou, S.H. Chen, H.K. Liao, K.Y. Wang, Y.J. Chen. *Small* **2** (2006) 485–489.
150. Y.S. Lin, P.J. Tsai, M.F. Weng, Y.C. Chen. *Anal. Chem.* **77** (2005) 1753–1760.
151. M. Brinkley. *Bioconj. Chem.* **3** (1992) 2–13.
152. B. Dubertret, P. Skourides, D.J. Norris, V. Noireaux, A.H. Brivanlou, A. Libchaber. *Science* **298** (2002) 1759–1762.
153. Y.M. Huh, Y.W. Jun, H.T. Song, S. Kim, J.S. Choi, J.H. Lee. *J. Am. Chem. Soc.* **127** (2005) 12387–12391.

154. M.E. Gindy, S.X. Ji, T.R. Hoyer, A.Z. Panagiotopoulos, R.K. Prud'homme, *Biomacromolecules* **9** (2008) 2705–2711.
155. M.A. White, J.A. Johnson, J.T. Koberstein, N.J. Turro. *J. Am. Chem. Soc.* **128** (2006) 11356–11357.
156. T.H. Galow, A.K. Boal, V.M. Rotello. *Adv. Mater.* **12** (2000) 576–579.
157. G. Han, C.C. You, B.J. Kim, R.S. Turingan, N.S. Forbes, C.T. Martin, V.M. Rotello. *Angew. Chem. Int. Ed.* **45** (2006) 3165–3169.
158. D. Li, G.P. Li, W.W. Guo, P.C. Li, E.K. Wang, J. Wang. *Biomaterials* **29** (2008) 2776–2782.
159. N. Nagatani, M. Shinkai, Y. Nagase, H. Honda, K. Hata, H. Mizuno. *Biotechnol. Lett.* **22** (2000) 999–1002.
160. K.C. Ho, P.J. Tsai, Y.S. Lin, Y.C. Chen. *Anal. Chem.* **76** (2004) 7162–7168.
161. C.E. Mora-Huertas, H. Fessi, A. Elaissari. *Int. J. Pharm.* **385** (2010) 113–142.
162. D.E. Owens, N.A. Peppas. *Int. J. Pharm.* **3** (2006) 93–102.

**FABRICATING *IN VITRO* NANOMATERIAL
SCAFFOLDS THROUGH INTEGRATED CIRCUIT
COMPATIBLE MICROFABRICATION TO
MODULATE MAMMALIAN CELLULAR
BEHAVIORS**

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4.1. INTRODUCTION

The past several decades have brought about an ever-increasing requirement for nanomaterial-based scaffolds that could be used to: a) explore cellular behaviors by mimicking *in vivo* extracellular matrix conditions, or b) modulate cellular behaviors by manipulating them on material interfaces. This chapter describes the development of easy-to-handle, high-throughput methods – a combination of common integrated circuit (IC)-compatible manufacturing procedures with features of mass production capacity—to create *in vitro* nanomaterial scaffolds and platforms to modulate cellular behavior and growth micropatterning for a variety of mammalian cells. Microfabrication has already been integrated into techniques of photolithography, reactive-ion etching, metal nanoparticle-assisted etching, and chemically-based surface modification. These approaches enable us to sufficiently design and create artificial scaffolds with small-scale features to probe cell-substrate interactions.

In the natural environment, cell-to-cell or cell-to-milieu interactions are crucial for the formation of organs and tissues *in vivo*. Exploring the interactions between materials and mammalian cells is a meaningful and interesting subject in various research communities including tissue engineering, regenerative medicine, and biosensors [1]. The ability to manipulate mammalian cell-material surface interactions has offered us a myriad of profound knowledge into biology-related research including mechanotaxis, cell viability, and stem cell differentiation [2,3]. Cell-material surface interactions are largely and intimately determined by the interplay of adhesive molecules at the cell-material interface. These adhesive molecules affect various cellular functions, such as division, proliferation, cell migration, differentiation, and structural protein distribution [4-6]. Various molecular mechanisms influencing the way that cells discern and react to their surrounding milieus have been explored, including ligand-integrin interactions [7-10], surface hydrophobicity or hydrophilicity, [11-13] and topography [14-21]. Because biomedical engineering places great significance on cell adhesion, the development of biomimetic materials to enable cell attachment and sustain desirable architecture has been widely investigated [22,23]. Notably, it has been shown that the actin cytoskeleton is a critical and dynamic structural component of the mammalian cell milieu that responds to changes in surface modification and, in doing so, alters characteristics of adhesion. Substrate-related modifications (often stimulations), for instance, can enhance actin polymerization and affect focal adhesion complexes [24]. Various collaborations between biology and engineering researchers have sought to develop biocompatible scaffolds to modulate cellular responses *in vitro*. As a result, controlling cellular environments using artificial scaffolds has become

an area of devoted, albeit difficult, research that carefully and thoughtfully integrates the regulation of both scaffold function and cell development. Most research in this field makes use of plastic cell culture dishes to perform experiments, but such surfaces are morphologically dissimilar to the natural extracellular matrix. Recent advances in the development of three-dimensional environments that can provide a more natively relevant surrounding support scaffold for cells have spawned crucial advances in understanding various physiological and pathological conditions [25-29]. In other literature, the differences between *in vitro* and *in vivo* outcomes have been extensively discussed [30].

Several approaches have been used to create nanomaterial-based scaffolds for *in vitro* experiments. Research exploring cell-material surface interactions at the nanometric scale can be catalogued into two groups, the examination of cellular responses to i) adhesion molecules on various substrates (chemically based approach) [31,32], or to ii) surface stiffness [33-37] and topographic features (physically based approach) [38-41]. With regard to chemical approaches, various chemicals or proteins micropatterns can be used to efficiently modulate cellular function. For example, cell adhesion and neurite outgrowth can be modified by chemically patterned surfaces [42-44]. Further, self-assembled monolayers of alkanethiols have been shown to influence the action potential of neurons [8]. Physically-based approaches, which use micro-/nano-topographically altered surfaces, can influence cytoskeletal remodeling, cellular morphology, and cellular spreading [45,46]. The effects of topographic features, including the effects of a variety of micro-/nano-scale geometries on cellular behaviors, have been investigated. Such investigations include experimental review of microposts [47-50], nanogratings [51,52], microgrooves [53], microperiodic structure [54,55], micropillars [56,57], nanopillars [58], and nanofibers [59]. In a notable example of both physical and chemical influences working together to affect cellular response, substrate topography has been shown to work in concert with chemical signaling to regulate cell behaviors [53]. The topographical dimension of adhesive molecules, such as collagen fibers, laminin, and fibronectin exists in the nanometric scale in nature; hence, to mimic *in vivo* conditions, it is necessary to examine the physiological and physical effects of nanoscale surface modifications, especially with regard to the manner by which they affect chemical contact sites for the regulation of cell activity. At present, our understanding of the physical and chemical properties that, together, affect interactions between the cell and the substrate surface and modulate specific cell behavior remains immature. This chapter, which discusses nanomaterials including oxidized silicon nanosponges and functionalized chitosan membranes coated with different monolayers of functional groups, may provide a suitable preliminary platform to further investigate cellular behaviors using biocompatible and biomimetic conditions that resemble *in vivo* milieus. The development of suitable artificial structures *in vitro* is

crucial in several medical fields including nerve regeneration, small-scale biomedical devices, and nanofabrication methodology.

Another challenging issue in biomedical engineering is determining how to array cells with a desired pattern on artificial scaffolds. The specific arrangement of cells is crucial to understand cell-to-scaffold interaction, but it is also important as a means of mimicking *in vivo* conditions. For example, native neural cell cultures are composed of a random arrangement of neural cells, a situation that is hard for researchers to emulate because neural cells prefer to aggregate into non-uniform groups. Using microfabrication, scientists can control neural cell adhesion and growth, which allows for experiments that better illuminate genuine neurobiological mysteries [42,60-63]. Current promising progress in microfabrication has allowed for cellular patterning in desired regions. The common microfabrication techniques for creating precise, solid surface micropatterns include nanoimprint lithography [64], microcontact printing [65-68] and microfluidic-based processes [69]. Microcontact printing technology, developed by Dr. Whitesides' research group (Harvard University), has become the most common approach for transferring protein patterns onto cultural substrates [70]. However, disadvantages of this method, namely surface sticking and an unstable yield rate, need to be overcome before it is suitable for mass production. Also, because the elastomeric stamp in this process is flexible, deformation frequently occurs when pressing the stamp onto a solid surface.

Studies have examined a variety of surface property changes that can be leveraged to specifically arrange cells on desired regions [11,71]. The capacity for mammalian cell micropatterning is a preliminary requirement for the development of artificial stents, suitable biosensors, and implantable artificial tissues. For this reason, the demand for nanomaterial-based *in vitro* scaffolds that can micropattern mammalian cells has been increasing, especially in the fields of regenerative medicine and tissue engineering. The core of this chapter, which is based on a selection of the past five-years' published research, includes an examination of the following nanomaterial-based devices: 1) artificial scaffolds to be used as *in vitro* platforms for studying cellular biology while mimicking the natural environment of the extracellular matrix (*e.g.*, silicon wafer and glass materials); and, 2) implantable materials (*e.g.*, chitosan membranes) to assist regenerative medicine, including organ transplant and nerve conduit procedures.

4.2. OXIDIZED SILICON NANOSPONGES

To investigate cellular behaviors in biomimetically and physiologically-relevant situations *in vivo*, oxidized silicon nanosponges have been fabricated using inorganic, oxidized silicon wafers, a commonly used material in IC manufacturing processes. It is worth noting that IC manufacturing industries using silicon-based platforms have the following characteristics: 1) robustness; 2) ease-of-use; and, 3) expandability to mass production. Therefore, silicon-based platforms have considerable potential for various applications in the field of biomedical engineering. Furthermore, oxidized silicon substrate can be modified with various functional group monolayers to detect cell responses, including organization of the cytoskeleton, biochemical changes, and cell adhesion. Moreover, because photolithographically-based microfabrication can be used to create specifically micropatterned regions and hydrophobic nanosponges on silicon surfaces, micropatterning of mammalian cells can be achieved to facilitate the insightful examination of cellular morphology. Sections 4.2 and 4.3 largely describe Yang's studies examining mammalian cell response and patterning on oxidized silicon nanosponges [72,73].

4.2.1. Surface modification of silicon substrates

Nanosponges have been fabricated on monocrystalline silicon surfaces *via* Ag-nanoparticle-assisted etching (Figure 1a) [74,75]. In these experiments, a silicon wafer was immersed into 0.01 M silver nitrate solution for approximately 5 min. Following formation of a metallic catalyst layer, the substrate was soaked in etchant containing hydrogen fluoride (HF, 49 % wt) and hydrogen peroxide (H₂O₂, 30 % wt) at a mixture ratio of 3 : 1 (v / v) for 3 min. The procedure for fabricating nanosponges on a silicon surface was formerly developed by Peng *et al.* [75]. In order to enhance biocompatibility, the silicon surfaces were fabricated to create a 20 nm-thick silicon dioxide layer. The wafer surfaces were hydroxylated by O₂ plasma treatment for 10 min *via* vapor deposition of silanol groups in a vacuum chamber (Figure 1b). Following this, a silanol-hydroxyl reaction was used to create a self-assembled molecular layer on the surface [76]. In total, six types of surfaces containing various chemical modifications and nano-topography were created, including pristine oxidized silicon surfaces, perfluorodecyltrichlorosilane (FDTS)-grafted oxidized silicon surfaces, (aminopropyl)trimethoxysilane (APTMS)-grafted oxidized silicon surfaces, pristine oxidized silicon nanosponge surfaces, FDTS-grafted oxidized silicon nanosponge surfaces, and APTMS-grafted oxidized silicon nanosponge surfaces.

Figure 1. Fabrication of modified silicon substrates. (a) Ag nanoparticle-assisted etching was used to fabricate silicon nanosponges. Through vapor deposition process, different chemical functional groups were self-assembled on silicon nanostructures. (b) The scheme of the self-assembled functional groups. Surface chemical modification was created by O₂ plasma treatment for surface hydroxylation. Next, the reaction between hydroxyl and silanol reacted. Finally, the monolayer was self-assembled on the interface [72].

4.2.2. Substrate surface characteristics

The hydrophobicity of the aforementioned surfaces was measured using a water contact angle meter. The results indicate that the water contact angles were $29^\circ \pm 2^\circ$, $110^\circ \pm 3^\circ$, $41^\circ \pm 2^\circ$, $1^\circ \pm 1^\circ$, $148^\circ \pm 4^\circ$ and $2^\circ \pm 1^\circ$ for pristine oxidized silicon substrate, FDTS-grafted oxidized silicon substrate, APTMS-grafted oxidized silicon substrate, pristine oxidized silicon nanosponge, FDTS-grafted oxidized silicon nanosponge, and APTMS-grafted oxidized silicon nanosponge, respectively (Figure 2). Note, FDTS functional groups can increase substrate surface contact angle on more hydrophobically coated surfaces. While, the nano-topography of surfaces coated with functional groups may enhance hydrophobicity, silicon surfaces grafted with APTMS functional groups were still hydrophilic and displayed a contact angle of less than 90° .

Figure 2. Contact angles and SEM images of modified substrates. (a) Pristine oxidized silicon substrate; (b) FDTS-grafted oxidized silicon substrate; (c) APTMS-grafted oxidized silicon substrate; (d) Pristine oxidized silicon nanosponge; (e) FDTS-grafted oxidized silicon nanosponge; (f) APTMS-grafted oxidized silicon nanosponge [72].

4.2.3. Cellular morphology on oxidized silicon surfaces

Chinese hamster ovary (CHO) cells were selected to study the interaction between cells and the aforementioned substrates. Figure 3 displays morphological images of CHO cells following 2 h of culture. The CHO cells grown on oxidized silicon surfaces appeared analogous to those grown on culture plates (Figure 3a). Figure 3d displays CHO cells stretched out and showing a rounded-up shape on pristine oxidized silicon nanospices. Figures 3b and 3e show CHO cells cultured on FDTS-grafted substrates but, in these images, nanospices are no longer observed and the cells have a smaller rounded-up shape. On the APTMS-grafted substrates, CHO cells were known to spread up to $100\ \mu\text{m}$ in size (Figure 3c, 3f). This morphology is caused by the NH_2^+ functional group of APTMS [77]. In comparison, CHO cells were seeded on

pristine oxidized silicon nanosponges and pristine oxidized silicon substrate for durations of 30, 60, 120, and 240 min. Figure 4 shows that, despite the fact that CHO cells showed flat morphology on the pristine oxidized silicon substrates, the cells appeared to be rounded-up on the pristine oxidized silicon nanosponges, and differentiated numerous nanospikes visibly emanate from the somas attached to the nanoposts of the oxidized silicon nanosponges. Nanosponges were thus seen to offer physical support for cells to adhere to the oxidized silicon surfaces.

Figure 3. SEM images of CHO cells seeded on functionalized surfaces after 2 hours of culture. (a) Pristine oxidized silicon substrate; (b) FDTS-grafted oxidized silicon substrate; (c) APTMS-grafted oxidized silicon substrate; (d) Pristine oxidized silicon nanosponge; (e) FDTS-grafted oxidized silicon nanosponge; (f) APTMS-grafted oxidized silicon nanosponge [72].

Figure 4. SEM images of the contact morphology of cells seeded on pristine oxidized silicon substrate and oxidized silicon nanosponge after different culturing durations (a-30: after 30 min of culture on pristine oxidized silicon substrate, b-30: after 30 min of culture on oxidized silicon nanosponge). Cells cultured on pristine oxidized silicon substrate for 30, 60, 120 and 240 min, respectively [72].

4.2.4. Cytoskeleton remodeling on different surfaces

Cytoskeletal orientation and focal adhesion formation are products of the interactions between cell shape and the extracellular matrix [78]. Cytoskeletal organization of CHO cells was examined to determine the influence of nanospoenges *via* staining with rhodamine-conjugated phalloidin (Figure 5). Note, the CHO cells seeded on pristine oxidized silicon nanospoenges showed

filamentous actin clustered in a punctuate manner, which could represent the adhesive dots of cell-nanoposts. These results indicate that actin polymerization is restricted along a specific direction when CHO cells encounter nanoscale adhesive dots on nanosponges.

Figure 5. Confocal microscope images of the nucleus and cytoskeleton of CHO cells seeded on silicon surfaces. (a) Pristine oxidized silicon substrate; (b) FDTs-grafted oxidized silicon substrate; (c) APTMS-grafted oxidized silicon substrate; (d) Pristine oxidized silicon nanosponge; (e) FDTs-grafted oxidized silicon nanosponge; (f) APTMS-grafted oxidized silicon nanosponge [72].

4.2.5. Cell attachment assays

Cell attachment assays have been carried out by re-culturing CHO cells for different time durations. Greater cell adhesion was observed in cultures using nanosponges compared to pristine oxidized silicon substrates, FDTS-, and APTMS- grafted substrates (Figure 6). These outcomes suggest that nano-topography is a determining factor in cell immobilization.

Figure 6. Cell attachment after 30, 60, 120 and 240 min of culture on silicon substrates with various surface modification. The error bars represent the standard deviations of cell number. The *p* values were compared with the pristine oxidized silicon substrates [72].

4.3. MICROPATTERNED SILICON SUBSTRATES

AZ4620 photoresist was micropatterned onto a monocrystalline silicon wafer surface to act as the protective layer during photolithography. The micropatterned silicon wafers were selectively etched using Ag nanoparticle-assisted etching. Subsequently, the surface was chemically modified as previously mentioned. Finally, photoresist was removed with acetone, and the silicon substrate with micropatterned features was prepared for further cell behavior study (Figure 7).

Figure 7. Schematic of the integrated circuit (IC)-based microfabrication for fabricating micropatterned silicon substrates. The designed patterns were fabricated through photolithography and Ag nanoparticle assisted etching. And then, the chemical surface modification was created via vapor deposition. Finally, mammalian cells were seeded on a micropatterned silicon surface [73].

4.3.1. Cell response to micropatterned silicon substrates

Because CHO cells could cross over the nanosponge gap between two flat silicon stages, the distance between two flat silicon stages was adjusted (Figure 8a, 8b). The maximum distance between two flat silicon stages that CHO cells could cross was approximately 40 μm (Figure 8c, 8d). For this reason, the desired distance of nanosponge gap for two flat silicon stages was set to 80 μm to prevent cells from crossing. The results indicate that HIG-82 fibroblasts and CHO cells prefer to attach onto a flat oxidized silicon surface rather than onto FDTs-grafted oxidized silicon nanosponges. With increasing cell culture time, cells gradually migrated from FDTs grafted oxidized silicon nanosponges. Further, Figure 9 shows that when HIG-82 fibroblasts and CHO cells were arranged on pristine oxidized silicon stages, HIG-82 fibroblasts were prone to connect together but CHO cells were not. CHO cells re-arranged on pristine oxidized silicon stages but the cell-to-cell connection was not discovered.

Figure 8. SEM images of CHO cells crossed over two silicon stages; (a) top view, (b) cross-section view. (c) Schematic of CHO cells crosses over two silicon stages. (d) The distance between two silicon stages with nanosponge gap that CHO cell would cross over versus the suspended percentage. The largest distance between two silicon stages was 40 μm for CHO cell to cross over. The error bars represent the standard deviations of the suspended percentage [73].

Figure 9. Staining images of F actin (red) and nucleus (blue). CHO cells and HIG-82 fibroblasts were at different culture durations on micropatterned silicon substrates [73].

4.3.2. Cell fusion analysis

Membrane fusion, including endocytosis and intracellular transport, is an integrative biological procedure [79]. In this research, micropatterned silicon substrate with a hydrophilic-hydrophobic boundary (created using pristine oxidized silicon surfaces and FDTS-grafted oxidized silicon nanosponges) was used to examine whether the cell-to-cell connections of HIG-82 fibroblasts were due to membrane fusion. To verify whether membrane fusion occurred when HIG-82 fibroblasts connected together, NBD C6-HPC was used to mark the phospholipid membrane. The results indicate that few fibroblasts fused together when cultured on micropatterned oxidized silicon substrates after 3 days of culture (Figure 10a). Further, when the width of flat regions was reduced to 5 μm , more HIG-82 fibroblasts were likely to fuse together; indicating that cell density may play a critical role in membrane fusion, since HIG-82 fibroblasts were not prone to stay on FDTS-grafted nanosponges. On the other hand, single CHO cells placed on 5 μm width flat regions re-arranged quite quickly, but did not fuse together (Figure 10b).

Figure 10. Cell membrane staining. The picture area is about 590 μm by 440 μm ; the total cell number in one picture was approximately 300 *via* calculating the nucleus in a staining picture. (a) Staining pictures of nucleus (blue), F actin (red) and membranes (green). (b) Fusion ratios of CHO cells and HIG-82 fibroblasts after 3 days of culture.

The error bars represent the standard deviations [73].

4.4. FUNCTIONALIZED CHITOSAN MEMBRANES

Chitosan-based biomaterials (chitin-based biopolymers) derived from nature, have been approved by the US Food and Drug Administration (US FDA). Typically, chitosan is used in a myriad of biotechnologically-related applications for implantable materials, including neural regeneration, and drug carriers [80-84]. Additionally, chitosan is a biodegradable polymer that is a suitable carrier of implantable materials for regenerative medicine [85,86]. Despite technological advances, it is still challenging to modify physically-based factors (*e.g.*, topography, geometry, and stiffness) on chitosan membranes. Recent advances in the development of chitosan membranes (a naturally derived biomaterial or “soft” material) prepared *via* microfabrication may help overcome existing restrictions and achieve several objectives in the field of tissue engineering. Chitosan-based platforms possess the following unique advantages: 1) they can be used to easily control cell function, and 2) they are suitable for tissue regeneration, due to mechanical properties, biological properties, and biomimetic characteristics. Furthermore, nanostructures on chitosan surfaces can provide uniform but non-periodic nanostructures that more closely mimic physiologically relevant microenvironments. Note, micro/nanoscaled features can be integrated onto chitosan membranes *via* multiple IC-based manufacturing processes. These modified chitosan membranes may be used to study cellular behaviors and micropatterning of mammalian cells. Paragraphs four and five describe Shuai’s studies, which display mammalian cell response and patterning on chitosan membranes with surface modification [87,88].

4.4.1. Surface modification of chitosan membranes

The scheme of microfabrication for chitosan membranes with flat and nanostructured surfaces is displayed in Figure 11. Chitosan solution contains 1 % (w/v) chitosan powder (190–310 kDa) in 1 % v/v acetic acid. Flat chitosan membranes were prepared by soaking diced silicon wafers in chitosan solution. After casting chitosan solution onto flat or nanostructured silicon wafers, the chitosan membranes were generated by drying for 6 h in an oven at 60 °C. Finally, diced treated silicon wafers were immersed in 0.1 M NaOH so that newly cast chitosan substrate could be peeled from the silicon wafer material. Functionalized chitosan membranes were modified through molecular vapor deposition on flat chitosan surfaces [72,73], and four types of chitosan membranes were created, including flat chitosan membranes, O₂-plasma-treated chitosan membranes, FDTS-grafted chitosan membranes and nanostructured chitosan membranes, to regulate cellular behaviors.

Figure 11. (A) Fabrication of chitosan membranes. The flat chitosan membranes were created *via* soaking chitosan solution onto a silicon mold, and then flat chitosan membranes were peeled off of the silicon mold in 0.1 M NaOH solution. (B) Fabrication of nanostructured chitosan membranes. The nanostructured chitosan membranes were created by soaking chitosan solution onto silicon nanosponges. After solvent evaporation, chitosan thin film processed a nanostructured surface that duplicated the silicon nanosponge [88].

4.4.2. Surface characteristics of chitosan membranes

Various chitosan membrane morphologies are shown in Figure 12. As evidenced by measurements of water contact angle for each of the chitosan surfaces examined, those chitosan surfaces treated with O_2 plasma demonstrated more hydrophilicity because there were more hydroxyl groups on their surface. FDTS-grafted surfaces produced a rise in measurable contact angle due to the presence of more fluorine groups. Note, the hydrophilicity of chitosan membranes was also enhanced by nano-topographically scaled features on the chitosan membranes. To closely examine surface differences, flat and nanostructured chitosan surfaces were examined *via* atomic force microscopy (AFM). Due to size restrictions of the AFM cantilever probe, accurate measurement of nanostructured chitosan surface was difficult, but differences between flat and nanostructured chitosan surfaces (relatively rough surface) could be distinguished.

Figure 12. Surface morphologies of physically / chemically modified chitosan membranes (a) SEM images and contact angle examination of chitosan surfaces with different surface modifications including flat, O₂-plasma-treated, FDTS-grafted and nanostructured. (b) Atomic force microscopy images of flat and nanostructured chitosan membranes [88].

4.4.3. Cellular morphology on modified chitosan surfaces

To examine cellular response to surface modification, NIH/3T3 fibroblasts were cultured on various chemically/physically modified chitosan membranes. Figure 13 displays phase contrast and staining image results from these experiments, which indicate the following: 1) cell proliferation was inhibited when NIH/3T3 fibroblasts were seeded onto nanostructured (nanotextured) chitosan surfaces, 2) NIH/3T3 fibroblasts cultures grew to a larger projected cell area on O₂ plasma-treated surfaces than on FDTS-grafted and nanostructured surfaces, and 3) NIH/3T3 fibroblasts spread and proliferated on O₂-plasma-treated chitosan surfaces. Our study suggests that, because the distance between chitosan-based nanostructures (about 80–100 nm) was consistent with the distance between two integrin clusters of cells, cell spreading was limited. Therefore, the extension of actin fibers was restricted as well.

Figure 13. NIH-3T3 fibroblasts immobilized on pristine, O₂-plasma-treated, FDTS-grafted and nanostructured chitosan surfaces after 12, 24 and 48 h of culture. (A) Phase contrast images of NIH-3T3 fibroblasts attached on chitosan surfaces with different culture time. (B) Staining images of F actin (red) and nucleus (blue) of NIH-3T3 fibroblasts. (C) Statistics of projected cell areas, and (D) cell densities of NIH-3T3 fibroblasts after 12, 24 and 48 h of culture. Data are mean $\pm$ standard deviation (N = 6, n = 20) [88].

To probe the interactions between integrin $\alpha 1 \beta 1$ in NIH/3T3 fibroblasts and chitosan-based nanostructures, rhodamine fibronectin was immobilized onto chitosan-based nanostructures to specifically bond with integrin $\alpha 1 \beta 1$ on cell membranes. Figure 14 shows that fibroblasts adhered and spread out on the nanostructured chitosan membranes after 1 h of culturing due to the fibronectin coating, which promoted both cell spreading and attachment. Results indicate that integrin clusters truly connected to chitosan-based

nanostructures. Furthermore, we observed more NIH/3T3 fibroblast integrin $\alpha1\beta1$ expressed in cultures grown on the flat chitosan membranes than in cell cultures grown on nanostructured chitosan membranes (Figure 15).

Figure 14. Staining images of nucleus (blue) and integrin $\alpha1\beta1$ (green) of NIH-3T3 fibroblasts seeded on nanostructured chitosan membranes with rhodamine fibronectin (red) treatment after 1 h (a and b) and 24 h (c and d) of culture. The results display that fibroblasts spread out on the nanostructures after 1 h of cell culture due to fibronectin coating, an extracellular matrix protein, support cell attachment and spreading out. These figures suggest that integrins truly attached onto single chitosan-based nanostructures and several integrins could gather onto single nanostructures. (e) Scheme of integrin attached onto chitosan-based nanostructures (nanosponges) with rhodamine fibronectin coating [88].

Figure 15. (a) Staining images of integrin of NIH-3T3 fibroblasts on flat and nanostructured chitosan surfaces after 48 h of culture. (b) Staining intensity of expressed integrin $\alpha1\beta1$ of single fibroblast *per* its adhesive area on flat and nanostructured chitosan membranes. Data are mean $\pm$ standard deviation (N = 5, n = 10) [88].

4.5. SINGLE-CELL CHITOSAN MICROARRAY

Micropatterning single cells at specific locations would be beneficial for probing cellular responses to drugs. A single-cell chitosan microarray with mixed flat and nanostructured surfaces was cast from a silicon mold that was fabricated through microfabrications including photolithography and Ag nanoparticle-assisted etching. First, photoresist was removed from the silicon mold, and then chitosan solution was cast onto the mold. This chitosan membrane was dried in an oven at 60 °C for approximately 6 h. The single-cell chitosan microarray was then peeled off of the silicon mold after soaking in 1 M NaOH. HeLa cells (cancer cells) were subsequently cultured on the surface of these microarrays for drug screening experiments (Figure 16). HeLa cells were treated with a chemical compound, cytochalasin D, which inhibited actin polymerization by disturbing actin microfilaments and causing cell apoptosis. Finally, in order to determine the percentage of apoptotic cells per total cells, annexin V-FITC was applied to label cells (Figure 17). The results show that approximately 59 % of the HeLa cells underwent apoptosis after 1 h with 10 mM cytochalasin D treatment, compared to merely 4 % apoptosis when HeLa cells were treated with cytochalasin D-free medium. In a final experiment, cytochalasin D was cleared away from cell cultures *via* washing with phosphate-buffered saline (PBS). Subsequently, these cells were incubated in cytochalasin D-free medium again for 1 h and for 24 h to examine actin microfilament morphology. The results show that, after just 1 h, recovered cells were still apoptotic. Nevertheless, the apoptosis percentage was reduced to approximately 30 % because the recovery duration was increased. This finding suggests that cells may re-enter the cell cycle and may have been able to reconstruct their filamentous actins.

Figure 16. The fabrication of single-cell chitosan microarray. Micropatterned silicon mold was fabricated *via* photolithography and chemical etching. And then the single-cell patterns on chitosan membrane were transferred from silicon mold *via* solution casting. After peeling from mold with NaOH solution, single-cell chitosan microarray was prepared for cell culture [88].

Figure 17. Drug screening applications on single-cell chitosan microarray. (a) Staining images of HeLa cells on microsquared chitosan microarrays. Color representation: F-actin (green), G-actin (red) and nucleus (blue). (b) Apoptotic cell percentage of HeLa cells cultured on a single-cell chitosan microarray and pristine chitosan membranes after 1 h of incubation with cytochalasin D treatment. Then, cytochalasin D was cleared away for next 1 h and 24 h incubation (recovery period). In the control group, HeLa cells were seeded in the medium without cytochalasin D treatment. Data are mean $\pm$ standard deviation. ($n = 20$, $N = 6$ (six samples)). (c) Staining images combined phase contrast images. HeLa cells were labeled with DAPI and Annexin V-FITC [88].

4.6. NANOROUGH GLASS SURFACES

Because the mechanosensitivity of embryonic stem cells (ESCs) to the physiological milieu remains unclear, some researchers have recently shown interest in regulating cell-nanoscale surface interactions to further explore stem cell response on material interfaces [89-91]. Among these pieces of research, Chen *et al.* developed an easy-to-handle microfabrication method for precisely controlling and micropatterning the nanoroughness of glass surfaces. The glass material, a common material used in cell culture, possesses the following advantages: 1) chemical ligands can be easily modified on its surface to promote cell affinity, 2) glass material is transparent, so it is beneficial for immediate observation *via* optical microscope, and 3) topographical modifications are easily fabricated on silica-based glass wafer surfaces *via* IC-compatible microfabrication. This study indicates that nanoroughness on glass surfaces could be useful for examining resultant regulatory signaling for different human embryonic stem cell (hESC) behaviors. Section 4.6 describes Chen's studies regarding the microfabrication of nanorough glass surfaces and their application for probing stem cell responses [92,93].

4.6.1. Microfabrication method for creating local nanoroughness

Specifically nanoroughed regions can be created on a glass wafer *via* IC-compatible microfabrication, including photolithography and reactive-ion etching (RIE) techniques. In Chen's studies, the photoresist layer was micropatterned onto each glass surface using photolithography. Then, the non-photoresist-protected regions with variously shaped designs were bombarded with RIE over different durations to generate randomly nanoscaled surfaces [94]. Note, the reactive ion species generated *via* SF_6 and C_4F_8 gases striking the unreactive glass substrate resulted in some defects, causing the glass surface to be reactive toward the etchant species. Finally, photoresist was removed by solvents, and the nanorough glass substrate was prepared for further stem culture purposes (Figure 18).

Figure 18. Fabrication of nanorough glass surface. The photoresist layer was micropatterned on a glass wafer surface *via* photolithography. Then, the glass wafer was fabricated *via* RIE process to generate the nano-scale surface. After the RIE process, photoresist was removed (stripped) with solvents. The nanorough glass substrate was prepared for further stem culture purposes [93].

4.6.2. Surface characterization of nanorough glass surfaces

Glass surfaces were processed with RIE at different durations in order to generate various levels of surface nanoroughness. Figure 19 shows statistical and scanning electron microscope (SEM) images displaying the different levels of nanoroughness produced by variations in glass substrate etching time. The roughness of the unprocessed glass surfaces, measured *via* atomic force microscope (AFM), was about 1 nm (the root-mean-square roughness, R_q). After the glass wafers were fabricated *via* RIE, the R_q ranged from 1–150 nm, which corresponded to different treatment durations. Furthermore, by precisely designing masks for photolithography, variously shaped nanorough islands could be achieved.

Figure 19. (a) The glass surfaces were processed with RIE at different durations in order to generate various levels of surface nanoroughness. (b) SEM images of micropatterned glass surfaces, including nanorough characters, square, circle, and triangle islands. (c) SEM images of glass surfaces with (middle and bottom) and without (top) treatments of RIE processes, with their rms nanoroughness indicated [93].

4.6.3. Cellular responses of hESCs on nanorough surfaces

Nanorough glass surfaces with different extents of nanoroughness were used as a growth substrate to probe functional responses of hESCs. First, the glass surfaces were coated with vitronectin to enhance self-renewal of hESCs. In terms of observation *via* scanning electron microscopy, hESCs showed highly branched, spreading morphology on a smooth glass surface ($R_q = 1$ nm) after 24 h of culture. However, hESCs showed short cytoplasmic extensions and less spreading on a nanorough glass surface ($R_q = 150$ nm). Furthermore, hESCs

displayed selectivity of cell immobilization between different extents of glass substrate nanoroughness. The results show that hESCs preferred to selectively adhere and aggregate onto the available smooth islands ($R_q = 1$ nm) rather than onto the nanorough regions ($R_q = 70$ nm) after 24 h of culture. Further, hESCs kept their stemness and showed positive expression of Oct3/4+ during the selective adhesion process (Figure 20a). Note, Oct3/4- hESCs, which expressed spontaneous differentiation, did not show any adhesion preference and preferred to randomly immobilize on the micropatterned glass surfaces (Figure 20b). With increased surface roughness, Oct3/4+ hESCs adhered less to surfaces regardless of treatment with Y27632, which is typically applied to promote survival of hESCs (Figure 20c). Figure 20d shows that hESCs were prone to self-renewal on the non RIE-treated glass surfaces after 7 days of culture.

Figure 20. (a) Phase-contrast images show that Oct3/4+ hESCs displayed selectivity of cell immobilization between different degrees of nanoroughness after 24 h of culture. (b) Phase-contrast images shows that Oct3/4- hESCs randomly attached on a micropatterned nanorough glass surface. (c) Adhesion percentage of hESCs on different roughness surfaces after 24 h of culture. (d) Rate of Oct3/4+ hESCs on the different level of nanoroughness glass surfaces after 7 days of culture [93].

4.6.4. Coculture system on nanorough glass surfaces

To further explore whether other cell types displayed responses similar to those of hESCs regarding glass surface roughness, NIH/3T3 fibroblasts were seeded onto the nanorough glass surface for examination. The results show that NIH/3T3 fibroblasts preferred to attach to the micropatterned nanorough islands with different geometries (Figure 21a, 21b). The micropatterned

nanorough glass surfaces could be used to control the adhesion location of NIH/3T3 fibroblasts and the adhesion selection of NIH/3T3 fibroblasts was contrary to that of hESCs. Intriguingly, these differences in cell preference, *i.e.*, the desire to attach onto flat or nanorough surfaces, offered a strategy to part different cell types for coculture systems. The results show that hESCs and NIH/3T3 fibroblasts selectively attach onto flat and nanorough regions after 48 h of culture (Figure 21c). Statistical analysis indicates high cell separation efficiency when using micropatterned nanorough glass surfaces to separate hESCs and NIH/3T3 fibroblasts (Figure 21d).

Figure 21. (a) Phase-contrast images of clusters of NIH/3T3 fibroblasts adhering onto nanorough islands ($R_q = 70 \text{ nm}$) with various patterns after 24 h of culture. (b) Cell adhesion percentage of NIH/3T3 fibroblasts on the flat glass surface ($R_q = 1 \text{ nm}$) and the nanorough glass islands ($R_q = 70 \text{ nm}$). (c) Merged optical microscopic image of coculture Oct3/4+ hESCs and NIH/3T3 fibroblasts spatially separating on a micropatterned nanorough glass surface after 48 h of culture. Cells were stained for nucleus (DAPI, blue) and Oct3/4+ (red). (d) Percentages of NIH/3T3 fibroblasts and hESCs located on the flat ($R_q = 1 \text{ nm}$) and nanorough ($R_q = 70 \text{ nm}$) regions of the micropatterned nanorough glass surfaces after 48 h of culture [93].

4.7. CONCLUSION

This chapter describes three types of new nanomaterial-based, *in vitro* cell culture platforms for modifying surfaces through IC-based microfabrication techniques, all of which display mass production potential. First, oxidized silicon nanosponges can be easily fabricated with various micro-/nano-surface patterns and have been shown to provide an easy-to-use, high-throughput *in vitro* platform for obtaining comprehensive insight into cell morphogenesis and exploring cellular responses in biomimicking environments. Secondly, chitosan material, a US FDA / US Environmental Protection Agency (US EPA)-approved biomaterial, demonstrates a wide range of potential applications as an implantable material for organ transplants and wound dressing. More importantly here, we found that single-cell chitosan microarrays, which can be cast from silicon molds, are useful for generating micro-/nano-structures on soft material surfaces that can subsequently be used for drug screening applications. Furthermore, chitosan membranes can also be functionalized through chemical modification to probe cellular behaviors. Thirdly, nanorough glass surfaces can be fabricated *via* microfabrication strategies to provide an efficient regulatory signal over different hESC behaviors as well as provide a selectively separating coculture system. We believe that silicon wafers, chitosan, and glass nanomaterial-based platforms are beneficial for creating inexpensive but stable *in vitro* cell culture devices for biomedical engineering and regenerative medicine.

REFERENCES

1. U. Vermesh, O. Vermesh, J. Wang, G.A. Kwong, C. Ma, K. Hwang, J.R. Heath. *Angew. Chem. Int. Edn. Engl.* **50** (2011) 7378–7380.
2. M. Sato, T.J. Webster. *Nanobiotechnology* **1** (2004) 105–114.
3. M.M. Stevens, J.H. George. *Science* **310** (2005) 1135–1138.
4. R.M. Bellin, J.D. Kubicek, M.J. Frigault, A.J. Kamien, R.L. Steward Jr., H.M. Barnes, M.B. DiGiacomo, L.J. Duncan, C.K. Edgerly, E.M. Morse, C.Y. Park, J.J. Fredberg, C.M. Cheng, P.R. LeDuc. *Proc. Natl. Acad. Sci. U. S. A.* **106** (2009) 22102–22107.
5. X.Y. Jiang, D.A. Bruzewicz, A.P. Wong, M. Piel, G.M. Whitesides. *Proc. Natl. Acad. Sci. U. S. A.* **102** (2005) 975–978.
6. C.S. Chen, M. Mrksich, S. Huang, G.M. Whitesides, D.E. Ingber. *Science* **276** (1997) 1425–1428.
7. D. Pesen, D.B. Haviland. *Appl. Mater. Interfaces* **1** (2009) 543–548.
8. E.V. Romanova, S.P. Oxley, S.S. Rubakhin, P.W. Bohn, J.V. Sweedler. *Biomaterials* **27** (2006) 1665–1669.
9. B.G. Keselowsky, D.M. Collard, A.J. García. *Biomaterials* **25** (2004) 5947–5954.
10. J.A. Neff, K.D. Caldwell, P.A. Tresco. *J. Biomed. Mater. Res.* **40** (1998) 511–519.
11. Y. Arima, H. Iwata. *Biomaterials* **28** (2007) 3074–3082.
12. K. Webb, V. Hlady, P.A. Tresco. *J. Biomed. Mater. Res.* **41** (1998) 422–430.
13. P. Van der Valk, A.W.J. Van Pelt, H.J. Busscher, H.P. De Jong, C.R. Wildevuur, J. Arends. *J. Biomed. Mater. Res.* **17** (1983) 807–817.

14. S. Qi, C. Yi, S. Ji, C.C. Fong, M. Yang. *Appl. Mater. Interfaces* **1** (2009) 30–34.
15. S.P. Low, N.H. Voelcker, L.T. Canham, K.A. Williams. *Biomaterials* **30** (2009) 2873–2880.
16. C.H. Choi, S.H. Hagvall, B.M. Wu, J.C.Y. Dunn, R.E. Beygui, C.J. Kim. *Biomaterials* **28** (2007) 1672–1679.
17. A.V. Sapelkin, S.C. Bayliss, B. Unal, A. Charalambou. *Biomaterials* **27** (2006) 842–846.
18. M.J. Dalby, M.O. Riehle, D.S. Sutherland, H. Agheli, A.S.G. Curtis. *Eur. Cells Mater.* **9** (2005) 1–8.
19. M.J. Dalby, S. Childs, S.J. Yarwood, M.O. Riehle, H.J.H. Johnstone, S. Affrossman, A.S.G. Curtis. *Biomaterials* **24** (2003) 927–935.
20. A.S. Andersson, F. Backhed, A. von Euler, A. Richter-Dahlfors, D. Sutherland, B. Kasemo. *Biomaterials* **24** (2003) 3427–3436.
21. K.B. Lee, S.J. Park, C.A. Mirkin, J.C. Smith, M. Mrksich. *Science* **295** (2002) 1702–1705.
22. R. Langer, J.P. Vacanti. *Science* **260** (1993) 920–926.
23. R. Singhvi, A. Kumar, G. Lopez, G.N. Stephanopoulos, D.I.C. Wang, G.M. Whitesides. *Science* **264** (1994) 696–769.
24. I. Firkowska, M. Olek, N. Pazos-Perez, J. Rojas-Chapana, M. Giersig. *Langmuir* **22** (2006) 5427–5434.
25. J.C. Culver, J.C. Hoffmann, R.A. Poché, J.H. Slater, J.L. West, M.E. Dickinson. *Adv. Mater.* **24** (2012) 2344–2348.
26. K. Pataky, T. Braschler, A. Negro, P. Renaud, M.P. Lutolf, J. Brugger. *Adv. Mater.* **24** (2012) 391–396.
27. D.F. Williams. *Biomaterials* **30** (2009) 5897–5909.
28. S. Even-Ram, K.M. Yamada. *Curr. Opin. Cell Biol.* **17** (2005) 524–532.
29. P. Friedl, K.S. Zänker, E.B. Brücker. *Microsc. Res. Tech.* **43** (1998) 369–378.
30. S.C. Marks Jr. *Am. J. Anat.* **141** (2005) 329–340.
31. R.D. Mundo, M. Nardulla, A. Milella, P. Favia, R. d’Agostino, R. Gristina. *Langmuir* **27** (2011) 4914–4921.
32. D.H. Kim, P. Kim, I. Song, J.M. Cha, S.H. Lee, B. Kim, K.Y. Suh. *Langmuir* **22** (2006) 5419–5426.
33. W.H. Chen, S.J. Cheng, J.T.C. Tzen, C.M. Cheng, Y.W. Lin. *PLOS ONE* **8** (2013) 8:e83394.
34. S.Y. Chou, C.M. Cheng, P.R. LeDuc. *Biomaterials* **30** (2009) 3136–3142.
35. N.D. Leipzig, M.S. Shoichet. *Biomaterials* **30** (2009) 6867–6878.
36. A.J. Engler, S. Sen, H.L. Sweeney, D.E. Discher. *Cell* **126** (2006) 677–689.
37. S. Even-Ram, V. Artym, K.M. Yamada. *Cell* **126** (2006) 645–647.
38. L. Prodanov, J. Te Riet, E. Lamers, M. Domanski, R. Luttge, J.J. van Loon, J.A. Jansen, X.F. Walboomers. *Biomaterials* **31** (2010) 7758–7765.
39. C.M. Cheng, R.L. Steward Jr, P.R. LeDuc. *J. Biomech.* **42** (2009) 187–192.
40. S.Y. Chou, C.M. Cheng, P.R. LeDuc. *Biomaterials* **30** (2009) 3136–3142.
41. B.G. Keselowsky, A.W. Bridges, K.L. Burns, C.C. Tate, J.E. Babensee, M.C. LaPlaca, A.J. García. *Biomaterials* **25** (2007) 3626–3631.
42. I. Poudel, J.S. Lee, L. Tan, J.Y. Lim. *Acta. Biomater.* **9** (2013) 4592–4598.
43. S.B. Jun, M.R. Hynd, N. Dowell-Mesfin, K.L. Smith, J.N. Turner, W. Shain, S.J. Kim. *J. Neurosc. Meth.* **160** (2007) 317–326.
44. A.K. Vogt, L. Lauer, W. Knoll, O. Andreas. *Biotechnol. Progr.* **19** (2003) 1562–1568.

45. S.V. Graeter, J. Huang, N. Perschmann, M. López-García, H. Kessler, J. Ding, J.P. Spartz. *Nano. Lett.* **7** (2007) 1413–1418.
46. S. Jungbauer, R. Kemkemer, H. Gruler, D. Kaufmann, J.P. Spatz. *Chem. Phys. Chem* **5** (2004) 85–92.
47. Y. Shao, J.M. Mann, W. Chena, J. Fu. *Integr. Biol.* **6** (2014) 300–311.
48. Z. Fan, Y. Sun, D. Chen, D. Tay, W. Chen, C.X. Deng, J. Fu. *Sci. Rep.* **3** (2013) 2176.
49. R.H.W. Lam, S. Weng, W. Lu. J. Fu. *Integr. Biol.* **4** (2012) 1289–1298.
50. J.M. Mann, R.H.W. Lam, S. Weng, Y. Sunab, J. Fu. *Lab. Chip* **12** (2012) 731–740.
51. A. Ferrari, M. Cecchini, M. Serresi, P. Faraci, D. Pisignano, F. Beltram. *Biomaterials* **31** (2010) 4682–4694.
52. M. Cecchini, A. Ferrari, F. Beltram. *J. Phys. Conf. Ser.* **100** (2008) 012003.
53. A. Bédier, C. Vieu, F. Arnauduc, J.C. Sol, I. Loubinoux, L. Vaysse. *Biomaterials* **33** (2012) 504–514.
54. J.N.H. Shepherd, S.T. Parker, R.F. Shepherd, M.U. Gillette, J.A. Lewis, R.G. Nuzzo. *Adv. Funct. Mater.* **21** (2011) 47–54.
55. R.A. Barry III, R.F. Shepherd, J.N. Hanson, R.G. Nuzzo, P. Wiltzius, J.A. Lewis. *Adv. Mater.* **21** (2009) 2407–2410.
56. M. Ghibaudo, L. Trichet, J.L. Digabel, A. Richert, P. Hersen, B. Ladoux. *Biophys. J.* **97** (2009) 357–368.
57. O.D. Roure, A. Saez, A. Buguin, R.H. Austin, P. Chavrier, P. Silberzan, B. Ladoux. *Proc. Natl. Acad. Sci. U.S.A.* **102** (2005) 2390–2395.
58. F. Haq, V. Anandan, C. Keith, G. Zhang. *Int. J. Nanomed.* **2** (2007) 107–115.
59. Z.Q. Feng, X.H. Chu, N.P. Huang, M.K. Leach, G. Wang, Y.C. Wang, Y.T. Ding, Z.Z. Gu. *Biomaterials* **31** (2010) 3604–3612.
60. M. Merz, P. Fromherz. *Adv. Funct. Mater.* **5** (2005) 739–744.
61. M. Merz; P. Fromherz. *Adv. Mater.* **14** (2002) 141–144.
62. S.K.W. Dertinger, X. Jiang, Z. Li, V.N. Murthy, G.M. Whitesides. *Proc. Natl. Acad. Sci. U.S.A.* **99** (2002) 12542–12547.
63. D.W. Branch, B.C. Wheeler, G.J. Brewer, D. Leckband. *IEEE Eng. Med. Biol. Mag.* **47** (2000) 290–300.
64. J.D. Hoff, L.J. Cheng, E. Meyhofer, L.J. Guo, A.J. Hunt. *Nano Lett.* **4** (2004) 853–857.
65. D.J. Kim, K.B. Lee, T.G. Lee, H.K. Shon, W.J. Kim, H.J. Paik, I.S. Choi. *Small* **1** (2005) 992–996.
66. K.Y. Suh, J. Seong, A. Khademhosseini, P.E. Laibinis, R. Langer. *Biomaterials* **25** (2004) 557–563.
67. R. Kane, S. Takayama, E. Ostuni, D.E. Ingber, G.M. Whitesides. *Biomaterials* **20** (1999) 2363–2376.
68. E. Kim, Y. Xia, G.M. Whitesides. *Nature* **376** (1995) 581–584.
69. A. Folch, M. Toner. *Biotech. Prog.* **14** (1998) 388–392.
70. Y. Xia, G.M. Whitesides. *Angew. Chem. Int. Ed.* **37** (1998) 550–575.
71. T. Matsuda, T. Sugawara. *J. Biomed. Mater. Res.* **29** (1995) 749–756.
72. C.Y. Yang, L.Y. Huang, T.L. Shen, J.A. Yeh. *Eur. Cells Mater.* **20** (2010) 415–430.
73. C.Y. Yang, T.C. Liao, H.H. Shuai, T.L. Shen, J.A. Yeh, C.M. Cheng. *Biomaterials* **33** (2012) 4988–4997.
74. J.Y. Chyan, W.C. Hsu, J.A. Yeh. *Opt. Express* **17** (2009) 4646–4651.
75. K. Peng, J. Hu, Y. Yan, Y. Wu, H. Fang, Y. Xu, S.T. Lee, J. Zhu. *Adv. Funct. Mater.* **16** (2006) 387–394.

76. B. Kobrin, J. Chinn, W.R. Ashurst, R. Maboudian. *Proc. SPIE* **5716** (2005) 151–157.
77. K. Webb, V. Hlady, P.A. Tresco. *J. Biomed. Mater. Res.* **49** (2000) 362–368.
78. L. Chou, J.D. Firth, V.J. Uitto, D.M. Brunette. *J. Cell Sci.* **108** (1995) 1563–1573.
79. R. Jahn, T. Lang, T.C. Südhof. *Cell* **112** (2003) 519–533.
80. Q. Ao, C.K. Fung, Y. Tsui. *Biomaterials* **32** (2011) 787–796.
81. I.Y. Kim, S.J. Seo, H.S. Moon, M.K. Yoo, I.Y. Park, B.C. Kim, C.S. Cho. *Biotech. Adv.* **26** (2008) 1–21.
82. K.E. Crompton, J.D. Goud, R.V. Bellamkonda, T.R. Gengenbach, D.I. Finkelstein, M.K. Horne, J.S. Forsythe. *Biomaterials* **28** (2007) 441–449.
83. V.R. Sinha, A.K. Singla, S. Wadhawan, R. Kaushik, R. Kumria, K. Bansal, S. Dhawan. *Int. J. Pharm.* **274** (2004) 1–33.
84. M. Cheng, J. Deng, F. Yang, Y. Gong, N. Zhao, X. Zhang. *Biomaterials* **24** (2003) 2871–2880.
85. A. Saber, S.P. Strand, M. Ulfendahl. *Eur. J. Pharm. Sci.* **39** (2010) 110–115.
86. Q. Hu, B. Li, M. Wang, J. Shen. *Biomaterials* **25** (2004) 779–785.
87. C.Y. Yang, C.Y. Sung, H.H. Shuai, C.M. Cheng, J.A. Yeh. *Sci. Technol. Adv. Mater.* **14** (2013) 044406.
88. H.H. Shuai, C.Y. Yang, H.I.C. Harn, R.L. York, T.C. Liao, W.S. Chen, J.A. Yeh, C.M. Cheng. *Chem. Sci.* **4** (2013) 3058–3067.
89. R.J. McMurray, N. Gadegaard, P.M. Tsimbouri, K.V. Burgess, L.E. McNamara, R. Tare, K. Murawski, E. Kingham, R.O.C. Oreffo, M.J. Dalby. *Nat. Mater.* **10** (2011) 637–644.
90. E.K.F. Yim, E.M. Darling, K. Kulangara, F. Guilak, K.W. Leong. *Biomaterials* **31** (2010) 1299–1306.
91. S. Oh, K.S. Brammer, Y.S.J. Li, D. Teng, A.J. Engler, S. Chien, S. Jin. *Proc. Natl. Acad. Sci. U. S. A.* **106** (2009) 2130–2135.
92. W. Chen, S. Weng, F. Zhang, S. Allen, X. Li, L. Bao, R.H.W. Lam, J.A. Macoska, S.D. Merajver, J. Fu. *ACS Nano* **7** (2013) 566–575.
93. W. Chen, L.G.V. Diaz, Y. Sun, S. Weng, J.K. Kim, R.H.W. Lam, L. Han, R. Fan, P.H. Krebsbach, J. Fu. *ACS Nano* **6** (2012) 4094–4103.
94. E. Metwalli, C.G. Pantano. *Nucl. Instrum. Meth. B* **207** (2003) 21–27.

A CHITIN NANOFIBRIL-BASED NON-WOVEN TISSUE AS A MEDICAL DRESSING: THE ROLE OF BIONANOTECHNOLOGY

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5.1. INTRODUCTION

The *Stratum Corneum* (SC), the outermost layer of keratinized cells, called corneocytes, is a specific skin protective barrier consisting of an intercellular lipid-like substance with a lamellar crystalline gel structure, which acts as a mortar between the corneocytes. It is highly active in lipid enzymatic synthesis and has the ability to adapt to the environment (Figure 1) [1].

Figure 1. Lamellar structure of *Stratum Corneum*

Barrier recovery and skin homeostasis, in fact, are the result of restoration of these lipids, that also involves control and normalization of keratinocyte turnover (Figure 2) [2]. When barrier perturbation becomes persistent, the processes of lipid and keratin synthesis can escalate, and if it becomes chronic, a pathogenic sequence of hypertrophic scars and keloids may appear in wound healing [3].

Figure 2. Skin homeostasis with keratinocyte turnover

Defects created by severely damaged skin caused by large burns or chronic wounds, modify the synthesis of the extra cellular matrix (ECM) and therefore impede the skin's ability to breathe, retain or expel water, and defend itself against harmful bacteria, oxidants and toxins. Thus, the main function of the SC is to provide protection for preserving the body from external insult. In fact, the skin represents the first line of defence and is the guardian of the body's organs; as a consequence, when the skin is burned beyond a certain percentage of total body area, death may occur. This is the reason why injured skin needs to be immediately covered with a dressing capable of restoring tissue integrity, maintaining homeostasis, and preventing invasion of toxic substances and pathogens. The four main goals of burn wound care are, in fact: (a) prevention of infection, (b) maintenance of a moist environment, (c) protection of the wound from external aggressions, and (d) reduction of scar formation [4].

Therefore, a medical dressing has to establish a barrier to environmental irritants, impede microbial growth, maintain a moist environment, and allow exchange of gaseous and nutritive ingredients. Moreover, it should not adhere to the wound, in order to allow new tissue growth, and it must be easily removed. For this purpose, specialized non-woven tissues made from engineered biomaterials are used that are biocompatible, non-allergenic, and non-toxic. They also promote wound healing because they can modulate ECM synthesis and regulate microbial growth.

In addition, the application of appropriate fibres capable of making the non-woven tissues free of binder and chemicals, and of carrying active ingredients for tissue repair and tissue regeneration, are becoming important concerns in the field of medical textiles. For better skin tissue regeneration, in fact, natural

polysaccharides like chitin or chitosan are required (Figure 3), since they seem to promote production and deposition of ECM in a tissue-specific way [5].

Figure 3. The polysaccharide chemical structures of cellulose, chitin and chitosan

Chitin, in fact, is the second most abundant biopolymer after cellulose and is found mainly in the exoskeletons of shellfish and insects, and in the cell walls of mushrooms. When nanostructured, it is considered to have great potential for application in tissue engineering scaffolds, drug delivery and dressings, as well. For this purpose, Morganti *et al.* developed and patented a single closed method for the preparation of chitin nanofibrils (CN) as shown in Scheme 1.

Scheme 1. Chitin nanofibril production cycle

It has been shown that this natural polymer, when nanostructured and electrospun, organized as a porous ECM-like structure (Figure 4), allowing cells to be seeded; thus having favourable biological properties for tissue regeneration [6-8].

Figure 4. ECM-like porous structure of a non-woven tissues based on the use of CN at scanning electron microscope (SEM)

For these reasons, our group has produced non-woven tissues based on CN cross-linked with hyaluronic acid (HA) by use of the gelation method and electrospinning technology (Figure 5) [9].

Figure 5. Non-woven tissues have been obtained by the gelation method and electrospinning technology, using HA and CN block-copolymeric nanoparticles.

The advantage of these natural CN-based non-woven tissues over the others is the capacity to control the thickness and composition of nanofibres, as well as the porosity of the obtained meshes. It is possible, in fact, to synthesize fibres of the desired diameters by the simplest and most economical method. The pore size and density, in reality, play an important role in many biomedical applications, resembling the architecture of the ECM [10]. The main parameters in controlling the diameter and uniformity of the electrospun nanofibres are: polymer molecular weight and concentration, viscosity, surface tension and conductivity of the solution / suspension, and the voltage selected. Thus, electrospinning, which also provides the possibility of combining different nanomaterials (NM) to produce polymeric nanocomposites, offers enormous advantages over traditional non-woven tissues made by macro-or-micro sized polymers.

5.2. THE MARKET

As recently reported [11], the available medical non-woven market is projected to reach US \$20 billion by 2017, and the market size for nanotechnology is expected to grow to over US \$3 trillion [12]. In these growing markets, "non-woven tissues remain the component of choice for providing appropriate protection to the skin, due to their ability to create barriers either from the structure of the non-woven itself or from an additional active coating for personal protective apparel" [13].

According to this report, advanced medications are used to a large extent in healthcare for making products designed to provide appropriate "*protective barriers*" between the patient and himself, patient and physician, and between different patients. The reason for such barriers is to reduce the spread of hospital infections, which are still rising worldwide along with antibiotic-resistance.

What about the production locations? Even though China is the current leading global exporter of non-woven roll goods, for the future of manufacturing, converting and using these medical dressings will represent potential opportunities of growth for the home markets, primarily because of geopolitical instabilities, unpredictable fuel costs, and increasing regulations [14,15]. In any case, to be competitive in the global market, product innovation is necessary, especially to enhance the quality of life of elderly or disabled people [16]. In the wound care industry, the goal is to create thinner dressings that are made by sustainable bionanotechnology with the use of active and green nanoparticles that have similar absorption properties as foam dressings.

Incoming wound care dressings must provide the ability to assess the speed and progress of wound healing, and permit the use of antimicrobial agents to minimize the potential for infections. In this field, nanotechnology may have a positive and strong impact. To this end, the possibility of directly binding

antimicrobial components, such as Ag^+ ions or other active ingredients, into the CN fibre before producing the non-woven tissue has been shown by electrospinning technology [17]. With this new technique it has been possible to reduce the concentration of Ag^+ ions in the final product, and strongly reduce its potential toxicity, and its environmental impact while maintaining its antibacterial effectiveness. However, the industrial sustainability of the entire life cycle of any medical dressing has to involve the use of safe, biodegradable, and recyclable active ingredients and fibres, causing less burden overall on human beings and the environment.

5.3. CHITIN NANOFIBRIL-HYALURONIC ACID NANOPARTICLES

The use of CN, HA and their derivatives are gaining popularity because they are natural polymers produced from the by-products of fisheries, according to the European Union (EU) and Organisation for Economic Co-operation and Development (OECD) programs for a forthcoming greener economy [18,19]. Both of these polymers are totally biodegradable, biocompatible, ecologically-friendly, and compostable. It is interesting to underline that, while HA is a polymer distributed along our body, as a fundamental component of the ECM, chitin has the same backbone (Figure 6) and is an important component of human cartilage that is necessary for bone articulation. For instance, chitin's reduced synthesis during the aging process is one of the causes of osteoporosis (Figure 7).

Figure 6. Chitin has the same backbone of HA

Figure 7. The reduced synthesis of glucosamine is one of the cause of osteoporosis: right normal tissue, left osteoblasts tissue of a patient affected by osteoporosis

Nonetheless, it is important to remember that while the environment is rich in chitinase enzymes, capable of metabolizing chitin-derived compounds, the human body possesses 18 families of the same enzyme, named chitotridase [20], which are necessary to catatabolize chitin / chitosan into its components: glucosamine and acetyl glucosamine. These important molecules, useful for the synthesis of glycosaminoglycans (Figure 8), are also involved in the process of glycosylation, which is necessary to bond amino acids and glycoside compounds (Figure 9).

This is the reason that CNs are non-toxic and completely safe for both humans and for the environment, when recovered as composites of the micro dimension or as single components of nanodimensions.

Figure 8. Glucuronic acid, a component of HA, and glucosamine, a component of chitin are molecules necessary to produce glycosaminoglycans

Figure 9. Physiological process of glycosylation involving glucose and *N*-acetyl glucosamine

The block copolymeric micro / nanoparticles CN-HA have been obtained by the gelation method in distilled water followed by refinement of the micro lamellae, as the result of the combination of electropositive CN and electronegative HA (Figure 10). Control of their size and dimensions has been verified by a Zetasizer and by SEM [21,22]. It is interesting to underline that during the gelation method, before mixing together the two water suspensions of CN/HA, different active ingredients, both water and oil-soluble, may be solubilised in advance by the use of surface active agents. According to the designed method, the nanoparticles can entrap the ingredients into their structure before the refinement process. Moreover, it is possible to regulate the size of the particles and the surface electrical charges, thereby modulating their penetrability towards targeted delivery. The obtained nanoparticles, in fact, have shown an ability to disturb the SC *lamellae* and increase their diffusion through the skin layers, because of the cationic character of CN.

Figure 10. Copolymeric nano *lamellae* and nanoparticles obtained by complexing CN with HA, by SEM

During the patented chitin nanocrystals (nanofibrils) production process, the final fibril composition is about 30 units of glucosamine / acetyl glucosamine in about a 1 : 1 ratio, in contrast to natural chitin in crustaceans where acetyl glucosamine is predominant (Figure 11).

Figure 11. Chemical structures of CN and Chitosan. When the degree of *N*-acetylation (DA) is greater than 50 %, the polysaccharide is considered to be CN. When the DA is less than 50 %, the polysaccharide is considered to be chitosan.

For this reason, both CN and chitosan, which are positively charged, can form a complex with many types of negatively-charged polymers and large molecules, such as HA or lignin.

This property of CN-based composites has been shown to be significant in the modulation of cell behaviour during tissue regeneration [23-25]. It seems possible that positively-charged CN, interacting with negatively charged glycosaminoglycans and proteoglycans, fundamental components of the ECM, may have an important role in tissue regeneration. This could be the reason for

the regenerative activity in repairing burned skin that was recently shown by the use of non-woven tissues made by CN as the main component (Figure 12) [26].

Figure 12. Repairing activity on a burned skin obtained by the use of a non-woven tissue made by CN bonded with Ag^+ ions

Of course the final composition of the obtained nanoparticles is fundamental to their activity, for example, at the skin level. Following exposure, in fact, some NM have been shown to penetrate beyond the skin, with the extent of penetration being dependent on the ability of the NM to cross biological barriers. However, skin penetration is also a function of NM size and other properties, such as surface charge, as well as formation of the protein or lipid corona on the NM surface [27]. Moreover, the excellent mechanical properties of CN combined with its good gas barrier properties also may find many applications in the food packaging industry. Thus, as a producer of CN, our research group participated in the EU project n-CHITOPACK (www.n-Chitopack.eu).

5.4. CONCLUSIVE REMARKS

Nanoscience constitutes a challenging scientific frontier capable of engineering materials on the scale of a nanometer, *i.e.* a billionth of a meter (Figure 13.), revolutionizing modern life. Thus, nanotechnology, as one of the key enabling technologies (KETs) identified by the EU commission [28] for contributing to sustainable development of high-tech applications, is expected to stimulate industrial growth innovation and development, not only in electronics but also in plant growth, in food packaging, and in cosmetic and pharmaceutical fields (Scheme 2). It has been predicted, therefore, that by 2020 nearly every industry will be affected by this new scientific frontier.

Figure 13. The nanometer scale

On the nanoscale, in fact, common materials can take on new physicochemical and biological properties, opening up new possibilities of exploitation for commercial small, medium enterprises (SMEs). For these reasons, the use of bionanomaterials is steadily increasing day by day due to the new properties addressed by the reduced dimensions of the ingredients used.

Scheme 2. Display of expected nanotechnology's stimulation of industrial growth innovation and development in electronics, in plant growth, in food packaging, and in cosmetic and pharmaceutical fields

This new technology particularly impacts industrial sectors, such as cosmetics, drugs and advanced medications, where safety and social health are important elements.

To this end, the last published data show a significant increase in the production of the most representative nanomaterials with an expected growth of 2 billion jobs by the end of 2015, and more than 1000 nano-enabled products currently available on the market in more than 20 different countries.

In this context, the EU is responsible for 30 % of nanomaterial manufacturing and use, including polymers containing nano-reinforcements. It is important to underline, in fact, that one of the main applications of nanotechnology in material science is the development of polymer nanocomposites, which are polymers reinforced with a low quantity of nanosized organic or inorganic ingredients dispersed into the polymer matrix.

5.5. ROLE OF CN AND CHITIN-DERIVATIVES IN COMPOSITE DEVELOPMENT

Thus, in composite manufacturing for the development of biocomponent fibres by meltdown (casting) and spunbound (electrospinning) technologies, the use of CN or CN-HA nanoparticles offers enormous advantages over traditional macro- or micro-sized fillers and applications across a wide range of industrial sectors, such as food packaging and advanced medications, as revealed by the recent results of the EU research projects n-CHITOPACK (www.n-Chitopack.eu), Bio-Mimetic (www.biomimetic.eu), and Chitofarma (www.mavicosmetics.it).

Through these projects, in which the MAVI Nanoscience Centre has been involved as an owner of CN technology and processes, thin transparent and flexible food-packaging based on the casting technology (Figure 14), hard food packaging for meat and / or coffee-caps (Figure 15), advanced medications and beauty masks (Figure 16) have been obtained.

Step by step preparation procedure of n-CHITOPACK films

Note: Poly(ethylene terephthalate) and Dura Lar films or stainless steel sheets were used as supports for casting the (CS/CN)/glycerol slurries

Figure 14. The casting production at lab level (By courtesy of G. Tischenko)

Figure 15. Coffee-caps and hard food containers made by PLA and CN

Figure 16. Advanced medications (right) and beauty masks (left) made by the use of CN

All of these containers, based on the use of CN, are totally biodegradable and compostable, and are safe for both humans and the environment. Moreover, it is interesting to underline how the mechanical properties of the films made of CN, chitosan and glycerol, generally used to temporary pack sandwiches, have shown more than three times higher elasticity and mechanical strength compared with those of cellulosic paper. On the other hand, hard containers made of poly(lactic acid) (PLA) reinforced by CN, have shown better resistance to the aging process, but still possess the same interesting compostability, and easy biodegradability recovered from the advanced medications also.

Thus the one-off use of this film and daily use of these hard food containers could decrease environmental pollution by the major utilization of chitin waste and reduced consumption of petrol-derived polymers. In addition, deforestation and the presence of greenhouse gas emissions also will be reduced because of the major use of the plant biomass.

In conclusion, the intricate composite network obtained by the meltdown and electrospun natural fibers, such as CN from crustacean waste and lignocellulosic compounds and manmade PLA from plant biomass, open new perspectives to produce promising biomimetic candidates for practical applications in biomedicine and tissue engineering [29], as well as in the food packaging field.

As previously reported, chitin-based biomaterials have favourable biological properties for tissue regeneration, since they are capable of restoring function and architecture of both aged and damaged skin [30]. These polysaccharide polymers play major roles in maintaining body contours by providing mechanical cushion and protection in recovering epithelium and soft tissue for their regeneration [31,32], making them safe for the human body and the environment.

In addition, it is important to underline the capacity that CN have shown for delivering active ingredients in the optimum dosage, increasing their bioavailability at skin level, and enhancing their efficacy compared to normal chitin, probably because of CN's nanodimensions of $240 \times 7 \times 5$ and needle-like crystal form (Figure 17).

Figure 17. CN by SEM

According to our first obtained results on the use of CN as a skin regenerative ingredient [33], new efforts of our group are focused on better understanding the activity of CN when used to make block copolymeric nanoparticles for pharmaceutical and cosmetic use, or to produce polymers for making

nanocomposites with other natural fibres for making biomedical non-woven tissues and/or innovative food packaging.

Our future goal is to use CN, lignin, and other lignocellulosic compounds to produce innovative anti-aging emulsions / beauty masks and regenerative advanced medications, as well as to use PLA for making innovative food packaging that is completely biodegradable and compostable.

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REFERENCES

1. P.M. Elias, G.K. Menon. *Adv. Lipid. Res.* **24** (1991) 1–26.
2. G.K. Menon, K.R. Feingold, M.P. Elias. *J. Invest. Dermatol.* **98** (1992) 279–289.
3. P.M. Elias, J.C. Ansel, L.C. Woods, K.R. Feingold. *Arch. Dermatol.* **132** (1996) 1505–1506.
4. M. Cho, T.K. Hunt, in *Cutaneous Wound Healing*, V. Falanga (Ed.) London, UK, Martin Dunitz, 2001, pp. 141–154.
5. T.L. Yang, T.H. Young. *Biomaterials* **29** (2008) 2501–2508.
6. M.V. Risbud, M. Sittering. *Trends Biotech.* **20** (2002) 351–356.
7. A. Di Martino, M. Sittering, M.V. Risbud. *Biomaterials* **26** (2005) 5983–5990.
8. C.E. Ayers, B.S. Jha, S.A. Sell, G.L. Bowlin, D.G. Simpson. *Wiley Interdiscip. Rev. Nanomed. Nanobiotech.* **2** (2010) 20–34.
9. D.H. Remember, I. Chun. *Nanotechnology* **7**(3) (1996) 216–223.
10. S.D. McCullen, S. Ramaswamy, L.I. Clarke, R.E. Gorga. *Wiley Interdiscip. Rev. Nanomed. Nanobiotechnol.* **1** (2006) 369–390.
11. O. Gia. Medical Nonwoven Disposables: A Global Strategic Business Report. Global Industry Analysts, Inc., San Jose, CA, March 2012.
12. Lux Research Inc., Nanomaterials of the Market. www.luxresearchinc.com (2009).
13. V.A. Barbur. The Future of Nonwovens in Medical Markets, http://www.nonwovens-industry.com/issues/2012-08/view_features/the-future-of-nonwovens-in-medical-markets (2012)
14. J. Prigneaux. Nonwoven Trade Reaches Record in 2011. *EDANA*. Nonwoven Industry, May 2012.
15. E. Krenzer, M. Evans. Stay Relevant in a High Volume, Commoditized, Medical Device Product Line in Manufacturing (Insourced and Outsourced). *Medical Device Manufacturing Summit*, Las Vegas, 2012.
16. H. Meinander, M. Varheenma. Clothing and Textiles for Disabled and Elderly People. *VTT Tiedotteita Research Notes*, 2002.
17. P. Morganti, G. Tishchenko, M. Palombo, I. Kelnar, L. Brozova, M. Spirkova, E. Pavlova, L. Kobera, F. Carezzi, in *Marine Cosmeceuticals. Trends and Prospects*, S.K. Kim (Ed.), New York, CRC-Press, 2013, pp. 681–715.

18. European Commission (EC), *Innovation for Sustainable Growth: A Bioeconomy for [europe.COM](#) final*, EC, Brussels, Belgium, 2012.
19. Organization for Economic Cooperation and Development (OECD), *The Bioeconomy to 2030: Designing a Policy Agenda, Main Findings*, OECD, Paris, France, 2009.
20. K.B. Eide, A.L. Norberg, E.B. Heggeset, A.R. Lindbom, K.M. Varum, V.G.H. Eijsink, M. Sorlie. *Biochemistry* **51** (2012) 487–495.
21. P. Morganti, P. Del Ciotto, G. Fabrizi, F. Guarneri, A. Cardillo, M. Palombo, G. Morganti. *SOFW-Journal* **139**(1/2) (2013) 12–23.
22. P. Morganti, G. Di Massimo, C. Cimini, P. Del Ciotto. *Pers. Care Eur.* **6**(9) (2013) 61–66.
23. P. Mezzana. *Acta Chirurgiae Investig. Dermatol.* **3** (2008) 5–13.
24. M. Mattioli-Belmonte, A. Zizzi, G. Lucarini, F. Giantomassi, G. Biagini, M.G. Tucci, F. Orlando, M. Provinciali, F. Carezzi, P. Morganti. *J. Bioact. Compat. Polym.* **22** (2007) 525–538.
25. P. Morganti, M. Mattioli-Belmonte, M.G. Tucci, G. Ricotti, G. Biagini. *J. Appl. Cosmetol.* **18** (2000) 125–130.
26. M. Palombo, Innovative Solutions for Advanced Medications: Biochemical and Clinical Data, *Proceeding of the 11th ISCD International Congress of Cosmetic Dermatology*, Beijing, China, 2014, p. 38.
27. F. Got Schalk, B. Nowack. *J. Environ. Monitoring* **13** (2011) 1145–1155.
28. E. Van de Velde, C- Rammer, P. Padilla, P. Scliessler, O. Slivke, B. Gehrge, V. Bilsen, R. Lukach, *Key Enabling Technologies*, European Union, Brussels, Belgium, 2012.
29. M. Wysokowski, A.L. Stelling, D. Stawski, T. Jesionowski, H. Ehrlich. *Polymers* **7** (2015) 235–265.
30. K. Hemmrich, D. Von Heimburg, R. Rendchen, C. Di Bartolommeo, E. Milella, N. Pallua. *Biomaterials* **26** (2005) 7025–7037.
31. K. Kohima, Y. Okamoto, K. Zkojima, K. Miyatake, H. Fujise, Y. Shigemasa, S. Minami. *J. Vet. Med. Sci.* **66** (2004) 1595–1598.
32. J. Sharma, M. Lizu, M. Stewart, K. Zygula, Y. Lu, R. Chaiman, X. Yan, Z. Guo, E.K. Wujcik, S. Wei. *Polymers* **12** (2015) 1936–1963.
33. P. Morganti, F. Carezzi, P. Del Ciotto, G. Morganti, M.L. Nunziata, Y.H. Gao, H.D. Chen, G. Tischenko, W.E. Yudin, in *Nanobiotechnology*, D.A. Phoenix, W. Ahmed (Eds.), Manchester UK, One Central Press, 2014, pp. 1–31.

**MAGNETOACTIVE ELECTROSPUN
NANOFIBRES IN TISSUE ENGINEERING
APPLICATIONS**

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6.1. OVERVIEW OF THE ELECTROSPINNING PROCESS

After the first observation of electrospinning (electrostatic spinning) by L. Rayleigh in 1897, followed by its first patenting in 1902 by J.F. Cooley and W.J. Morton, in 1914 J. Neleny reported that a liquid jet could be emitted from a charged liquid droplet in the presence of an electrical field [1]. Twenty years later, when A. Formhals patented a process that allowed the spinning of synthetic fibres using electric charges [2,3], electrospinning became a valid technique to produce small-sized fibres. Following Formhals' pioneering work, researchers have focused on an in-depth understanding of the electrospinning process. In 1969, D.G. Taylor published on the jet formation process, examining the behaviour of the polymer solution droplet at the edge of a capillary under an electric field. From his studies he obtained the characteristic value of the elongated conical fluid structure known as the Taylor cone [4], generated at the tip of the needle due to the electrostatic forces being exerted at the fluid's surface. Since the early 1990s, the work of D.H. Reneker and co-workers focusing on the use of electrospinning for generating one-dimensional (1D) polymer nanostructures has attracted the attention of many researchers [5-7].

Although other methods can also be employed towards the fabrication of 1D nanomaterials, namely drawing [8], self-assembly [9,10], melt-blow [11], phase-separation [10], and template synthesis [12], electrospinning is considered to be the most popular and versatile fibre fabrication method [13]. It can be used to produce continuous synthetically and naturally-derived polymer nanofibres [6,14,15] with diameters ranging from micrometers down to a few nanometers [14]. Its simplicity, cost-effectiveness and applicability, not only to polymers [14,16] and ceramics [17,18] but also to composites, enable the development of polymer-based fibrous nanocomposites *via* the combination of polymers with inorganic nanofillers [19].

Although experimentally the electrospinning process is relatively simple and straightforward, the electrospinning mechanisms are rather complicated, including among others the Taylor cone theory [20], the bending instability [21] and the electrically forced jet-stability theory [22].

A simple electrospinning set-up consists of four major components: A high-voltage power supply, a syringe with a metallic needle, known as a spinneret, a syringe pump used for delivering the solution through the spinneret at a constant and controllable rate, and a grounded metallic (conductive) collector, on which the produced fibres accumulate (Figure 1).

Direct current (DC) power supplies are typically used in electrospinning, although the use of alternating current (AC) potential is also feasible. A high-voltage power supply is connected with both the needle and the collector. The positive electrode (anode) is connected to the needle and the negative electrode (cathode) is placed onto the collector, which is usually grounded. In a

typical fibre-generating process, a syringe is filled with a polymer solution and a high voltage (up to 30 kV) is applied between the syringe nozzle and the collector. The syringe may be placed perpendicularly, letting the polymer fluid drop with the help of gravity, or horizontally in respect to the grounded collector.

By applying a high voltage, the pendent drop of the polymer solution at the needle tip of the spinneret is highly electrified and the induced positive charges are evenly distributed over the droplet surface. The electrostatic repulsion forces developed between the surface charges and the electrostatic force exerted by the external electric field are the major electrostatic forces acting on the droplet. Under these electrostatic interactions the hemispherical surface of the fluid at the tip of the needle's syringe elongates, forming the Taylor cone. At a critical voltage, *i.e.*, once the strength of the electric field has surpassed a threshold value, the electrostatic repulsive forces overcome the surface tension of the solution and force a jet to erupt from the tip of the Taylor cone. The jet follows a direct path towards the grounded collector for a very short distance from its origin and reaches a bending instability point. After this point the jet undergoes a stretching and a rapid whipping process, as illustrated in Figure 1.

As the charged jet accelerates towards lower-potential regions, it dries in flight upon solvent evaporation, whereas the increase in the electrostatic repulsion forces generated between the charged polymer chains results in fibre elongation and consequently reduction in fibre diameters. With the combination of jet-bending instability and solvent evaporation, the jet eventually becomes solidified on the collector in the form of randomly oriented nanofibres.

Figure 1. Schematic presentation of a basic electrospinning set-up

As long as a polymer can be electrospun into nanofibres, ideally the diameters of the fibres should be consistent and controllable, the fibre surface defect-free or defect-controllable, and continuous single fibres should be collectable. However, research performed during the last 15 years on polymer processing *via* electrospinning has shown that the aforementioned are by no means easily achievable [14] and that the success of the whole process is governed by different parameters. The latter are classified in terms of: (a) Solution parameters, including viscosity / concentration [16,23-25], conductivity / solution charge density [15,26-29], surface tension [16,23,30-32], polymer molecular weight [23,33,34], dipole moment, and dielectric constant [14,23,35,36]; (b) Processing parameters, including electrical potential [36-38], solution flow rate [23,39], needle diameter [7,40,41], and distance between the syringe needle tip and the grounded collector [14,15,24,42,43]; (c) Ambient parameters, including solution temperature, humidity and air velocity in the chamber; and finally (d) Collector composition, geometry and motion [15,16,23,35]. Based on the above parameters, different results may be obtained using the same polymer system and electrospinning set-up. Thus, it is difficult to provide quantitative relationships that can be applied across a broad range of polymer / solvent systems. However, there are general trends which are useful when determining the optimum conditions for a certain system, which are summarized in Table 1 [44].

Table 1. Influencing parameters on the fibre morphology in the electrospinning process

Solution properties	
<u>Concentration / Polymer molecular weight / Viscosity</u> Low concentration / viscosity leads to beaded fibres and droplets. Fibres with fewer beads and free of droplets are obtained by increasing the concentration / viscosity. Low polymer molecular weight / viscosity (regardless of concentration) also generates beaded fibres, whereas polymers of extra high molecular weight are more difficult to spin. Fibres generated from viscous solutions appear to be relatively continuous and thicker compared to fibres generated from low viscosity solutions, which tend to be shorter and finer.	[14,26,45,46] [23,32-34] [25,36,47]
<u>Conductivity / Solution charge density</u> Thinner fibre diameters with fewer beads are obtained upon increasing conductivity whereas the tendency of droplet formation during the process is reduced. The increase of the net charge density can be realized <i>via</i> the addition of salts in the polymer solution.	[27-29] [16,35,39]
<u>Surface tension</u> By reducing the surface tension of the polymer solution, fibres can be	[5,14,46,48]

Solution properties	
obtained without beads.	[16]
Solutions with lower surface tension lead to the effective elimination of beads and the generation of fibres with larger diameters.	[14,36]
Different solvents may contribute differently to the surface tension.	
<u>Dipole moment and dielectric constant</u>	
Solvents with high dipole moment values result in the successful fabrication of continuous electrospun fibres.	[49]
Process parameters	
<u>Applied voltage</u>	
The increase of the applied field (related to the change in the instability mode – change in the shape of the jet initiating point) causes an increase in fibre length.	[7,28,36]
The jet diameters are also affected by applied voltage changes.	
Although initially they seem to decrease with increasing voltage, further increase results in thicker fibre diameters, due to a higher mass flow from the needle tip.	[7,24,37,50]
The increase of the voltage leads to the decrease of the beads, while further increase results in the formation of beaded fibres due to the decrease in the stability of the initiating jet.	[36,38]
<u>Polymer flow rate</u>	
At too high flow rates beaded defects can be observed.	[7,23,43]
Upon increasing the flow rates both diameters and pore size increase.	
<u>Needle tip-to-collector distance</u>	
The fibre diameter decreases by increasing the distance, whereas the formation of wet fibres and beaded structures is usually obtained by shortening the distance between the needle-tip and the collector.	[15,24,42,43]
<u>Collector composition and geometry</u>	
The more conductive collectors dissipate the charge of the fibres, whereas when this charge is not dissipated the fibres repel one another, resulting in the generation of a more porous structure.	[23,48]
Ambient parameters	
<u>Temperature</u>	
The increase in temperature results in the generation of fibres with smaller diameters due to the decrease of the polymer solution viscosity.	[7,23,28]
<u>Humidity</u>	
An increase in the humidity level results in the appearance of small circular pores on the surface of the fibres, while a further increase leads to pore coalescing, and the drying of the fibres is prevented.	[7,23,28]

6.2. ELECTROSPINNING TECHNOLOGY IN TISSUE ENGINEERING

Tissue engineering or regenerative medicine deals with the development and application of biological and therapeutic substitutes that restore, maintain, or improve tissue function by using matrix scaffolds derived from natural or synthetic polymers. These scaffolds need to be viable with human cell systems for the repair or regeneration of damaged or failed cells or tissues provoked by injury, disease, or congenital defects [51,52]. Significant considerations include the nanoscale dimensions and the three-dimensional (3-D) structure of these scaffolds, since many biologically functional molecules, extracellular matrix (ECM) components, and cells interact in the same range operating in 3-D, and mimic the properties of certain fibrous components of the native ECM in tissues [23,53].

General characteristics that are considered to be essential in scaffold design include the acceptable shelf-life and biocompatibility without eliciting undesirable responses such as inflammation and toxicity upon implantation in the body [51,54]. The degradation rate of the material should match the time required for the healing or regeneration process. The scaffold should be fully biodegradable and the degradation products should be non-toxic, and able to be metabolized and cleared from the body. Moreover, for maximum cell loading and cell-matrix interactions, high porosity and pore size are essential parameters for an appropriate scaffold destined for use in tissue engineering applications [51,54,55]. The mechanical properties, which depend mainly on the chemical structure and crosslinking density, play an important role in the adhesion and gene expression of the cells and their matching with the tissue at the implantation site [55].

Fibrous nanomaterials have been exploited in many biological applications, such as biosensing [56,57], drug delivery [58-60], bioseparation [61] and tissue engineering [62-64], thus opening new possibilities in the biomedical arena. As aforementioned, one of the most popular and versatile nanofibre fabrication techniques used for the production of synthetically or naturally-derived nanofibrous materials is electrospinning [38]. The materials derived from this technique exhibit a range of unique features and properties, including extremely long length, very small diameters resulting in high surface-to-volume ratios, highly porous structures, lightweight properties, and low cost [15,16,65,66]. Moreover, these materials are characterized by very good mechanical properties especially in the case of fibrous nanocomposites, derived from the incorporation of a variety of functional micro / nano particles within the fibres. More precisely, different nanoparticles or nanofiller types may be dispersed in polymer solutions, which are then electrospun to generate composites in the form of continuous nanofibres and nanofibrous assemblies [52,67].

These outstanding properties render electrospun polymer-based fibrous materials promising candidates for many applications, not only in the biomedical field, such as scaffolds in tissue engineering and drug delivery systems [38,68-70], but also in environmental applications including filtration and water remediation processes [71] and in catalysis [72] (Figure 2). Since the major challenges in the field of nanofibres is their utility as biomaterials and specifically in tissue engineering applications, many researchers have exploited the recent advances of this technology for producing a wide range of nanofibrous polymeric materials, both natural and synthetic, that have been used as scaffolds in tissue engineering [68].

In particular, electrospun nanofibres derived from biopolymers such as collagen [73,74], alginate [75,76], hyaluronic acid [77], chitosan (CS) [78], and starch [79], synthetic polymers such as polyurethanes, polymethacrylates, aliphatic polyesters such as poly(lactic acid) (PLLA) [80,81], polycaprolactone (PCL) [24,82] and poly(glycolic acid) (PGA) [83], poly(vinylpyrrolidone) (PVP), poly(acrylonitrile) (PAN), poly(vinyl alcohol) (PVA), poly(ethylene oxide) (PEO) [84], poly(ethylene terephthalate) (PET) [85] and their combinations have demonstrated great potential as scaffold platforms for musculoskeletal and connective tissue engineering (including bone, and skeletal muscles) [86], skin tissue engineering, vascular tissue engineering and neural tissue engineering, provided that they possess the correct design parameters as mentioned above [87-89].

6.3. ELECTROSPUN MAGNETOACTIVE NANOCOMPOSITES IN TISSUE ENGINEERING

One of the most exciting and developing classes of advanced materials are nanocomposites, which have gained considerable interest due to their potential applications in many technological and scientific fields [90]. These materials comprise two or more phases of different chemical constituents or structures, with at least one phase having nanometric dimensions [91]. The combination of functional inorganic / organic fillers at the nanometer scale with polymer-based fibres produced by electrospinning leads to novel and attractive nanocomposite systems which exhibit promising features for many applications including biomedicine [68,92,93], catalysis [72,94], in environmental [95], and in energy-related applications [27,91]. The combination of the properties of the different components and the enhanced materials' properties derived from the development of organic-inorganic interfacial interaction phenomena has led to an improved performance compared to pristine polymer fibres [26,35,91,96].

Figure 2. Schematic presentation of electrospun polymer-based fibrous materials employed in different applications

Among other inorganic nanoparticulates, magnetic nanoparticles (MNPs) offer attractive possibilities in biomedicine and are more beneficial compared to microparticles, since they exhibit controllable size ranging from a few nanometers up to tens of nanometers, and dimensions smaller than or comparable to those of cells (10–100 μm), viruses (20–450 nm), proteins (5–50 nm) or genes (2 nm wide and 10–100 nm long) [97,98], improving tissular diffusion [99,100]. Moreover, therapeutic NPs with diameters ranging from 10–100 nm can be distributed throughout the circulatory system and penetrate small capillaries [62]. Surface modification provides additional functions rendering them ideal candidates as contrast enhancement agents in magnetic resonance imaging (MRI), in biomolecular detection, cell tracking, and for targeted drug delivery in tumor therapy [100,101]. Additionally, MNP destined for use in drug delivery must retain sufficient hydrophilicity and must not exceed 100 nm in size (including the surface coating), so as not to be recognized by the reticuloendothelial system (RES). RES is a class of cells existing in different locations within the human body that are phagocytic, *i.e.*, they can engulf and destroy bacteria, viruses, and other foreign substances such as nanoparticles [102].

To date, numerous examples have appeared in the literature dealing with the fabrication of electrospun magnetoactive polymer-based fibrous nanocomposites that mainly focus on synthetic and characterization aspects [103]. In such materials different types of polymers including natural polymers, biopolymers and synthetic polymers, have been combined with magnetic nanoparticles (MNPs) including iron oxide (magnetite) (Fe_3O_4) [60,104-106], maghemite ($\gamma\text{-Fe}_2\text{O}_3$) [107-109], cobalt (Co) [110], nickel (Ni) [111], iron-platinum (FePt) [112,113] NPs *etc.* From all the above, iron oxide NPs have been the most widely studied due to their biocompatibility, non-toxicity, and stability and are, by far, the most commonly employed MNPs in biomedical applications [114].

MNP-loaded therapeutic systems have shown promising results in tissue engineering applications. More precisely, it has been demonstrated that magnetoactive tissue engineering scaffolds can be used in the treatment of bone diseases by promoting the proliferation and differentiation of osteoblasts, increasing osteointegration, and accelerating new bone formation [115]. According to previous reports, a possible mechanism explaining this effect might be that the presence of MNPs embedded within a tissue engineering scaffold may induce mechanical stresses on the bone tissue, which in turn results in an enhancement in cell growth, proliferation and differentiation. Moreover, it was suggested that the Fe^{2+} ions released from the Fe_3O_4 nanoparticles may also promote cell growth and differentiation. Osteoblasts have the ability to incorporate metal ions, where phagocytosis of metal oxide NPs with diameters smaller than 1000 nm by osteoblasts was also observed. Y. Wu and co-workers (2010) have reported that osteoblast-like cells of the series MG63 incorporated Fe_3O_4 magnetic NPs. The response of the metalloproteinases found within the ECM of the osteoblasts to the NPs resulted in the modulation of the ECM. Additionally, it was demonstrated that controlled bone growth may be realized by magnetically promoting osteoblast proliferation and differentiation in Fe_3O_4 -containing hydroxyapatite [116]. Furthermore, it has been reported that the Fe_3O_4 NPs stimulate mesenchymal stem cell growth owing to their ability to suppress intracellular H_2O_2 , thus leading to the acceleration of the cell cycle progression [117-119]. Based on previous literature reports, the applied static magnetic field promotes cell proliferation even in the absence of magnetic nanoparticles [119], thus stimulating bone tissue regeneration [120,121]. X. Ba *et al.* (2011) reported on the enhanced proliferation of osteoblasts under moderate static magnetic fields (SMFs) on magnetic-free scaffolds [122]. The authors stated that even in the presence of moderate SMF osteogenic differentiation an activity of the osteoblasts is promoted. The introduction of magnetic nanoparticles results in further enhancement in cell growth, proliferation and differentiation, thus demonstrating that the applied magnetic field in combination with the embedded magnetic nanoparticles acts in a synergistic manner [119].

Figure 3. Schematic presentation of different routes employed for the fabrication of magnetoactive electrospun nanofibres

Electrospun magnetoactive polymer-based nanofibres can be generated either by the direct mixing of MNPs (pristine or pre-stabilized by polymers or surfactants) with the polymer solution followed by electrospinning [108,123,124], through the electrospinning-electrospraying process [55,66,103,125] or *via* post-magnetization processes (Figure 3).

Examples include PAN / Fe_3O_4 [126], poly(ϵ -caprolactone)/FePt [112], PEO / PLLA / Fe_3O_4 [60], PVP / PLLA / Fe_3O_4 [127], poly(methyl methacrylate) (PMMA) / Fe_3O_4 [128,129], and polyurethane (PU) / Fe_2O_3 [123].

However, only a limited number of literature examples so far have dealt with the evaluation of electrospun magnetoactive polymer-based membranes as tissue engineering scaffolds, focusing more on bone regeneration processes.

Bone is a complex, highly organized living organ forming the structural framework of the body. It is composed of an inorganic mineral phase, namely hydroxyapatite, and an organic phase of mainly type I collagen. The treatment of bone injuries through tissue engineering depends, among others, on the mechanical properties of the bone tissue, the porosity, hardness and the overall 3-D architecture.

J. Meng *et al.* (2010) studied cell proliferation, differentiation and ECM secretion of osteoblast cells in the presence of magnetoactive electrospun nanofibrous composite mats under a static magnetic field, providing a system with promising application potential in bone tissue engineering and bone regeneration treatment [109]. More precisely, nanofibrous scaffolds composed

of $\gamma\text{-Fe}_2\text{O}_3$ nanoparticles coated with *meso*-2,3-dimercaptosuccinic acid (DMSA), poly(D,L-lactide) (PLA), and hydroxyapatite nanoparticles (nHA) were fabricated by electrospinning. Upon applying a static magnetic field (0.9–1.0 mT), a significantly higher proliferation rate and faster differentiation of osteoblast cells were displayed.

PCL-based magnetoactive electrospun nanofibrous scaffolds were fabricated by J.T. Kannarkat and co-workers (2010) for potential use in bone healing and regeneration [130]. From their studies they concluded that the MNPs do not alter the stability of the fibrous PCL scaffold. Moreover, they demonstrated that the MNPs did not prohibit cell growth; on the contrary, cell clustering was observed after 9 days of cell culture. Cell elongation was also observed, indicating cell differentiation. As stated by the authors, such cell elongation phenomena may be attributed to the high surface area of the electrospun fibrous scaffold, facilitating multiple site attachment.

Electrospun superparamagnetic aligned fibrous bundles were developed by W.Y. Lee *et al.* (2011) to be tested as biomaterial scaffolds. The development of aligned tissue engineering scaffolds is of paramount importance since the alignment of cells plays an important role in the skeletal muscle tissue characterized by a well-oriented architecture. This material consisted of highly oriented electrospun poly(L-lactide-*co*-glycolide) (PLGA) fibres with embedded superparamagnetic iron oxide NPs (SPION). Cell alignment and differentiation by using mouse C2C12 myoblasts (ATCC CRL-1772) demonstrated that C2C12 myoblasts proliferate along the direction of the aligned fibres, similarly to native skeletal muscle tissues [131]. In the presence of an externally applied magnetic field the generated cell rods self-assembled into highly ordered 3-D tissues. These results demonstrate the high potential of magnetoactive polymer-based fibrous bundles as a scaffold promoting cell growth and as a cargo for the magnetic field-induced generation of highly oriented 3-D cell-dense tissues.

Y. Wei *et al.* (2011) have reported on the fabrication of magnetic biodegradable fibrous mats by means of the electrospinning technique, with potential use in bone regeneration. More precisely, they have prepared a magnetoactive nanofibrous scaffold based on native polysaccharide CS, PVA, and Fe_3O_4 NPs. MG-63 cells were cultured on the Fe_3O_4 / CS / PVA nanofibrous membranes to evaluate the cell growth dynamics. The obtained results demonstrated that cell adhesion and proliferation increased in the presence of the MNPs [132].

The performance of magnetoresponsive fibrous mats on bone regeneration was also studied by K. Lai *et al.* (2012) who have reported on the fabrication of magnetic biocompatible fibrous scaffolds consisting of poly(lactic-*co*-glycolic acid) (PLGA) and superparamagnetic Fe_3O_4 NPs with variable nanoparticle loading. The authors studied their effect on different bone cells (Rps17/1.8 and MC3T3-E1) in the absence of an external magnetic field. The magnetite-containing nanocomposite scaffolds exhibited excellent biocompatibility,

enhanced osteoblast cell attachment and proliferation at an early culture time in comparison to the pristine PLGA fibrous analogues [133].

Aligned superparamagnetic nanofibres comprising poly(lactic-co-glycolide) (PLGA) and MNPs (Fe_3O_4) have been developed by H. Hu *et al.* (2013) by employing magnetic electrospinning. This electrospinning variation utilizes a magnetic field that causes the parallel stretching of magnetoactive polymer fibres. The effect of the fibre alignment on the cell performance was studied by using C2C12 myoblast cells. The magnetically aligned nanofibres exhibited superior cell attachment and proliferation in comparison to their randomly oriented analogues, offering guide cell growth along the longitudinal axis of the nanofibres [134].

J. Meng *et al.* (2013) reported on the fabrication of a novel nanofibrous composite scaffold by means of the electrospinning technique. The magnetoactive scaffold composed of superparamagnetic $\gamma\text{-Fe}_2\text{O}_3$ NPs, nHA NPs and PLA resulted in the acceleration and induction of a higher amount of osteocalcin positive cells *in situ*, under a static magnetic field [135].

A nanocomposite fibrous substrate for bone regeneration was also prepared by D. Shan *et al.* (2013). The magnetoresponsive matrix composed of PLLA and Fe_3O_4 NPs was prepared by using a modified chemical co-precipitation method. The dispersion of the MNPs within the fibrous mat showed a positive effect in cell attachment, while enhanced cell attachment was obtained when compared with the pristine PLLA nanofibres [136].

R.K. Singh *et al.* (2014) have reported on magnetic electrospun nanofibrous scaffolds, based on PCL and iron oxide MNPs, with mechanical and biological properties applicable for bone regeneration. Among others, the authors demonstrated that the presence of MNPs within the non-woven fibrous mats results in an enhancement of their mechanical performance. Moreover, it was shown that osteoblastic cells favoured the MNPs-incorporated nanofibres, providing excellent cellular interactions compared to the pristine PCL [137].

Since the presence of a magnetic field significantly influences the cell behaviour, in a last example L. Li *et al.* (2014) have studied the influence of a SMF of moderate intensity on osteoblast and 3T3 fibroblast cultures by using randomly oriented and aligned electrospun magnetic composite nanofibrous mats consisting of a biodegradable polyester, namely PLA and iron oxide MNPs [138].

6.4. CONCLUSIONS

The unique properties of magnetic nanoparticles, in combination with the special characteristics of electrospun polymer nanofibres have led to the development of novel, multifunctional fibrous nanocomposites with outstanding properties for tissue engineering applications, focusing more on bone regeneration processes. Recent studies in this field have demonstrated that the use of polymer-based magnetoactive electrospun fibres leads to an enhancement in cell growth, proliferation and differentiation in comparison to their pristine fibrous polymer analogues. The exact mechanism by which magnetic scaffolds promote cell proliferation is still under investigation [132,133,137] and the area of magnetically-triggered tissue engineering is wide open for further development in the near future.

REFERENCES

1. J. Zeleny. *Phys. Rev.* **3** (1914) 69–91.
2. A. Formhals. US Patent 1-975-504 (1934).
3. J.K. Hong, S.V. Madhally. *Tissue Eng. B* **17** (2011) 125–141.
4. G. Taylor. *Proc. Roy. Soc.* **313** (1969) 453–475.
5. J. Doshi, D.H. Reneker. *J. Electrostat.* **35** (1995) 151–160.
6. D.H. Reneker, I. Chun. *Nanotechnology* **7** (1996) 216–223.
7. T. Subbiah, G.S. Bhat, R.W. Tock, S. Parameswaran, S.S. Ramkumar. *J. Appl. Polym. Sci.* **96** (2005) 557–569.
8. T. Joachim, C. Ondarcuhu. *Europhys. Lett.* **42** (1998) 215–220.
9. G.M. Grzybowski, B. Whitesides. *Science* **295** (2002) 2418–2421.
10. L.A. Smith, P.X. Ma. *Colloid. Surface. B* **39** (2004) 125–131.
11. J.C. Ellison, A. Phatak, D.W. Giles. *Polymer* **48** (2007) 3306–3316.
12. M. Charles. *Accounts Chem. Res.* **28** (1995) 61–68.
13. W.E. Teo, S. Ramakrishna. *Nanotechnology* **17** (2006) R89–R106.
14. Z.M. Huang, Y.Z. Zhang, M. Kotaki, S. Ramakrishna. *Compos. Sci. Technol.* **63** (2003) 2223–2253.
15. J. Venugopal, Y.Z. Zhang, S. Ramakrishna. *Proc. Inst. Mech. Eng. N. J. Nanoeng. Nanosyst.* **218** (2005) 35–45.
16. J. Yang, S. Zhan, N. Wang, X. Wang, Y. Li, Y. Li, W. Ma, H. Yu. *J. Disper. Sci. Technol.* **31** (2010) 760–769.
17. H. Wu, W. Pan, D. Lin, H. Li. *J. Adv. Ceram.* **1** (2012) 2–23.
18. D. Li, Y. Wang, Y. Xia. *Nanoletters* **3** (2003) 1167–1171.
19. H.S. Wang, G.D. Fu, X.S. Li. *Recent Pat. Nanotechnol.* **3** (2009) 21–31.
20. A.L. Yarin, S. Koombhongse, D.H. Reneker. *J. Appl. Phys.* **90** (2001) 4836–4846.
21. D.H. Reneker, A.L. Yarin, H. Fong, S. Koombhongse. *J. Appl. Phys.* **87** (2000) 4531–4547.
22. M.M. Hohman, M. Shin, G. Rutledge, M.P. Brenner. *Phys. Fluids* **13** (2001) 2201–2220.
23. Q.P. Pham, U. Sharma, A.g. Mikos. *Tissue Eng.* **12** (2006) 1197–1211.
24. T.J. Sill, A.H. von Recum. *Biomaterials* **29** (2008) 1989–2006.

25. C. Huang, S. Chen, C. Lai, D.H. Renker, H. Qiu, Y. Ye, H. Hou. *Nanotechnology* **17** (2006) 1558–1563.
26. V. Pillay, C. Dott, Y.E. Choonara, C. Tyagi, L. Tomar, P. Kumar, L.C. du Toit, V.M.K. Ndesendo. *J. Nanomater.* **2013** (2013) 1–22.
27. A. Greiner, J.H. Wendorff. *Adv. Polym. Sci.* **219** (2008) 107–171.
28. P. Baumgarten. *J. Colloid Interf. Sci.* **36** (1971) 71–79.
29. I. Hayati, A.I. Bailey, T.F. Tadros. *J. Colloid Interf. Sci.* **117** (1987) 205–221.
30. M.M. Hohman, M. Shin, G. Rutledge, M.P. Brenner. *Phys. Fluids* **13** (2001) 2221–2236.
31. A.K. Haghi, M. Akbari. *Phys. Status Solidi.* **204** (2007) 1830–1834.
32. N. Bhardwaj, S.C. Kundu. *Biotechnol. Adv.* **28** (2010) 325–347.
33. P. Guptaa, C. Elkinsb, T. E. Longb, G.L. Wilkes. *Polymer* **46** (2005) 4799–4810.
34. M. Pradny, L. Martinova, J. Michalek, T. Fenclova, E. Krumbholcova. *Cent. Eur. J. Chem.* **5** (2007) 779–792.
35. A. Frenot, I.S. Chronakis. *Curr. Opin. Colloid Interface Sci.* **8** (2003) 64–75.
36. J.M. Deitzel, J. Kleinmeyer, D. Harris, N.C. Beck Tan. *Polymer* **42** (2001) 261–272.
37. K. Garg, G.L. Bowlin. *Biomicrofluidics* **5** (2011) 1–19.
38. P. Supaphol, O. Suwantong, P. Sangsanoh, S. Srinivasan, R. Jayakumar, S.V. Nair. *Adv. Polym. Sci.* **246** (2012) 213–240.
39. X. Zong, K. Kim, D. Fang, S. Ran, B.S. Hsiao, B. Chu. *Polymer* **43** (2002) 4403–4412.
40. D. Li, Y. Xia. *Adv. Mater.* **16** (2004) 1151–1170.
41. Z. Li, C. Wang, *SpringerBriefs in Materials*, Springer, 2013, p.p. 29–73, p.p. 75–139.
42. R. Jaeger, M.M. Bergshoeff, C. Martin Battle, H. Schlinherr, G.J. Vancso. *Macromol. Symp.* **127** (1998) 141–150.
43. S. Megelski, J.S. Stephens, D. Bruce Chase, J.F. Rabolt. *Macromolecules* **35** (2002) 8456–8466.
44. I. Savva. PhD Thesis (2014).
45. C. Drew, X. Wang, L.A. Samuelson, J. Kumar. *J. Macromol. Sci. A* **40** (2003) 1415–1422.
46. H. Fong, D.H. Reneker. *J. Polym. Sci. B* **37** (1999) 3488–3493.
47. H.L. Simons, Patented 3-280-229 (1966).
48. H.Q. Liu, Y.L. Hsieh. *J. Polym. Sci. B* **40** (2002) 2119–2129.
49. L. Wannatong, A. Sirivat, P. Supaphol. *Polym. Int.* **53** (2004) 1851–1859.
50. M.M. Demir, I. Yilgor, E. Yilgor, B. Erman. *Polymer* **43** (2002) 3303–3309.
51. X. Wang, B. Ding, B. Li. *Mater. Today* **16** (2013) 229–241.
52. R. Sridhar, S. Sundarajan, J. Reddy Venugopal, R. Ravichandran, S. Ramakrishna. *J. Biomater. Sci. Polym. Ed.* **24** (2013) 365–385.
53. Z. Xu, X. Huang, L. Wan. *Adv. Topics Sci. Technol.* (2009) 306–328.
54. X. Zhang, M.R. Reagan, D.L. Kaplan. *Adv. Drug Deliv. Rev.* **61** (2009) 988–1006.
55. Q. Hou, P.A. De Bank, K.M. Shakesheff. *J. Mater. Chem.* **14** (2004) 1915–1923.
56. G. Ren, X. Xu, Q. Liu, J. Cheng, X. Yuan, L. Wu, Y. Wan. *React. Funct. Polym.* **66** (2006) 1559–1564.
57. D. Li, M.W. Frey, A.J. Baeumner. *J. Membr. Sci.* **279** (2006) 354–363.
58. C.S. Brazel. *Pharm. Res.* **26** (2009) 644–656.
59. L. Wang, M. Wang, P.D. Tophamc, Y. Huang. *RSC Adv.* **2** (2012) 2433–2438.

60. I. Savva, A.D. Odysseos, L. Evaggelou, O. Marinica, E. Vasile, L. Vekas, Y. Sarigiannis, T. Krasia-Christoforou. *Biomacromolecules* **14** (2013) 4436–4446.
61. I. Munaweera, A. Aliev, K.J. Balkus. *ACS Appl. Mater. Interfaces* **6** (2014) 244–251.
62. G.G. Walmsley, A. McArdle, R. Tevlin, A. Momeni, D. Atashroo. *Nanomed. Nanotechnol.* NANO-01083 (2015).
63. Y.M. Ha, T. Amna, M.H. Kim, H.C. Kim, M.S. Hassan, M.S. Khil. *Colloids Surf. B* **102** (2013) 795–802.
64. C. Zhang, M.R. Salick, T.M. Cordie, T. Ellingham, Y. Dan, L.S. Turng. *Mater. Sci. Engin. C* **49** (2015) 463–471.
65. D.G. Yu, L.M. Zhu, K. White, C. Branford-White. *Health* **1** (2009) 67–75.
66. F.E. Ahmed, B.S. Lalia, R. Hashaikeh. *Desalination* **356** (2015) 15–30.
67. R.L. Dahlin, F.K. Kasper, A.G. Mikos. *Tissue Eng. B* **17** (2011) 349–364.
68. N. Khan. *Studies by Undergraduate Researchers at Guelph* **5** (2012) 63–73.
69. M. Prabakaran, R. Jayakumar, S.V. Nair. *Adv. Polym. Sci.* **246** (2012) 241–262.
70. M. Zamani, M.P. Prabhakaran, S. Ramakrishna. *Int. J. Nanomed.* **8** (2013) 2997–3017.
71. M. Putti, M. Simonet, R. Solberg, G.W.M. Peters. *Polymer* **63** (2015) 189–195.
72. I. Savva, A.S. Kalogirou, A. Chatzinicolaou, P. Papaphilippou, A. Pantelidou, E. Vasile, E. Vasile, P.A. Koutentis, T. Krasia-Christoforou. *RSC Adv.* **4** (2014) 44911–44921.
73. J.A. Matthews, G.E. Wnek, D.G. Simpson, G.L. Bowlin. *Biomacromolecules* **3** (2002) 232–238.
74. K.S. Rho, L. Jeong, G. Lee, B.M. Seo, Y.J. Park, S.D. Hong, S. Roh, J.J. Cho, W.H. Park, B.M. Min. *Biomaterials* **27** (2006) 1452–1461.
75. G. Ma, D. Fa, Y. Liu, X. Zhu, J. Nie. *Carbohydr. Polym.* **87** (2012) 737–743.
76. J.J. Chang, Y.H. Lee, M.H. Wu, M.C. Yang, C.T. Chien. *Carbohydr. Polym.* **87** (2012) 2357–2361.
77. R. Vasita, D.S. Katti. *Int. J. Nanomed.* **1** (2006) 15–30.
78. J. Venugopal, S. Low, A.T. Choon, S. Ramakrishna. *J. Biomed. Mater. Res. B* **84** (2007) 34–48.
79. L. Kong, G.R. Ziegler. *Food Hydrocolloid.* (2014) 20–25.
80. X.M. Mo, C.Y. Xu, M. Kotaki, S. Ramakrishna. *Biomaterials* **25** (2004) 1883–1890.
81. H. Yoshimoto, Y.M. Shin, H. Terai, J.P. Vacanti. *Biomaterials* **24** (2003) 2077–2082.
82. C.Y. Xu, R. Inai, M. Kotaki, S. Ramakrishna. *Biomaterials* **25** (2004) 877–886.
83. J.R. Porter, T.T. Ruckh, K.C. Popat. *Biotechnol. Progr.* **25** (2009) 1539–1560.
84. J.G. Fernandes, D.M. Correia, G. Botelho, J. Padrao, F. Dourado, C. Ribeiro, S. Lanceros-Mendez, V. Sencadas. *Polym. Test.* **34** (2014) 64–71.
85. J.D. Schiffman, C.L. Schauer. *Polym. Rev.* **48** (2008) 317–352.
86. A. Abdal-hay, H. Raj Pant, J. Kyoo Lim. *Eur. Polym. J.* **49** (2013) 1314–1321.
87. T. Jiang, E.J. Carbone, K.W.-H. Lo, C.T. Laurencin. *Prog. Polym. Sci.* (2015).
88. R. Vasita, D.S. Katti. *Int. J. Nanomed.* **1** (2006) 15–30.
89. L.S. Nair, C.T. Laurencin. *Adv. Biochem. Eng. Biotechnol.* **102** (2006) 47–90.
90. F. Liu, M.W. Urban. *Progr. Polym. Sci.* **35** (2010) 3–23.

91. R. Sahay, P. Suresh Kumar, R. Sridhar, J. Sundaramurthy, J. Venugopal, S.G. Mhaisalkar, S. Ramakrishna. *J. Mater. Chem.* **22** (2012) 12953–12971.
92. B. Jeong, A. Gutowska. *Trends Biotechnol.* **20** (2002) 305–311.
93. D. Liang, B.S. Hsiao, B. Chu. *Adv. Drug Deliv. Rev.* **59** (2007) 1392–1412.
94. G. Nie, Z. Li, X. Li, J. Lei, C. Zhang, C. Wang. *Appl. Surf. Sci.* **284** (2013) 595–600.
95. S. Xiao, M. Shen, R. Guo, Q. Huang, S. Wang, X. Shi. *J. Mater. Chem.* **20** (2010) 5700–5708.
96. T. Krasia Christoforou, *Organic-inorganic polymer hybrids: synthetic strategies and applications*, Springer, 2015, p.p. 11–63.
97. Q.A. Pankhurst, J. Connolly, S.K. Jones, J. Dobson. *J. Phys. D* **36** (2003) 167–181.
98. R. Singh, J.W. Lillard Jr. *NIH Public Access* **86** (2009) 215–223.
99. P. Tartaj, M. del Puerto Morales, S. Veintemillas-Verdaguer, T. Gonzalez-Carreno, C.J. Serna. *J. Phys. D* **36** (2003) R182–R197.
100. M. Mahmoudi, S. Sant, B. Wang, S. Laurent, T. Sen. *Adv. Drug Deliv. Rev.* **63** (2011) 24–46.
101. D.L.J. Thorek, A.K. Chen, J. Czupryna, A. Tsourkas. *Ann. Biomed. Eng.* **34** (2006) 23–38.
102. J. Chomoucka, J. Drbohlavova, D. Huska, V. Adam, R. Kizek, J. Hubalek. *Pharmacol. Res.* **62** (2010) 144–149.
103. I. Savva, T. Krasia-Cristoforou, *Magnetic nanoparticles: Synthesis, physicochemical properties and role in biomedicine*, Nova Publishers Inc., New York, 2014, p.p. 163–199.
104. H.Y. Li, C.M. Chang, K.Y. Hsu. *J. Mater. Chem.* **22** (2012) 4855–4860.
105. T. Gong, W. Li, H. Chen. *ACTA Biomater.* **8** (2012) 1248–1259.
106. J.T. Kannarkat, J. Battogtokh, J. Philip. *J. Appl. Phys.* **107** (2010).
107. S.T. Tan, J.H. Wendorff, C. Pietzonka, Z. Hong Jia, G. Qing Wang. *ChemPhysChem* **6** (2005) 1461–1465.
108. J. Meng, B. Xiao, Y. Zhang, J. Liu, H. Xue, J. Lei, H. Kong, Y. Huang. *Sci. Rep.* **3** (2013) 1–7.
109. J. Meng, Y. Zhang, X. Qi, H. Kong, C. Wang, Z. Xu, S. Xie, N. Gu, H. Xu. *Nanoscale* **2** (2010) 2565–2569.
110. O. Kriha, M. Becker, M. Lehmann. *Adv. Mater.* **19** (2007) 2483–2488.
111. X. Chen, S. Wei, C. Gunesoglu. *Macromol. Chem. Phys.* **211** (2010) 1775–1783.
112. T. Song, Y.Z. Zhang, T.J. Zhou. *J. Magn. Magn. Mater.* **303** (2006) E286–E289.
113. T. Song, Y. Zhang, T. Zhou, C.T. Lim, S. Ramakrishna, B. Liu. *Chem. Phys. Lett.* **415** (2005) 317–322.
114. V.I. Shubayev, T.R. Pisanic, S. Jin. *Adv. Drug Deliv. Rev.* **61** (2009) 467–477.
115. X.B. Zeng, H. Hu, L.Q. Xie, F. Lan, W. Jiang, Y. Wu, Z.W. Gu. *Int. J. Nanomed.* **7** (2012) 3365–3378.
116. Y. Wu, W. Jiang, X. Wen, B. He, X. Zeng, G. Wang, Z. Gu. *Biomed. Mater.* **5** (2010) 015001 (7p).
117. G. Yin, Z. Huang, M. Deng, J. Zeng, J. Gu. *J. Colloid Interface Sci.* **363** (2011) 393–402.
118. D-M. Huang, J-K Hsiao, Y-C. Chen, L-Y. Chien, M. Yao, Y-K. Chen, B.S. Ko, S.C. Hsu, L.A. Tai, H.Y. Cheng, S.W. Wang, C.S. Yang, Y.C. Chen. *Biomaterials* **30** (2009) 3645–3651.
119. Q. Cai, Y. Shi, D. Shan, W. Jia, S. Duan, X. Deng, X. Yang. *Mater. Sci. Eng. C* **55** (2015) 166–173.

120. P. Singh, R.C. Yashroy, M. Hoque. *Indian J. Biochem. Biophys.* **43** (2006) 167–172.
121. B. Strauch, M.K. Patel, J.A. Navarro, M. Berdichevsky, H.L. Yu, A.A. Pilla. *Plast. Reconstr. Surg.* **120**(2) (2007) 425-430.
122. X. Ba, M. Hadjiargyrou, E. DiMasi, Y. Meng, M. Simon, Z. Tan, M.H. Rafailovich. *Biomaterials* **32** (2011) 7831–7838.
123. C.H. Park, S.J. Kang, L.D. Tijing, H.R. Pant, C.S. Kim. *Ceram. Int.* **39** (2013) 9785–9790.
124. J. Zhu, S. Wei, X. Chen. *J. Phys. Chem. C* **114** (2010) 8844–8850.
125. L. Francis, J. Venugopal, M.P. Prabhakaran, V. Thavasi, E. Marsano, S. Ramakrishna. *Acta Biomater.* **6** (2010) 4100–4109.
126. B. Wang, Y. Sun, H. Wang. *J. Appl. Polym. Sci.* **115** (2010) 1781–1786.
127. I. Savva, D. Constantinou, O. Marinica, E. Vasile, L. Vekas, T. Krasia-Christoforou. *J. Magn. Magn. Mater.* **352** (2014) 35–40.
128. I. Savva, G. Krekos, A. Taculescu, O. Marinica, L. Vekas, T. Krasia-Christoforou. *J. Nanomater.* **2012** (2012) 1–9.
129. V.S. Pavan Kumar, V. Jagadeesh Babu, G.K. Raghuraman, R. Dhamodharan, T.S. Natarajan. *J. Appl. Phys.* **101** (2007) 114317.
130. J.T. Kannarkat, J. Battogtokh, J. Philip, O.C. Wilson, P.M. Mehl. *J. Appl. Phys.* **107** (2010) 09B307.
131. W.Y. Lee, W.Y. Cheng, Y.C. Yeh. *Tissue Eng. C* **117** (2011) 651–661.
132. Y. Wei, X. Zhang, Y. Song, B. Han, X. Hu, X. Wang, Y. Lin, X. Deng. *Biomed. Mater.* **6** (2011) 1–15.
133. K. Lai, W. Jiang, J.Z. Tang, Y. Wu, B. He, G. Wang, Z. Gu. *RSC Adv.* **2** (2012) 13007–13017.
134. H. Hu, W. Jiang, F. Lan, X. Zeng, S. Ma, Y. Wu, Z. Gu. *RSC Adv.* **3** (2013) 879–886.
135. J. Meng, B. Xiao, Y. Zhang, J. Liu, H. Xue, J. Lei, H. Kong, Y. Huang, Z. Jin, N. Gu, H. Xu. *Sci. Rep.* **3**(2655) (2013) 1–7.
136. D. Shan, Y. Shi, S. Duan, Y. Wei, Q. Cai, X. Yang. *Mater. Sci. Eng. C* **33** (2013) 3498–3505.
137. R.K. Singh, K.D. Patel, J.H. Lee, E.J. Lee, J.H. Kim, T.H. Kim, H.W. Kim. *PLOS one* **9** (2014) e91584 (1–16).
138. L. Li, G. Yang, J. Li, S. Ding, S. Zhou. *Mater. Sci. Eng. C* **34** (2014) 252–261.

MAGNETIC NANOPARTICLES IN CELL-BASED THERAPIES

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7.1. INTRODUCTION

The use of magnetic nanoparticles (MNPs) in medicine can be broadly sub-classified into four themes. Firstly, the use of nanoparticle-bound antibodies to magnetically separate or guide small molecules and drugs; secondly, imaging techniques based on the local magnetic field created by the nanoparticles (magnetic resonance imaging, magnetic particle imaging, and magnetoresistive biosensing); thirdly, nanoparticle-guided delivery of therapeutic cells to different organs using applied magnetic field; and fourthly, magnetic thermotherapy where magnetic fluids are used to heat up a local environment through heat dissipation triggered by an alternating current (AC) magnetic field [1,2]. This chapter will focus on using magnetic nanoparticle to deliver therapeutic cells to target specific organs.

The great advantage of cell based therapies is their regenerative capacity to replace lost tissue, a capacity not achievable in conventional drug therapy. A major challenge in the field of regenerative medicine, however, is to develop efficient methods of cell delivery to damaged areas of the body. This problem can be broken down into issues of: the number of cells to be targeted; how to make surgical procedures as minimally invasive as possible; and how to facilitate adequate retention and integration of cells in the area of interest.

In cell therapy, magnetic nanoparticles have been used to label cells both for the purposes of tracking their passage through the body and also to enhance their targeting to a particular site [3]. This involves labelling cells with particles prior to loading into an organism and then exposing that organism to a magnetic field. This approach relies on the fact that the normal human body contains relatively little magnetic material (like iron) and so exposure to magnetic fields usually has little impact. Recent studies have shown that magnetic labelling of cells is relatively easy and safe [4].

7.2. TYPES OF NANOPARTICLES

MNPs can be classified as superparamagnetic, paramagnetic, ferromagnetic or ferromagnetic depending on their magnetic cores, and contain manganese, gadolinium or, most commonly, iron oxide [5]. As iron oxide based nanoparticles have been used most widely for cellular interventions, we will discuss about only iron based particles in the rest of the chapter. Superparamagnetic iron oxide nanoparticles (SPIONs) are the most widely used magnetic nanoparticles among other iron oxide particles. The core is usually made of magnetite (Fe_3O_4) or maghemite ($\gamma\text{-Fe}_2\text{O}_3$). To prevent agglomeration of the colloidal suspension and to enhance biocompatibility the surface of the SPION is usually covered with a compatible coating. There are various types of SPIONs with different kinds of coatings; however the most appropriate type for a specific cell labelling remains to be determined. A number of synthetic and natural coatings have been used to date, such as dextran and carboxymethylated dextran, alginate, starch, polyethylene glycol (PEG), poly D,L-lactide-*co*-glycolide (PLGA), and organosilane. Small molecules with charged surface such as citrate, amino acids, hydroxamate, and dimercaptosuccinic acid have also been used as SPION coatings [6].

7.3. MECHANISM

Cell migration plays critical role not only in therapy but also in development, angiogenesis, immune response, wound healing and cancer metastasis. During these processes cells undergo directed migration in the presence of motogenic stimuli as an external guidance cue [7]. This steering mechanism coupled to the basic motility machinery has been exploited by sensing aligned fibers or gradients in concentration, mechanical properties or electric field [8]. It has been well established that cells undergo chemotaxis in response to soluble cues, haptotaxis in response to graded adhesion in the underlying substrate, electrotaxis in response to electric fields, and durotaxis in response to mechanical signals in the environment [9,10]. While the cell migration behaviour, signal transduction and cytoskeleton dynamics elicited by other directional cues has been largely determined, responses to magnetic directional cues are much less understood.

Figure 1. Schematic representation of magnetic nanoparticle labelled cellular migration toward the magnetic field. Internalized nanoparticles do not direct cellular migration if there is no external magnetic field (A). In presence of the external magnet (B), the cellular magnetic particles lined up towards the field and provide cells a mechanical cue to migrate (C). Image reconstructed from Bradshaw *et al.* (2015) [11] with permission from The Royal Society of Chemistry.

Upon internalization of the nanoparticles inside the cell, the MNPs get confined inside endosomes, which are submicrometric vesicles of the endocytotic pathway. Their movement can be modulated in response to an external magnetic field (Figure 1). The general mechanism of magnetic nanoparticles based cell migration involves these endosomes behaving as small magnets and attract each other *via* dipole–dipole interactions with the aid of the magnetic field. Eventually they will form small chains in the direction of the magnetic field and provide the cell a magnetic moment toward the field [11].

7.4. THERAPEUTIC CELL DELIVERY

Developing new ways of delivering cells to target tissue is a major challenge in translating cell therapeutics research into clinical use. The number of transplanted cells, the site of transplantation and the route of administration are the important parameters that influence effective implementation of cellular transplantation strategies in regenerative medicine. Less invasive cell transplantation, but sufficient cell retention and engraftment in the tissue of interest are essential to achieve a significant therapeutic benefit. Magnetic force based targeting enables delivery of significant numbers of therapeutic cells to key areas of specific organs. Here we report some of the preclinical studies where magnetic targeting has been used to deliver therapeutic cells to diseased tissue.

7.4.1. Eye disorders

Vision impairment is one of the commonest disabilities. Developed countries are facing the challenges of combating a number of progressive blinding disorders, such as age-related macular degeneration (more than 14 million people blind or severely impaired), retinitis pigmentosa ($\sim 1 / 3500$), and diabetic retinopathy (~ 4.2 million people over the age of 40 in the United States) [12-15]. With retinal degenerations so prevalent worldwide, new approaches of treatment would be widely applicable. Moreover, it will bring significant advancement in improving the quality of life as well as remove pressure from the healthcare systems. Cell based therapies, directing at genetic abnormalities or at modifying pathological processes, hold much promise in treating a wide variety of diseases that lead to vision loss [16]. The major approaches of cell based therapies for retinal degeneration includes cell replacement and providing neuroprotection. While cell replacement therapy is facing the challenges of developing functional photoreceptor cells from human embryonic stem cells/induced pluripotent stem cells (hESCs/iPSCs) and proper integration of them in the retina, the discovery of mesenchymal stem cells (MSCs) and their versatile characteristics open an attractive field of investigating neuroprotection in dystrophic retina [17].

Apoptotic cell death is a central mechanism in many blinding retinal diseases, such as retinitis pigmentosa (RP) and also the atrophic (dry) form of age-related macular degeneration [18,19]. Therefore, therapeutic approaches targeting apoptotic cell death could play major role in preventing vision loss. The concept of neuroprotection by inhibiting cell death has emerged over the last decade and a range of factors, such as ciliary neurotrophic factor (CNTF), brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), and basic fibroblast growth factor (bFGF), have been identified as potential neuroprotectants. Major concerns with the neurotrophic factors are either they are too large to cross the blood-retinal barrier (BRB) or are associated with undesirable systemic complication [20]. Cell-based therapies,

with their paracrine effects, have overcome the complexities associated with systemic delivery of these molecules. However, there are issues, like targeting cells to disease tissue, potential for collateral damage, breaching local tissue barriers, ease of delivery method and repeatability, which still require significant considerations [21]. Targeting cells to specific *loci* is particularly important in diseases of central nervous system (CNS). For instance, regional differences in tissue function in human retina can make randomly targeted therapy suboptimal. In age-related macular degeneration, cell therapy needs to be targeted specifically at the macular (cone rich central) region of the retina since cells delivered into the peripheral retina will have no functional benefit or might even disrupt normal tissue function. The direct injection of cells into the macula is feasible; however there is significant risk of complications, which increases with repeat injections. In a recent study, intravenous fluidMAG-D-labeled MSCs were magnetically targeted to upper hemisphere of the dystrophic rodent retina by placing a disc magnet in the orbit (Figure 2). These magnetized MSCs produced enhanced amount of neurotrophic factors (GDNF and CNTF) and anti-inflammatory factors [interleukin-10 (IL-10) and hepatocyte growth factor (HGF)] in the retina which, in turn provides better neuroprotection [21].

Figure 2. Flat-mount images of rat retinas (a, c, e) in control animals and (b, d, f) after magnetic targeting of mesenchymal stem cells (MSCs). Arrows indicate small areas of magnetic mesenchymal stem cells in the retinas without a magnet applied (a, c, e). The dashed white circle shows the position of the orbital disc magnet. MSCs have been labeled with Qtracker 655 and appear red/orange on the image. Image reconstructed from A. Yanai *et al.* (2012) [21] with permission of the author.

7.4.2. Spinal cord injury

Spinal cord injury (SCI) is a devastating traumatic injury that can lead to serious neurological deficit and permanent invalidity. The sensory deficits occur due to tissue damage, loss of neurons, axonal degeneration and the poor ability of axons to regenerate across the lesion [22]. This limited regeneration capability of the central nervous system poses the greatest challenge in

developing an effective therapy for SCI. Among the potential therapies that have been tested in preclinical and clinical studies, transplantation of stem cells showed promising results. Stem cells can replace lost neurons, provide permissive growth environment and thus enhance regeneration [23]. Despite the potential, stem cell approach has some serious limitations like low efficiency in delivery, retention and engraftment. A significant therapeutic benefit can be achieved from minimally invasive but highly effective delivery strategy. For SCI model, intrathecal delivery is such a technique where there is more cell retention and survival with higher delivery efficiency than intravenous route. Magnetic targeting can achieve higher efficacy by promoting the homing of cells to the site of injury. A number of groups have reported that a significant number of magnetic nanoparticles labeled cells can be accumulated at SCI lesion *via* magnetic targeting [3,24,25]. With magnetic targeting improved behavioral response was also recorded compare to conventional cell delivery.

Figure 3. Magnetic targeting of MSCs to the site of SCI. (A) *In vivo* model of the non-invasive magnetic targeting model. (B) Schematic representation of the magnetic targeting mechanism. Images reconstructed from D. Tukmachev *et al.* (2015) [24] with permission from The Royal Society of Chemistry.

Inappropriate focusing ability is one of the common disadvantages of the magnetic delivery strategies. Magnetic field gradient is at its highest value at the pole where maximum cell capturing occurs. However, most of the times the poles cannot be placed near or at the target site, which renders the system rather inefficient and limited. To overcome these problems, a Czech group has developed a minimally invasive magnetic targeting strategy for SCI model that enables efficient cell retention at a lesion site after intrathecal delivery [24]. This system consists of a ring shaped holder with two cylindrical magnets facing their same poles toward each other (Figure 3A). This arrangement

creates a focusing zone (named trapping area) where the vertical and the horizontal magnetic force components nullify each other and prevent cells from further movement. The system can be manipulated to place the focusing zone exactly on the lesion site. The cells can be guided to a lesion site in a rat model within two hours of intrathecal delivery with this system, which is significantly higher than the usual 10–12 h (Figure 3B) [24].

7.4.3. Cancer

A major difference in cell based therapeutic approach between cancer and regenerative medicine is that in cancer, the cells generally mean to cause necrosis rather than regeneration in the target site. For cancer therapy, wide variety of cells, including erythrocytes, bacterial ghosts, genetically engineered stem and dendritic cells, have been manipulated as novel drug-delivery systems. The goals of all sophisticated drug delivery systems, which are currently being investigated, are – to be nontoxic and biocompatible, have long half-life, better targeting and less remote accumulation, multi-drug loading and tracking capability. Cell-based systems have been proved to be a better drug carrier considering all these facts, than the synthetic nanoparticulate system. Red blood cells (RBCs) have been extensively investigated as a vector for cell-based drug-delivery system because of their easy access, inherent bio-compatibility, flexibility, *in vivo* stability and long systemic half-life. However, in some cases the small size of RBCs limits their chance to directly approach the tumor cells as well as their extravascular diffusion ability. To circumvent this problem a chimeric RBC, called RBC-IONP-Ce6-PEG, has been developed by a group of scientist where they attached iron oxide nanoparticle coated with Chlorin e6 (Ce6), – clinically used photodynamic agent, on the membrane of mouse RBCs and loaded the cells with doxorubicin (DOX) – a chemotherapy drug (Figure 4). For further stability the nanoparticles were coated with PEG. Intravenous delivery of these cells showed higher stability, longer systemic circulation and high efficient tumor penetration when an external magnetic field is applied. This strategy also demonstrated low remote organ retention and a synergistic tumor growth inhibition effect after performing a combined photodynamic and chemotherapy [26].

Figure 4. Preparation and delivery of magnetic RBC. Schematic diagram showing (A) the preparation steps of theranostic RBCs modified with magnetic nanoparticles, Ce6, DOX, and PEG; and (B) *in vivo* magnetic tumor targeting. Image Reconstructed from C. Wang *et al.* [26] with the permission of the author.

7.4.4. Heart diseases

Cardiovascular diseases, such as myocardial infarction, are the leading causes of death and disability worldwide [27]. Myocardial infarction (MI) is the irreversible damage of the healthy and contractile myocardium. The tissue becomes akinetic and fibrotic and the heart cannot pump blood at its full potential. Although the current available treatments have greatly impacted the trajectory of patient health following a MI, the rate of mortality and morbidity still remains very high [27,28]. In recent years, stem cell based regenerative therapy has emerged as a potential therapy. A number of preclinical studies have reported improved cardiac function after administering cells to treat MI through direct myocardial injection. However, this cell delivery method includes rapid cell loss caused by leakage of the injected cell suspensions and needle mediated direct tissue damage. Thus, alternative cell therapy strategies have been explored. A Japanese group has recently developed 300 μm thick cell sheet, comprised of 10–15 piled-up magnetized cells, using magnetic force (Figure 5). This cell sheet, comprising either mesenchymal stem cells or induced pluripotent stem cells, induced angiogenesis when transplanted into an ischemic mouse heart [29,30]. They termed this nanoparticles aided system as magnetic force based tissue engineering system (Mag-TE). Lipid coated iron oxide nanoparticles labeled free cells in a small plate with media, forms a sheet-like structure by piling up according to the magnetic force after 24 h of incubation. In this approach magnetic nanoparticles and force have been used for structural purpose rather than directional.

Figure 5. Development of iPS cell-derived cell sheet by combining magnetic nanoparticle and the extracellular matrix (ECM) precursor embedding systems. Fluorescence-activated cell sorting (FACS) sorted, purified cells were mixed with magnetic nanoparticles and incubated for 2 h. 10^6 labeled cells were mixed with ECM precursor and seeded onto ultra-low-attachment plates. When these plates were placed on a cylindrical neodymium magnet, a vertical magnetic force was applied to the plate. After washing and media replacement, the incubation was continued for an additional 24 h. Image reconstructed from K. Tetsutaro *et al.* (2013) [28] with the permission from the author.

7.4.5. Respiratory disease

The airway epithelium is the primary environmental barrier and has a number of important physiological functions which includes humidifying the air, regulating the airway smooth muscle, eliminating inhaled pathogens and particulates, and recruiting immune cells in response to injury. Chronic airway diseases, for instance chronic obstructive pulmonary disease (COPD), cystic fibrosis and lung cancer, cause a large number of morbidity and mortality worldwide [31]. Although lung transplant is considered as a viable treatment options for these patients, its aspect is severely limited by donor options and post-surgical complicacy [32]. A rapidly growing number of investigations of stem cells and progenitor cells based therapies as well as *ex vivo* lung bioengineering have offered exciting new avenues for providing novel potential therapeutic approaches for respiratory diseases [31]. Like other fields, failure to deliver significant number of cells to the target site severely undermines the utility of airway repair by cell therapy.

Magnetic nanoparticles based cell targeting has showed promising results so far in long term cell engraftment. Labelling endothelial progenitor cells with magnetic nanoparticles and targeting them with Halbach cylinder magnet has increased the number of retained cells in a tubular structure, which in principle, compatible with airway epithelium (Figure 6) [33]. In this technique the tubular scaffold, positioned inside the cylindrical Halbach magnet, faced an intense magnetic field that draw the nanoparticles labelled cells and facilitates an efficient and uniform delivery. In an early work, dynamic rotational cell seeding has been used to seed autologous cells onto the lumen of decellularized trachea, prior to transplantation into a human recipient [34]. Although the technique was clinically successful, the continuous movement between the cell suspension and scaffold surface over a period of 96 h made it inefficient. Compare to this technique, magnetic targeting produce an uniform cellular distribution on the graft luminal surface in only 2 h [33].

Figure 6. A schematic representation of the Halbach cylinder cell-seeding device. The Halbach cylinder consisted of 12 NdFeB rods, 75 mm long, with a 1208 rotation angle for the magnetization orientation set into aluminium housing. The bore of the cylinder was designed to house a 50 ml tube that contains the membrane scaffolds during the tissue engineering process. Image reconstructed from M. Gonzalez *et al.* (2012) [33] with the permission from the author.

7.5. SAFETY

Unlike other forces such as light, electric fields and ultrasound for manipulating therapies, magnetic fields have a very minimal effect on biological systems. Moreover, it can reach and penetrate safely and deliver cells to deep in to the tissue targets. Nonetheless there are negative effects of these particles and they can be divided into direct (immediate) and indirect (mediated). The direct effects are conferred by the influence of nanoparticles on cells – their accretion in the cytoplasm, docking on membranes, mechanical destruction of cell membranes, conformational changes of cytoskeleton and functional impairment of biopolymers [1].

The indirect influence of nanoparticles can be mediated by a number of factors, like peroxide modification of molecules with alteration of their properties, changes in homeostasis parameters, activation of endogenous damage factors, coagulating and kallikrein-kinin systems. These features appear in response to the exposure of nanomaterial into the cell. Nanoparticles could also be involved in stimulation of the immune system through hapten or adjuvant activity, as well as mediated by cytokines secreting activated phagocytes [1].

7.5.1. Toxicity

A number of studies have investigated the cytotoxic potential of several different magnetic nanoparticles on different cell types and generally have found no or low cytotoxicity at lower concentration ($> 100 \mu\text{g ml}^{-1}$). This cytotoxicity is mostly conferred by the fact that the core of the particle contains a transition metal [35]. Other factors such as composition of the coating or its breakdown products, cell-media composition *etc.* can also play a role in conferring the cytotoxicity. The core generally has high dispersion rate and the large specific surface area allows a large number of transition metal atom, such as Fe atoms, to interact with the cellular environment by accepting or donating electrons, thus stimulating the formation of ions and radicals [35]. Most commonly formed radicals are reactive oxygen species (ROS), such as the superoxide anion, hydroxyl radicals and the non-radical hydrogen peroxide. Higher dose or greater reactive transition metal leads to increased production of ROS. Higher ROS level has been associated with significant toxic effect such as peroxidizing lipids, disrupting DNA, modulating gene transcription, altering proteins and resulting in decline in physiological function and apoptosis. Aside from producing directly from the particle surface, ROS can also be generated from leaching of iron molecule, altering mitochondrial function and inducing cell signaling pathway (Figure 7) [36].

Figure 7. Schematic representation of potential SPION induced cellular toxicity. Image reconstructed from N. Singh *et al.* (2010) [36] with the permission from the author.

Table 1. Toxicity study of nanoparticles on different cell types

Coating	Size	Concentration	Cell	Incubation time	Toxicity
Dextran	100–150 nm	0.1 mg mL^{-1}	macrophages (human)	7 days	20 % viable [38]
	35 nm	10 mg mL^{-1}	macrophages (human)	14 days	mildly toxic [39]
	30 nm	10 mg mL^{-1}	malignant mesothelioma cells (human)	3 days	mildly toxic [40]
	15 nm	0.05 mg mL^{-1}	hTERT-BJ1 (human fibroblasts)	3 days	mildly toxic [41]
	10–100 nm	0.2 mg mL^{-1}	GL261 (mouse brain tumor cells)	1 day	mildly toxic [42]
poly(vinyl alcohol)	82 nm	0.2–20 mM	L929 (mouse fibroblasts) and K562 (human leukemia)	2 days	toxicity was dependent on nanoparticle shape and size [43,44]
silica	50 nm	4 mg mL^{-1}	A549 (human lung)		$\text{IC}_{50} = 4 \text{ mg mL}^{-1}$ [45]

Coating	Size	Concentration	Cell	Incubation time	Toxicity
			adenocarcinoma epithelial cells)		
	30–120 nm	10 $\mu\text{g mL}^{-1}$	macrophages and dendritic cells (human)	2 days	dose- and size-dependent damage [46]
amine-surface	61–127 nm	0.03 μg –3 mg mL^{-1}	HepG2 (human liver carcinoma cells)	5 days	high positive charge causes severe cytotoxicity [47]
	10–20 nm	36 mM	human heart (HCM), brain (BE-2-C), and kidney (293T) cell lines	1 day	cells were viable (< 40 %) after 1 day [48]
tetraheptylammonium	30 nm	2.5 $\mu\text{g mL}^{-1}$	K562 (human leukemia)	3 days	cells were viable (< 60 %) after 2 days [49]
Tween 80	30 nm	25–500 $\mu\text{g mL}^{-1}$	macrophages J774 (mouse)	1–6 h	dose- and time-dependent damage [50]
1-hydroxyethylidene-1,1-bisphosphonic acid	20 nm	0.1 mg mL^{-1}	mesenchymal stem cells (rat)	2 days	cells were viable (70 %) [51]
chitosan	13.8 nm	123.52 g mL^{-1}	SMMC-7721 (human hepatocellular carcinoma cells)	12 h	cells were viable (10 %) [52]
human like collagen	35.5 nm	25 to 250 $\mu\text{g mL}^{-1}$	NIH3T3 cells	24 h	Dose and coating dependent [53]
starch	200 nm	0.05–0.5 mg mL^{-1}	Mesenchymal stem cells (rat)	24 h	>95 % cells survived [21]
none	20–40 nm	1.0 mg mL^{-1}	aortic endothelial cells (porcine)	24 h	Dose dependent [54]
	50–75 nm	25–400 $\mu\text{g mL}^{-1}$	Mesenchymal stem cells (human)	24–72 h	Dose and time dependent [55]

Besides ROS mediated cytotoxicity, several other nanoparticle-induced cell injury mechanisms have been proposed, such as genotoxicity, actin cytoskeleton disruption [54] and loss of mitochondrial membrane potential. The exposure of nanoparticles can lead to deleterious DNA damage that may initiate carcinogenesis. It has been reported that intramuscular injection of iron dextran complex is associated with spindle cell and pleomorphic sarcoma in rat [37]. Exposure to iron oxide nanoparticles can also induce actin stress fiber formation. Actin has many important cellular functions, including morphological stability, adhesion, motility, and permeability. Actin stress fiber formation and increased cell elastic modulus may negatively impact one or more of these functions. These stress fiber formation can also be a secondary effect of ROS accumulation in response to nanoparticles. A study showed that ROS scavengers reduced actin stress fiber formation and cell death in endothelial cells after nanoparticles exposure [54].

Mitochondrial membrane potential loss increases in a dose dependent manner in human mesenchymal stem cells exposed to nanoparticles. Mitochondrial membrane potential ($\Delta\psi M$) plays important role in adenosine triphosphate (ATP) synthesis, the redox system, and cell defense mechanisms. The loss of $\Delta\psi M$ may activate oxidative stress response, which can lead to neurodegenerative disorder, metabolic diseases, aging and cancer. Down regulation of superoxide dismutase (SOD), glutathione S-transferase mu 3 (GSTM3), glutathione peroxidase (GPx), and TNFRSF1A expression in human mesenchymal stem cells (hMSC) may be attributed to nanoparticle induced loss of mitochondrial membrane potential. TNFRSF1A is associated with increase production of ROS, mitochondrial membrane damage and onset of apoptosis [55].

Although the dose of nanoparticle administered for cellular therapy is only 1.25–5 % of the total stored iron in the body, magnetic targeting can result in higher localized iron concentration in the target site. This excessive accumulation of nanoparticles can lead to imbalance of iron homeostasis and can cause aberrant cellular responses. The nanoscale size of the magnetic nanoparticles also allows them to diffuse across biological membrane and tissue barrier. That can potentially induce cytotoxicity by impairing the functions of the major component of the cell, like mitochondria, nucleus and DNA [36].

So far a large number of apparently contradicting results on toxicity of nanoparticles have been reported due to various cell types tested and the difference in concentrations used. Thus, the primary cells and established cell lines with different physiology would be ideal to generate standard which is more representative for a variety of cells. Table 1 gives an overview of the investigated toxicities of various cell types.

7.5.2. Biochemical effects

The biochemical effects of the magnetic nanoparticles have been studied extensively. The major type of nanoparticle, SPION, with or without its different surface coatings, may result in cellular alteration including actin cytoskeleton's modulation, alteration in gene expression profiles, disturbance in iron homeostasis, activation of signaling pathways and impairment of cell cycle [36]. As speculated the effects vary depending on the cell and particle type. For instance, SPION does not affect the viability, proliferation, multiple differentiation and membrane antigen of bone marrow derived MSCs [56]. But adipose derived MSCs showed higher osteogenic differentiation capability when incubated with SPION *in vitro* [57].

As discussed earlier, some cells showed increased ROS production when incubated with nanoparticles. Higher ROS activity can lead to a number of biochemical and physiological changes in the cells. For instance, in endothelial cells iron oxide nanoparticles induce ROS formation which disrupts the actin cytoskeleton and alters cell morphology, locomotion, chemotaxis and mechanics [54]. While not cytotoxic, a high dose of nanoparticle interferes with actin cytoskeleton, which decreased the cell proliferation in neural progenitor cells and human blood outgrowth endothelial cells [58]. In another study, exposure to nanoparticles rearranges the dynamic cortical meshwork of F-actin in human microvascular endothelial cells and increased permeability on the membrane [59]. The mechanism that changes actin dynamics involved ROS induced glycogen synthase kinase 3 beta (GSK-3beta) inhibition through activation of the Akt signaling pathway. Akt also plays important role in insulin signaling and in linking growth factor signaling *via* phosphoinositide (PI) 3-kinase to basic metabolic functions, such as synthesis of protein and lipid, carbohydrate metabolism and transcription. Therefore, iron oxide nanoparticles could potentially lead to perturbation of these normal cellular and physiological pathways through Akt signaling [37,59].

The change in actin cytoskeleton architecture also leads to modification of actin-associated genes. Microarray analysis of human fibroblast cells showed higher expression of genes associated with actin remodeling after 48 h exposure to nanoparticles. Genes of other pathways have also been influenced by nanoparticles in different cells. For instance signal transduction pathway genes such as integrin subunits, tyrosine kinases and several members of the protein kinase C family showed increase expression in presence of SPION. Other genes related to cell movement and interaction such as growth hormones, ion channels, and Ras-related proteins can also be upregulated by nanoparticles. SPION exposure also induce reorganization of fibroblast's matrix material by significantly increasing the expression of ECM proteins and matrix metalloproteinases [60].

Expression of endocrine hormones, such as insulin, glucagon and somatostatin, were observed on pancreatic islet cells after labeling them with Resovist

(carboxydextran-coated SPION), only insulin showed higher expression along with its transcription factor Beta-cell E-box trans-activator (BETA2). Higher expression of insulin in response to nanoparticle has two clinical significance. First, islet cells labeled with magnetic nanoparticle can produce more insulin for patient undergoing cell transplant; Second, higher insulin induces iron uptake by fat cells by increasing ferritin synthesis and localizing transferrin protein on the membrane. Under pathological conditions, such as cancer, atherosclerosis, hypertension and arthritis, iron can leave its ferritin bound steady state condition. Thus higher insulin can initiate a vicious cycle where higher iron uptake results in more insulin production and iron overloading in the cell. As we discussed before, this could lead to accumulation of highly toxic ROS [61,62]. Secretion of other paracrine factors and secreted proteins can also be modified by MNPs. Glial derived neurotrophic factor (GDNF) and ciliary neurotrophic factor (CNTF) from mesenchymal stem cells showed opposite secretion profile in presence of increasing SPION concentration. While higher concentration of SPION has an inhibitory effect on GDNF, it has a stimulatory effect on CNTF [63].

Exposure to magnetic nanoparticle augments cell cycle progression *via* upregulating cell cycle controlling proteins. For instance, mesenchymal stem cells showed higher accumulation of hyperphosphorylated retinoblastoma tumour suppressor protein pRb, cyclins and cyclin-dependent kinases (cyclins B, D1, E, CDK2 and CDK4), when labelled with Resovist (Ferucarbotran). At the same time negative regulators of the cell cycle, such as p21Cip1, and p27Kip1, members of the CIP / KIP family as well as tumor suppressing p53 showed decreased expression. Since iron oxide nanoparticles can also activate Akt pathway, it can be speculated that nanoparticle exposure induces proliferation and survival by PI3 / Akt mediated escaping apoptosis mechanism [59,64]. Cell-cycle analysis in another study indicated that high dose of Fe₃O₄ nanoparticles altered the cell-cycle progression in human mesenchymal stem cells. Although no significant arrest in the S-phase and the G2 / M compartment was evident, a dose dependent increase in the sub-G0–G1 population was observed [55].

7.5.3. Metabolism

The metabolism of the magnetic nanoparticles can be subdivided into cellular and physiological level. In the cellular level different types of cells handles the nanoparticles in more or less similar fashion nanoparticles enter the cells *via* endocytosis, accumulate in the endosomes and ultimately fuses with lysosome. The acidic environment of endosome accelerates the dissolution of the nanoparticles, which is slowly released to the cytoplasm and eventually contributes to the total cellular pool [35]. In the physiological level, cell death due to iron overload or any other reasons lead to accumulation of the nanoparticles in ECM. The absorption, metabolism and excretion of the particles in the ECM is actively conducted by the reticuloendothelial system

and its component like the mononuclear phagocytes. Any nanoparticles not engulfed by the RES system or leaked to the blood get picked up by the iron binding proteins. Transferrin, ferritin and lactoferrin are the major iron-binding proteins in the circulatory system of the vertebrate organisms. Iron released from the nanoparticles, carried by these proteins to various organs and tissues. The typical final bio-distribution of iron oxide nanoparticles is 80–90 % in liver, 5–8 % in spleen and 1–2 % in bone marrow. Majority of the nanoparticles are carried to liver for metabolism and subsequently used by RBC or excreted by kidney. Small iron nanoparticles (> 10 nm) are usually rapidly removed through extravasation and renal clearance, whereas large particles (> 200 nm) are sequestered by the spleen *via* mechanical filtration [35].

For the physiological metabolism, blood compatibility is an essential property. Blood compatibility and blood contact characteristics should always be evaluated before clinical trials to gauge the safety of the nanoparticles. There are several clinical assays available, such as prothrombin time, activated clotting time, activated partial thromboplastin time and thrombin time, which can determine the coagulation properties of the particles. Effective protein adsorption actively influences nanoparticles fate and bio-distribution inside the body. Generally, nanoparticle size, surface charge, surface coating, shape, and stability contribute the interaction of the nanoparticles with proteins. Sometimes the preliminary modifications of coating the nanoparticles with lipids or polysaccharides make them more amenable for bio-distribution [36].

7.6. CONCLUSION

In summary, magnetic nanoparticles exhibit unique properties which endow various advantages and opportunities in biomedical applications including targeted cellular therapy. Locating cells using magnetism and directing their passage along magnetic fields is evolving as a particularly useful, non-contact approach in solving targeting and tracking problems in regenerative medicine. Theoretical adverse effects have yet to be realized in preclinical studies showing large-scale, serious adverse effects at MNP doses that would be applicable in cell therapeutics. The consensus, however, is that while some nanoparticles are safe for certain biomedical applications, they need to be considered more carefully for other uses. Surface coatings and particle size seem to be crucial parameters for the observed MNP-induced adverse effects, as they are critical determinants of cellular responses and intensity of effects. There is an urgent need for more rigid, standardized nanoparticle toxicology research because many of the published toxicology studies report conflicting results. In addition, longer preclinical and clinical prospective studies, that are sufficiently powered, are needed to establish the most important therapeutic targets for this emerging technology.

REFERENCES

1. B. Shapiro, S. Kulkarni, A. Nacev, A. Sarwar, D. Preciado, D.A. Depireux. *Annu. Rev. Biomed. Eng.* **16** (2014) 455–481.
2. I. Šafařík, K. Horská, M. Šafaříková, Magnetic nanoparticles for biomedicine, in: A. Prokop (Ed.), *Intracellular Delivery: Fundamentals and Applications*, Springer, 2011, pp. 363–372.
3. V. Vanecek, V. Zablotskii, S. Forostyak, J. Ruzicka, V. Herynek, M. Babic, P. Jendelova, S. Kubinova, A. Dejneka, E. Sykova. *Int. J. Nanomedicine* **7** (2012) 3719–3730.
4. J. Riegler, K.D. Lau, A. Garcia-Prieto, A.N. Price, T. Richards, Q.A. Pankhurst, M.F. Lythgoe. *Med. Phys.* **38** (2011) 3932–3943.
5. K. Gregory-Evans, A.E. Bashar, C. Laver. *Regen. Med.* **8** (2013) 1–3.
6. S. Sharifi, H. Seyednejad, S. Laurent, F. Atyabi, A.A. Saei, M. Mahmoudi. *Contrast Media Mol. Imaging* **10** (5) (2015) 329–355.
7. R.J. Petrie, A.D. Doyle, K.M. Yamada. *Nat. Rev. Mol. Cell. Biol.* **10** (2009) 538–549.
8. C. Carmona-Fontaine, H. Matthews, R. Mayor. *Cell. Adh. Migr.* **2** (2008) 240–242.
9. L. Lara Rodriguez, I.C. Schneider. *Integr. Biol. (Camb).* **5** (2013) 1306–1323.
10. X. Trepát, J.J. Fredberg. *Trends Cell. Biol.* **21** (2011) 638–646.
11. M. Bradshaw, T.D. Clemons, D. Ho, L. Gutierrez, F.J. Lazaro, M.J. House, T.G. St Pierre, M.W. Fear, F.M. Wood, K.S. Iyer. *Nanoscale* **7** (2015) 4884–4889.
12. K.M. Gehrs, D.H. Anderson, L.V. Johnson, G.S. Hageman. *Ann. Med.* **38** (2006) 450–471.
13. J.A. Boughman, P.M. Conneally, W.E. Nance. *Am. J. Hum. Genet.* **32** (1980) 223–235.
14. X. Zhang, J.B. Saaddine, C.F. Chou, M.F. Cotch, Y.J. Cheng, L.S. Geiss, E.W. Gregg, A.L. Albright, B.E. Klein, R. Klein. *JAMA* **304** (2010) 649–656.
15. L.L. Molday, D. Hicks, C.G. Sauer, B.H. Weber, R.S. Molday. *Invest. Ophthalmol. Vis. Sci.* **42** (2001) 816–825.
16. P. Charbel Issa, M. Groppe, R.E. MacLaren. *Ophthalmologe* **109** (2012) 121–128.
17. A.W. Joe, K. Gregory-Evans. *Curr. Eye Res.* **35** (2010) 941–952.
18. G.H. Travis. *Am. J. Hum. Genet.* **62** (1998) 503–508.
19. J.L. Dunaief, T. Dentchev, G.S. Ying, A.H. Milam. *Arch. Ophthalmol.* **120** (2002) 1435–1442.
20. K. Gregory-Evans, K. Po, F. Chang, C.Y. Gregory-Evans. *Ophthalmic Res.* **47** (2012) 32–38.
21. A. Yanai, U.O. Hafeli, A.L. Metcalfe, P. Soema, L. Addo, C.Y. Gregory-Evans, K. Po, X. Shan, O.L. Moritz, K. Gregory-Evans. *Cell Transplant.* **21** (2012) 1137–1148.
22. S. Kubinova, E. Sykova. *Regen. Med.* **7** (2012) 207–224.
23. C.A. Lee-Kubli, P. Lu. *Neural Regen. Res.* **10** (2015) 10–16.
24. D. Tukmachev, O. Lunov, V. Zablotskii, A. Dejneka, M. Babic, E. Sykova, S. Kubinova. *Nanoscale* **7** (2015) 3954–3958.
25. H. Sasaki, N. Tanaka, K. Nakanishi, K. Nishida, T. Hamasaki, K. Yamada, M. Ochi. *Spine (Phila Pa 1976)* **36** (2011) 933–938.
26. C. Wang, X. Sun, L. Cheng, S. Yin, G. Yang, Y. Li, Z. Liu. *Adv. Mater.* **26** (2014) 4794–4802.

27. K.A. Gerbin, C.E. Murry. *Cardiovasc. Pathol.* **24** (2015) 133–140.
28. M. Ishii, R. Shibata, Y. Shimizu, T. Yamamoto, K. Kondo, Y. Inoue, N. Ouchi, T. Tanigawa, N. Kanemura, A. Ito, H. Honda, T. Murohara. *Int. J. Cardiol.* **175** (2014) 545–553.
29. M. Ishii, R. Shibata, Y. Numaguchi, T. Kito, H. Suzuki, K. Shimizu, A. Ito, H. Honda, T. Murohara. *Arterioscler. Thromb. Vasc. Biol.* **31** (2011) 2210–2215.
30. T. Kito, R. Shibata, M. Ishii, H. Suzuki, T. Himeno, Y. Kataoka, Y. Yamamura, T. Yamamoto, N. Nishio, S. Ito, Y. Numaguchi, T. Tanigawa, J.K. Yamashita, N. Ouchi, H. Honda, K. Isobe, T. Murohara. *Sci. Rep.* **3** (2013) 1418.
31. D.J. Weiss. *Stem Cells* **32** (2014) 16–25.
32. A.N. Lau, M. Goodwin, C.F. Kim, D.J. Weiss. *Mol. Ther.* **20** (2012) 1116–1130.
33. J. Gonzalez-Molina, J. Riegler, P. Southern, D. Ortega, C.C. Frangos, Y. Angelopoulos, S. Husain, M.F. Lythgoe, Q.A. Pankhurst, R.M. Day. *J. R. Soc. Interface* **9** (2012) 3008–3016.
34. P. Macchiarini, P. Jungebluth, T. Go, M.A. Asnaghi, L.E. Rees, T.A. Cogan, A. Dodson, J. Martorell, S. Bellini, P.P. Parnigotto, S.C. Dickinson, A.P. Hollander, S. Mantero, M.T. Conconi, M.A. Birchall. *Lancet* **372** (2008) 2023–2030.
35. I.V. Milto, A.Y. Grishanova, T.K. Klimenteva, I.V. Suhodolo, G.Y. Vasukov, V.V. Ivanova. *Biochem. (Mosc)* **79** (2014) 1245–1254.
36. N. Singh, G.J. Jenkins, R. Asadi, S.H. Doak. *Nano Rev.* **1** (2010).
37. G. Liu, J. Gao, H. Ai, X. Chen. *Small* **9** (2013) 1533–1545.
38. E. Pawelczyk, A.S. Arbab, A. Chaudhry, A. Balakumaran, P.G. Robey, J.A. Frank. *Stem Cells* **26** (2008) 1366–1375.
39. K. Muller, J.N. Skepper, T.Y. Tang, M.J. Graves, A.J. Patterson, C. Corot, E. Lancelot, P.W. Thompson, A.P. Brown, J.H. Gillard. *Biomaterials* **29** (2008) 2656–2662.
40. K. Muller, J.N. Skepper, M. Posfai, R. Trivedi, S. Howarth, C. Corot, E. Lancelot, P.W. Thompson, A.P. Brown, J.H. Gillard. *Biomaterials* **28** (2007) 1629–1642.
41. C.C. Berry, S. Wells, S. Charles, A.S. Curtis. *Biomaterials* **24** (2003) 4551–4557.
42. A.L. Martin, L.M. Bernas, B.K. Rutt, P.J. Foster, E.R. Gillies. *Bioconjug. Chem.* **19** (2008) 2375–2384.
43. M. Mahmoudi, A. Simchi, M. Imani, A.S. Milani, P. Stroeve. *Nanotechnology* **20** (2009) 225104.
44. M. Mahmoudi, V. Serpooshan, S. Laurent. *Nanoscale* **3** (2011) 3007–3026.
45. J.S. Kim, T.J. Yoon, K.N. Yu, M.S. Noh, M. Woo, B.G. Kim, K.H. Lee, B.H. Sohn, S.B. Park, J.K. Lee, M.H. Cho. *J. Vet. Sci.* **7** (2006) 321–326.
46. A. Kunzmann, B. Andersson, C. Vogt, N. Feliu, F. Ye, S. Gabrielsson, M.S. Toprak, T. Buerki-Thurnherr, S. Laurent, M. Vahter, H. Krug, M. Muhammed, A. Scheynius, B. Fadeel. *Toxicol. Appl. Pharmacol.* **253** (2011) 81–93.
47. G. Huang, J. Diakur, Z. Xu, L.I. Wiebe. *Int. J. Pharm.* **360** (2008) 197–203.
48. M. Mahmoudi, S. Laurent, M.A. Shokrgozar, M. Hosseinkhani. *ACS Nano* **5** (2011) 7263–7276.
49. G. Lv, F. He, X. Wang, F. Gao, G. Zhang, T. Wang, H. Jiang, C. Wu, D. Guo, X. Li, B. Chen, Z. Gu. *Langmuir* **24** (2008) 2151–2156.
50. S. Naqvi, M. Samim, M. Abdin, F.J. Ahmed, A. Maitra, C. Prashant, A.K. Dinda. *Int. J. Nanomedicine* **5** (2010) 983–989.
51. G.J. Delcroix, M. Jacquart, L. Lemaire, L. Sindji, F. Franconi, J.J. Le Jeune, C.N. Montero-Menei. *Brain Res.* **1255** (2009) 18–31.

52. Y. Ge, Y. Zhang, S. He, F. Nie, G. Teng, N. Gu. *Nanoscale Res. Lett.* **4** (2009) 287–295.
53. X. Liu, H. Zhang, L. Chang, B. Yu, Q. Liu, J. Wu, Y. Miao, P. Ma, D. Fan, H. Fan. *Nanoscale Res. Lett.* **10** (2015) 28.
54. K. Buyukhatipoglu, A.M. Clyne. *J. Biomed. Mater. Res. A* **96** (2011) 186–195.
55. V.S. Periasamy, J. Athinarayanan, M. Alhazmi, K.A. Alatiah, A.A. Alshatwi. *Environ. Toxicol.* (2014).
56. J.H. Sun, Y.L. Zhang, S.P. Qian, X.B. Yu, H.Y. Xie, L. Zhou, S.S. Zheng. *Mol. Med. Rep.* **5** (2012) 317–320.
57. H.T. Xiao, L. Wang, B. Yu. *Int. J. Clin. Exp. Med.* **8** (2015) 698–705.
58. S.J. Soenen, N. Nuytten, S.F. De Meyer, S.C. De Smedt, M. De Cuyper. *Small* **6** (2010) 832–842.
59. P.L. Apopa, Y. Qian, R. Shao, N.L. Guo, D. Schwegler-Berry, M. Pacurari, D. Porter, X. Shi, V. Vallyathan, V. Castranova, D.C. Flynn. *Part Fibre Toxicol.* **6** (2009) 1.
60. C.C. Berry, S. Charles, S. Wells, M.J. Dalby, A.S. Curtis. *Int. J. Pharm.* **269** (2004) 211–225.
61. H.S. Kim, Y. Choi, I.C. Song, W.K. Moon. *NMR Biomed* **22** (2009) 852–856.
62. D.W. Reif. *Free Radic. Biol. Med.* **12** (1992) 417–427.
63. A.E. Bashar, A. Metcalfe, A. Yanai, C. Laver, U.O. Hafeli, C.Y. Gregory-Evans, O.L. Moritz, J.A. Matsubara, K. Gregory-Evans. *IEEE Trans. Magn.* **49** (2013) 389–393.
64. D.M. Huang, J.K. Hsiao, Y.C. Chen, L.Y. Chien, M. Yao, Y.K. Chen, B.S. Ko, S.C. Hsu, L.A. Tai, H.Y. Cheng, S.W. Wang, C.S. Yang. *Biomaterials* **30** (2009) 3645–3651.

Chapter

8

M13 BACTERIOPHAGE NANOMATERIALS FOR REGENERATIVE MEDICINE

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8.1. INTRODUCTION

Recent advances in nanotechnology have enabled us to see, measure, and control nano-scaled objects at the desired level. This technology has allowed us to envision how to fabricate and regenerate tissues and organs by smart tissue scaffolding, and has facilitated the development of highly efficient clean and green energy conversion devices. Although we are now in the stage of utilizing various methodologies to manipulate atoms and molecules to get new functional materials, the design of novel materials with well-defined structures that perform particular functions is still challenging in materials science [1]. Many material researchers have focused their efforts on developing new materials through endless reiteration of rational design and performance characterization processes. However, nature may have already solved such material design issues through the process of evolution. Thus, mimicking natural functional materials may be crucial to developing the desired functional materials. Some examples includes glass sponges (optical fibers) [2], brittle stars (optical lens array) [3], diatoms (sophisticated periodic structures), abalone shells (fracture resistant materials) [4], bones (support structures for vertebrates) [5], and cells (exquisite self-replicating biomachines) [6-8]. Most bio-organisms have specific functions and self-templated hierarchical structures that are difficult to mimic. Most unique characteristics are dependent on genetic information, which works as a molecular machine to control many specific functions by translating the desired proteins and precisely programmed processes in biosystems. Protein-based “bottom-up” synthesis of nanoscale functional materials and devices is thus one of the most promising areas in the newly emerging field of nanobiomaterials [9-12]. Identifying active basic buildings blocks from biological examples is crucial in biomaterial design because of the complicated nature of biological materials.

Meanwhile, the genetic engineering of phage viruses has recently provided various opportunities for building novel bio-nanomaterials by integrating various disciplines, including biology, chemistry, physics, materials science, and electric engineering. By mimicking the evolutionary process in nature, phages can be used for identifying protein (or peptide) sequences that can specifically recognize desired (target) materials at the molecular level [6,8]. These recognition elements can be used as a basis to design unprecedented materials by synthesizing inorganic nanomaterials and developing sensory materials. Additionally, due to their well-defined shape, viral particles can be used to self-assemble various ordered structures which can create novel functional nanomaterials for various applications, including energy generation [13,14], biosensors [15,16], semiconductors, and tissue regenerating materials.

In this chapter, I would like to introduce the unique features of phage viral particles and the recent accomplishments in phage-based materials as a tool to fabricate functional nanomaterials and potential future applications in regenerative medicine.

8.1.1. The M13 bacteriophage

Bacteriophages (phages) are prokaryotic viruses that can infect bacterial host cells. The name “bacteriophage” can be translated to “bacteria eater” in Greek. Like its name, once the phage infects the bacterial host, the virus exploits the host biosynthetic machinery to produce many identical copies of the viral particle itself. Phages are also one of the most common organisms on earth. Phages are composed of protein capsids surrounding outer surfaces of the viral particles (shells) and genomic materials (DNA or RNA) inside of the protein shells (Figure 1). There are many different types of phages depending on their genomic material, life cycle, and shape. Genomic materials can be either DNA or RNA in a single stranded or double stranded form. Depending on the replication process, the phage can be lysogenic or lytic. Lysogenic phages infect host cells and inject their genomic materials. The genetic materials then overtake host cell metabolism to reproduce the identical genetic material and corresponding proteins. These products are delivered to the host cell membrane and the new phages are packaged and released. Therefore, lysogenic phages do not break the host cell wall. However, lytic phages invade the host cell and replicate inside of the host cells. Right after replication, the phages destroy the host cell wall and the newly amplified phages can infect other host cells. There are many different shapes of phages, such as linear (M13, Fd, F1, Ff) or spherical (MS2). Some phages possess very sophisticated shapes. For example, the T4 phage possesses an icosahedral head and a long tail connected by a cylindrical body. Although the shape, composition, and life cycles are different, phages can replicate exact copies of themselves because they possess the phenotype and genotype in the same body. Because of this property, phages are excellent candidates to develop nanomaterials (Figure 1). Thanks to commercially available genetic tool kits, the M13 phage has been extensively used for the further development of evolvable nanoscale materials for information mining, the synthesis of new materials, and the self-assembly of various nanostructures.

8.1.2. Phage structure

The M13 phage is a bacterial virus comprised of a single stranded DNA encapsulated by various major and minor coat proteins. It has a long rod filament shape that is approximately 880 nm in length and 6.6 nm in width (Figure 1A) [17,18]. The viral capsid is composed of 2,700 copies of helically arranged major coat protein, pVIII, and 5–7 copies of minor coat proteins, pIII, pVI, pIX and pVII, located at either of its ends [17,18]. The M13 phage can infect and propagate only within bacteria displaying F-pili, such as *Escherichia*

coli (*E. coli*) [19]. It is a non-lytic bacterial virus, meaning that it does not break the bacterial cell membrane upon exit, but instead is secreted through a protein pore channel in the bacterial membrane [17,20,21]. Bacterial host growth is slowed down due to increased metabolic demands of phage production, but continues after infection [22]. These qualities allow for easy mass amplification of the bacteriophage in bacterial culture. For the last two decades, the chemical or biochemical landscape of the phage structure was greatly expanded through genetic engineering of the phage [23-26] and site-specific bio-orthogonal organic synthesis approaches [27-30].

Figure 1. Schematic diagram of various distinct structures of various phages. (A) Long rod structure of the M13 bacteriophage with genomic schematic diagrams to show each protein expressed on the M13 phage surface. (B) Sophisticated structure of the T4 bacteriophage with an icosahedral head and long tail connected through the cylindrical body. (C) Spherical structure of the MS2 bacteriophage.

8.2. PHAGE ENGINEERING

Many foreign DNA or synthetic DNA sequences can be integrated into the phage genome and expressed at various sites of the phage body through genetic engineering [26,31]. Various non-natural amino acids have been expressed on the phage surface using amber codon tRNA approaches [32,33]. In addition, site-specific chemical reactions have been developed and enable further modification of the phage surface with various chemicals such as fluorescent dyes or chromophores for various applications, including biochemical imaging and energy harvesting applications [28-30].

8.2.1. Genetic engineering of phages

Phage engineering can be performed by utilizing the phage's genetic information on coat proteins. Phage display is a practical example of how to make engineered phages for different purposes. In terms of modification types, these can be divided according to the modified coat protein, *i.e.* type 3, type 8, type 88 and / or a combination of modifications (Figure 2). Using standard recombinant DNA technology, the expression of foreign peptides (or proteins) of interest can be achieved by the incorporation of corresponding coding sequences into replicable viral coat DNA. Table 1 summarizes representative constructed synthetic phages with different types of coat modifications and their applications [34].

Figure 2. Multifunctional synthetic phage construction. (A) Type 3 phage engineering, (B) Type 8 phage engineering, (C) Type 3+3 phage engineering, (D) Type 8+8 phage engineering.

Table 1. Selected reports of constructed engineered phages

Peptide sequences	Engineering Type	Targeted Protein	Ref.
RGD	3 and 8	Integrin $\alpha V\beta_1$	[35-38]
IKVAV	8	Integrin $\alpha_6\beta_4$	[35]
DGEA	8	Integrin $\alpha_2\beta_1$	[39]
HPQ	3 and 8	Streptavidin	[37,40]
RLIVGDPSSFQEKDADTL	3	Chlamydia	[38]
YWQPYALPL	3 and 8	IL-1R type I	[41]
KRTGQYKL	3	FGFR	[42]
GERWCFDGPRAWVCGWEI, GGNECDIARMWEWECFERL, RGWVEICAADDYGRCLTEAQ	8+8 and 3+3	VEGFR	[43]
GGCADGPTLREWISFCGG	8+8	TpoR	[44]
AFDWTFFVPSLIL	3	CCR5	[45]
QEVCMTSCDKLMKCNWMAA M	8+8 and 3+3	DR5	[46]
TAWSEVLDLLRR	3	PMCA4	[47-49]
SSCESPEVDYLECLY, LQCRYDQLIEEWRCHEY	8+8	B-cell maturation antigen	[50]

8.2.1.1. pIII minor coat, or pVIII protein engineering

pIII minor coat protein engineering (Type 3) is relatively well-known for the insertion of foreign peptides. Foreign peptides displayed on all five pIII subunits are constrained to lie very close to each other, but their attachment to the virion surface is probably quite flexible. For these reasons, it is likely that such displayed peptides can form multivalent interactions with immobilized selectors or cellular receptors. Phage display has been developed for use as an information mining tool [26,51,52], in which the diversity of the amino acid libraries presented by the phage provides information on binding between the peptide and its target [53-55]. Recently, a synthetic phage displaying the fragment antigen-binding (Fab) in the pIII region was utilized in phage display and expanded regarding its capacity to express peptides with variable size, selectivity and sensitivity [56]. Phage display has been used to identify the peptides mimicking many functional peptides, including chemokines or chemokine receptors, and was then utilized to study and target the roles of chemokines and their receptors [41,57-59] (see Table 1).

The Lee group [23,35-37,39] has mostly utilized pVIII engineering for tissue engineering purposes, but also performed pIII engineering to make a multifunctional synthetic phage for sensing and capturing purposes (Table 1).

Foreign peptides displayed on pVIII were introduced soon after pIII display was introduced. “Landscape” peptide presentation on the major coat protein of the filamentous phage has been utilized to template inorganic crystals for energy and memory storage devices [60-63] and to make stimulus-responsive materials [64]. Phages have also been exploited for medical applications, such as targeted drug [65,66], gene [67], and imaging agent [68] delivery, and as a tissue engineering scaffold material [35]. Merzylak *et al.* presented a cell signaling Arg-Gly-Asp (RGD) motif on pVIII proteins for a tissue engineering scaffold applications [23,35]. The approach used to display a foreign peptide on every copy of pVIII protein demonstrated how other functionally designed groups can be presented on the phage filament with quantitative analyses on the characteristics of the inserts and their constrained sequences expressed on a phage particle.

8.2.1.2. *pVI, pVII or pIX minor coat protein engineering*

Other minor coat proteins, *i.e.* pVI, pVII or pIX, have been used in phagemid format, with mosaic display of inserted foreign peptides [69,70]. Fusion proteins were expressed on pVII and pIV from the phagemid as procoats with *ompA* and *pelB* leaders. Since these proteins are likely to interact with one another in the phage capsid, this method may be useful for engineering antibodies or integrins, which are dimeric proteins. This technology was later extended to construct a large, human single-chain Fv (scFv) antibody library on pIX [71].

8.2.1.3. *NN type engineering*

Mosaic display using type 88 or 33 systems overcomes two potential disadvantages of pVIII major coat modification (Type 8) and pIII minor coat modification [72,73]. Type 88 vectors contain a synthetic recombinant pVIII gene beside wild type pVIII genes. To minimize recombination between the recombinant and wild type pVIII genes, the sequence of the recombinant pVIII gene is designed to be very different from the wild type pVIII gene, while encoding the same amino acid sequences. Similarly, type 33 system has two pIII genes, one full length and one truncated (amino acids 198–408). The former expresses a functional pIII, while the second gene produces a fusion protein.

8.2.2. Directed evolution of phages

One of the most remarkable features of phage-based materials versus other engineering materials is the ability to direct the evolutionary processes of the material. Evolutionary processes mainly consist of diversification, selection, and replication processes. In nature, mutation can occur during the replication process of a gene and diversify the species with various new functions. By mimicking the evolutionary process in nature, phages can be used as a

template to perform directed evolutionary processes, called phage display [26,31]. Phage display is a fast evolutionary screening process that allows for isolating specific binding peptides to various desired material targets. All of the phage coat proteins can be genetically modified to display relatively short (< 8–20 amino acid) peptide sequences. Insertion of randomized DNA sequences into specific gene locations of the phage genome generates a highly diverse library of peptides (up to 10^{11} random sequences) on the viral particles [26,51].

8.2.2.1. Phage display to select functional peptide sequences

To select a functional peptide sequence for a given target material, the engineered phage library pool goes through several rounds of selection processes (Figure 3). Mostly, these processes depend on the affinity for the target material. However, it is also possible to screen other functional proteins. As for affinity selection, the phages are allowed to bind to the target. The non-bound phages are then washed away, and the bound ones are eluted. The eluted phages are amplified through *E. coli* bacterial host infection. These processes are repeated several more times under more stringent binding conditions to enrich for phages with greater affinity for the target material in each consecutive round. Finally, the dominant binding peptides emerge and are identified through DNA analysis of the phage genome. Phage display on pIII is most often utilized since, as the biggest phage coat protein, it allows for greater peptide sequence variability and size, as well as the display of constrained libraries [26,55]. The lower valency of the protein (only 5 copies as opposed to 2700 for pVIII) allows for the selection of peptides with higher affinity towards the target, due to a lack of avidity effects [25]. Recently, a major coat engineered library has been developed and used for various inorganic material syntheses, such as semiconductor or electric materials and conjugating the phage with carbon nanotubes [6,8,13,14,74]. By combining with microelectro-mechanical system (MEMS) techniques, Liu *et al.* recently developed a microfluidic device to perform the phage display process in an automated manner on a small scale [75].

8.2.2.2. Phage display to identify protein interactions

The phage display technique was originally developed for small peptide antibodies or for identifying protein epitope-like ligands. The main advantage of this approach is that the use of an amino acid library allows for the identification of an epitope sequence in a protein, which is not necessarily in sequential order, but could assemble and become functional through protein folding [25]. Furthermore, presentation of such a library on a phage protein allows for an immediate connection between the identified peptide and its encoding genetic sequence [24,25]. Since the inception of this method, a variety of peptide ligands have been discovered for protein-protein interaction including specific protein binding [76–78], DNA binding [79], receptor binding

[80,81], and cell and tissue binding [82]. Furthermore, through the use of phage display, identified short peptide motifs can be further matured for better binding against the target of interest [83,84]. For example, phage display was instrumental in determining the best binding conformation for the well-known RGD motif, as well as for elucidating the different sequences of RGD flanking residues for specificity to a certain type of integrin binding [80].

Figure 3. Schematic diagram of the phage display process

8.2.3. Chemical engineering of phages

Although genetic engineering approaches have been widely used to design novel bionanomaterials, there are two main motives for the development of methods for the chemical functionalization of phages: 1) The functional groups expressed by genetically programmable bionanomaterials are limited to peptides composed of natural amino acids, which cannot incorporate the vast (bio)chemical diversity of natural or synthetic compounds. While genetic engineering is powerful for tuning every coat protein copy, excessive mutations diminish the packaging, replication, and assembly efficiency of the phage. 2) To expand the use of phages in novel functional applications such as (bio)chemical sensing, bioimaging, and tissue engineering, the chemical functionalization of the M13 phage is essential. The phage capsid is aligned along the shaft and is composed of 2,700 copies of pVIII and ~ 5 copies of the minor coat proteins pIII, pVI, pIX, and pVII located at either end [17,18]. The 50-residue pVIII (98 % by mass) is composed of three distinct domains, namely a negatively charged hydrophilic N-terminal domain (1–20), an intermediate hydrophobic domain [21–39], and a positively charged domain (40–50) that interacts electrostatically with phage genomic DNA. Only the N-terminal domain is exposed to the medium, allowing it to be targeted for

genetic or chemical functionalization. The final five residues of pVIII are structurally unconstrained, thus providing an optimal target for genetic engineering. Additionally, the minor coat protein pIII, which resides on one tip of the phage, has been extensively exploited in phage display owing to its flexibility and the accessibility of its N-terminus [85], which allows for the insertion of various peptide lengths, including larger proteins (> 100 amino acids) [86]. This section will focus on the chemical functionalization of pVIII and pIII, which have more tolerance to genetic mutation for inserting specific amino acids, thereby leading to facile chemo-selective modification. Incorporation of synthetic functional groups in a site-specific and quantitative manner is a challenging issue in chemical functionalization with further application in biomedical areas. With the growing expectations for phages as a potential bionanomaterial in the development of next-generation functional materials, the functionalization of phages by combined biological and chemical methods is gaining enormous interest.

The two most important challenges during the functionalization of M13 bacteriophages with synthetic functional groups are: 1) the incorporation of functional groups at the desired active sites under mild reaction conditions where the phage structure is retained, and 2) coupling synthetic functional groups with multiple copies of proteins that have abundant potential reactive groups. Therefore, the chemical conjugation strategy in phage modification requires mild and facile chemo-selective reactions. Researchers have recently begun to understand the site-specific chemical accessibility of amino acids within the coat proteins. To achieve a controlled and orthogonal chemistry on the phage, several amino acids (cysteine, N-terminal alanine, lysine, N-terminal serine / threonine, aspartic acid / glutamic acid, and tyrosine) are often exploited as target residues (see Table 2). Either wild-type or genetically engineered phages displaying specific amino acid(s) on the exposed coat protein domain were used for chemical functionalization. In the wild-type phage, amino groups in N-terminal alanine and in lysine (Lys8) and carboxylic acid groups in glutamic acid (Glu2) and aspartic acid (Asp4 and Asp5) on pVIII are considered viable targets for selective chemical functionalization such as amide bond formation. In order to utilize a wide range of chemo-selective modifications in phages, cysteine and other amino acids such as N-terminal serine / threonine or tyrosine, which do not appear in the solvent-exposed domain of the wild-type coat proteins, are used in genetic engineering. Several chemo-selective modification methods have been employed for each incorporated amino acid residue (see Table 2). This development of site-specific organic synthesis approaches [27,28,30,87] enables phage surfaces to be modified with various chemicals such as fluorescent dyes, chromophores, enzymes, and synthetic oligomers (*e.g.*, poly(ethylene glycol) (PEG)), and allows them to be used in various applications, including bioimaging, biosensing, tissue engineering, and energy harvesting [28,30,87].

Table 2. Selected reports of chemically functionalized phages [88,89]

Target amino acid	Chemical reaction	Incorporated materials
Cysteine (genetically incorporated) on pVIII	Alkylation [90-92]	Alkyl, proxyl, and fluorescein
Cysteine (genetically incorporated) on pIII	Benzylation [93]	Tris(bromomethyl)benzene
Alanine (N-terminal) and lysine on pVIII	Amide formation and 1,3-dipolar cycloaddition	Arg-Gly-Asp-Ser (RGDS) peptide [94], biotin [95], fluorophore [96,97], PEG [98], enzyme [99], and prodrug [100]
Alanine (N-terminal) on pVIII	Transamination and oximation	Fluorophore [101] and PEG [101]
Aspartic acid and glutamic acid on pVIII	Amide formation	Diamino compound or fluorophore [98]
Aspartic acid and glutamic acid on pVIII	Electrostatic interaction	Oligolysine [102]
Serine on pIII	Oxidation and oximation	Mannose and biotin [103]
Tyrosine on pVIII	Diazotization	Diazonium compound [104]

8.2.4. Self-assembly of phages

Controlling the assembly of basic structural building blocks in a systematic and orderly fashion is an emerging issue in various areas of science and engineering, including physics, chemistry, material science, biological engineering, and electrical engineering. The self-assembly technique can be used to develop functional nanostructures associated with organic-, inorganic-nanoparticles, copolymers, and proteins, and has several unique advantages, the most important of which is its easy processing [7,88,105-107]. Functional nanostructures can be achieved at low cost since the solvent-based procedure utilized in their creation can proceed without additional manipulation of the physics, chemistry, and vapor pressure of the solvent of the basic structural building blocks. The regular formation of highly packed building blocks increases device performance by increasing the density of building blocks per unit volume [108,109].

The construction of building blocks using self-assembly techniques is inspired by biological systems [2,110-112]. Although mimicking the biological process of assembly is still in its infancy due to the uncertainty and complexity of the structure, using natural building blocks can be a solution to overcome this obstacle. Of the natural materials, including DNA, collagen, yeast, and viruses, viruses were recently highlighted as unique natural building blocks for the

self-assembly process [113-116]. Furthermore, the M13 bacteriophage has attracted significant attention in the field because of its easy growth and handling properties. The use of biological materials as templates enables non-toxic synthesis at low cost. Phages are virus nanofibers approximately 880 nm in length and 6.6 nm in diameter and are safe for use in humans. They can be easily modified genetically and chemically to provide specific functions. Phages can also be used as a template to reveal the homogeneous distribution and percolated network structure of inorganic nanostructures under ambient conditions [13,89,117-118]. Inexpensive and environmentally friendly synthesis is possible through M13 bacteriophage engineering.

8.2.5. Fabrication of the M13 bacteriophage self-assembly (M13SA) building block

The traditional preparation of M13SA is based on the layer-by-layer (LBL) assembly technique. By using linear poly(ethylene imine) and poly(acrylic acid), this controlled molecular interaction leads to a regular orientation of the bacteriophage [119-121]. Recently, novel techniques for 2-D and three-dimensional (3-D) assembly of the M13 bacteriophage were introduced.

2-D structure fabrication: Low permeability has been a significant issue in 2-D fabrication because of the thickness of traditional membranes in the active area. Current research efforts are therefore primarily focused on making thin membranes that maintain separation efficiency with high permeability [122-125]. Interconnected basic structural building blocks can provide structural integrity and large-scale production with excellent permeability [126,127]. However, the range of the pore size distribution due to the non-uniform integration of the building blocks limits the application of this system; using the M13 bacteriophage could be a solution to this problem. Lee *et al.* generated a unidirectionally aligned 2-D M13 bacteriophage structure on a graphene oxide (GO) surface using a simple fabrication method [128]. The genetically programmed pIII protein placed at the end of the M13 bacteriophage had specific binding interactions with the carboxylate-functionalized edges of the GO. The relatively neutral body of the virus had a weaker connection with the GO surface, which was less chemically active than the end of the virus with the GO edge. The genetically modified pIII protein made strong salt-bridge type interactions with the GO edge *via* hydrogen bonding. The pIII protein, displayed with a disulfide bond-constrained peptide, made a strong cyclic structure with the edge of the GO and the aligned virus in the same direction owing to the energetic affinity of peptide geometry [129]. In contrast, the pVIII protein on the virus body demonstrated slight electrostatic repulsion due to the inherent negative charge. The fabricated M13 virus on the GO surface could be unidirectionally aligned by the external shear force of a water stream. This technique, which orients the direction of M13 by the dipping and sweeping

procedure, was previously reported [130]. Actual alignment of the M13 virus was observed using transmission electron microscopy (TEM). Through this approach, it is anticipated that highly orientated 2-D viral structures can be produced on both a large scale and at low cost, while maintaining high performance [129].

3-D structure fabrication: While 2-D phage formation is important for the application of thin structures such as ultrathin membranes, the 3-D structure of phage is also essential for engineering batteries [131], piezoelectric generators [132], and photovoltaics [133]. Chen *et al.* reported the generation of a polyaniline (PANI) and single walled carbon nanotube (SWNT) composite 3-D structure using the M13 bacteriophage as a template [134]. PANI is an excellent conductive polymer and exhibits enhanced performance when mixed with SWNT. PANI and SWNT composites have been studied extensively through different assembly methods such as colloidal mixtures [135], electrostatic deposition [136], electropolymerization [137,138], radical polymerization in solution [139], and direct polymerization on SWNT supports [140-143]. However, poor dispersion and aggregation of SWNTs in the composites has limited the progress of these studies. Chemical modification of SWNTs [139,141] by adding surfactants [140,143] or binders [137,144] was applied to overcome this hurdle. Although the fabrication of composites was successful in this approach, heavy loading of SWNTs to achieve efficiency created another obstacle for further applications due to the high associated costs. A phage was introduced as a template to generate a better PANI/SWNT composite because of its efficient dispersion ability. It was genetically engineered to bind SWNTs along the length of the bacteriophage, thereby allowing SWNTs to be clustered without aggregation. The M13/SWNTs composite was cross-linked to form a hydrogel type scaffold. The continuous 3-D porous M13/SWNTs scaffold was successfully combined with PANI using a direct polymerization method. This result represents one of the best cost-effective solutions, since the aqueous-based synthetic process allows for reduced cost and easy large-scale production.

8.2.6. Application of (M13SA) as an artificial extracellular matrix (ECM)

Wang *et al.* reported that specific fibronectin peptides (RGD and PHSRN) displayed on the bacteriophage matrix with unique topology serve as an ECM for differentiating mesenchymal stem cells (MSC) into osteoblasts [145]. The phage-based film can provide a unique rigid / groove nanostructure, and the ECM topology had a significant influence on cell behavior such as cell differentiation by cell shape elongation [146-148]. This demonstrates that the rigid / groove structure formed through self-assembly of the M13 bacteriophage significantly induced the elongation and parallel alignment of rat MSC. In addition, insertion of RGD and PHSRN peptides into the M13 bacteriophage increased cell adhesion and viability [145]. The simultaneous

functionality of ECM topology and peptides *via* the M13 bacteriophage could be a novel strategy in stem cell research.

Wang *et al.* also suggested a 3-D structure of M13 bacteriophage-based ECM for vascularized osteogenesis of MSC [149]. It is known that new bone formation is promoted by proper angiogenesis [150]. Thus, to induce bone tissue angiogenesis in an artificial ECM, RGD peptides that contain the blood vessel regeneration factor, α_v integrin, were introduced through the M13 bacteriophage. RGD peptides are highly unstable when mixed physically or chemically with ECM or medium. Therefore, an RGD-displayed virus matrix could be a possible solution for maintaining RGD peptides in the ECM for a long period in order to achieve successful bone regeneration *in vivo*. To test this, an RGD-displayed virus-based matrix was implanted into a rat radial bone defect. The vascular endothelial growth factor (VEGF) and wild type M13 were used as positive and negative controls, respectively, for comparison. Quantification of the bone volume of the RGD-displayed virus-based matrix sample showed comparable results in comparison to the normal bone tissue sample. The number of blood vessels formed was approximately 50 % greater than the positive control. The RGD-displayed virus-based matrix induced the formation of vascularized bone without VEGF [149]. Based on these results, the application of various peptide-displayed bacteriophages in nanomedicine and regenerative medicine is highly anticipated.

8.3. TISSUE ENGINEERING

The field of tissue engineering strives to fulfill a need to regenerate, repair, or replace biological tissue damaged due to injury or disease. The regulatory approval process for medical devices, and especially combination therapies that encompass products containing both biomaterials and cells, is staggeringly long. Therefore, most of the commercial products currently available are based on some of the initial efforts in tissue engineering that focused on cellular therapies, such as biomaterial skin substitutes and bioactive bone filler materials [151]. As the market and clinical penetration of tissue engineering-based technology is still extremely low, while the basic science knowledge of materials and molecular biology, as well as the know-how of technology transfer strategies for this field is rapidly growing, the creative space for improvement and innovation is vast. The blueprint design considerations of tissue engineers are based on the cellular microenvironment *in vivo*. There the cells are in close contact with the other cells, as well as ECM. The structure and composition of the ECM is highly dependent on the cell and tissue type it contains, but is generally an interconnected network of proteins, carbohydrates, and proteoglycans (highly branched combinations of the other two). The ECM is continuously synthesized and remodeled by the cells that it surrounds. The ECM scaffold is fibrillar in nature, and contains proteins with

diameters ranging from 5–300 nm [152]. This matrix provides physical support to cells, but also along with neighboring cells often provides topographical cues for cell polarization or migration. Furthermore, the components of the ECM, as well as the ligands and receptors displayed on other cells, serve to provide chemical signaling to cells. The signals for adhesion, migration, proliferation, and differentiation are provided by differential exposure of integrin binding sites, as well as growth factors and cytokines. These signaling motifs can either be presented directly upon contact, or be hidden and only exposed upon matrix remodeling, or in the context of tissue injury [153–155]. Furthermore, the matrix components can bind soluble growth factor molecules from the physiological milieu and present them to cells as needed, as seen with the laminin-bound heparan sulfate proteoglycan that binds and presents bFGF upon demand to neural cells [153,154]. Such a complicated and dynamic environment is nearly impossible to capture with current technologies. A highly simplified cellular environment is the goal of engineered materials that strive to preserve only the most important physiological functionalities.

8.3.1. Architecture for tissue engineering materials (physical cues)

In imitating tissue-specific architectures, many tissue engineering efforts have been directed towards making a biomimetic nanofibrous environment conducive to cell growth. Nanoscale features are much smaller than the size of a cell, and so allow cells to experience a more physiological 3-D environment and retain their normal shape. Materials that are in the microscale range are often bigger than the size of a cell, and present a 2-D surface for cell binding, which forces the cells to spread and flatten, therefore changing their natural morphology [156]. Nanoscale features more closely resemble the dimensions of receptors and extracellular proteins, and provide a greatly increased surface area for ligand immobilization and cell attachment. Filopodia, *i.e.* cell sensory units, have previously been observed to interact with islands as small as 10 nm in height [157]. Additionally, cells grown on substrates with nanoscale topographical features have been shown to upregulate a different set of genes than on substrates with microscale features [158], explaining differences in cell behavior, including increased proliferation and differentiation seen on nanoscale topographies [158,159]. Many tissues such as nerves, cornea, muscles, blood vessels and cartilage, and cell processes such as migration, contractility, and polarization are influenced by the underlying topography of their environments. Cell alignment, elongation, and migration have long been noted to occur in a preferential direction if grown on a material with a directionally oriented topography such as fibers, ridges, or steps [159–163]. This is in part due to the structural organization and assembly of the cytoskeleton that can convert the mechanical input stimuli of the surrounding substrate into the chemical response output of regulatory cell signaling pathways [164]. Cell alignment and elongation depend on a probability that a

cell will make a successful protrusion in a given direction. It is more favorable for actin and microtubule filaments to assemble in a plane, rather than over a physical obstacle such as an edge or a sharp turn [165,166]. Therefore, it is more likely that filopodia protrusions formed by polymerizing actin and microtubule filaments will also extend in a plane, initiating focal adhesions and leading the cell to polarize and elongate if an obstacle is present. Numerous studies have been designed to study the extent of cell alignment on steps and fibers of different dimensions, investigating factors such as the width of the feature, depth, and spacing [161,162]. Both the polarization of fibroblasts and the extension of neural cell processes have been shown to be guided by such features *in vivo* during development [167,168] and tissue remodeling [169], as well as in *in vitro* studies [159,161,162,165].

8.3.2. Receptor-ligand interactions for tissue engineering materials (chemical cues)

Cells respond to their surroundings in part by specific receptor-ligand interactions that occur at their plasma membrane. Both the display profile of cell receptors and the availability of surrounding ligands differ from tissue to tissue, cell to cell, and even at various stages of development for a given cell [153,155,170]. For example, the quantity of epidermal growth factor receptors (EGFR) expressed on neural progenitor cells differs during the different stages of development, and their number and engagement determines whether the cell will remain in a progenitor state or differentiate [155,171]. Additionally, whether this engagement is transient, as often occurs with soluble presentation of ligands, or sustained as in the example of covalently linked EGF molecules on a substrate, can determine the differentiation behavior of cells [155,172]. Furthermore, receptor activation is almost never a singular event. The importance of receptor density and even more so clustering on controlling cellular adhesion has been demonstrated by constructing spatially controlled patterns of RGD peptides [153,172,173]. Receptor aggregation is important for the phosphorylation of cytoplasmic receptor segments, which initiates the recruitment of other cytoplasmic molecules that in turn trigger an intercellular relay (*i.e.* the tyrosine kinase signaling pathway) transmitting ligand engagement events to gene transcription [153,155]. As discussed above, *in vivo*, the cell is often presented with a multitude of different signals simultaneously to elicit specific reactions. This synergy has also been demonstrated with tissue engineering substrates where a mix of ligands, *i.e.* adhesive molecules and growth factors, elicits stronger activation of cell behavior rather than each ligand presented individually [153,172,174].

8.3.3. Current technologies for tissue engineering materials

Several technologies have been utilized to create materials incorporating the biomimetic features described above to study cell responses and the utility of these materials as tissue engineering matrices. Nanotextured surface topographies have been created with a variety of lithography-based techniques. Lithographic fabrication methods such as nanoimprint lithography, e-beam lithography, microcontact printing, and embossing are based on the concept of transferring a pattern to a surface [175,176]. This is accomplished by using a mask or masking materials (such as in colloidal lithography) to shield a pattern, and then applying energy, either UV or electrons, to selectively strengthen or etch the exposed parts of the sensitive substrate material. Very precisely ordered patterns may be produced, and a resolution of 5 nm has been achieved with techniques such as nanoimprint lithography [161,162,177]. Even though such microfabrication methods provide incredible techniques to study cell behavior *in vitro*, they are difficult to transfer to large scale *in vivo* tissue engineering applications due to the high costs of production, small area of patterning, and difficulties in translating this method to three dimensions [162]. To create a more biomimetic 3-D scaffold for cell growth, both fibrous and gel materials have been implemented.

Nanofibrous networks have been made utilizing techniques such as electrospinning, peptide self-assembly and polymer phase separation. Electrospinning methodology uses a high strength electrical field to spin fibers from melted polymer solution droplets and deposit them on a substrate. The various parameters of the process, including polymer composition, field strength, and the distance between the spinneret and the substrate can be adjusted to produce fibers of various diameters. Furthermore, an electrically grounded rotating drum can be used as a collector to achieve orientation alignment of the deposited fibers [159,175,176,178]. A variety of biodegradable synthetic polymers (poly(L-lactide) (PLLA), poly(glycolic acid) (PGA), and polycaprolactone (PCL)) and biopolymers (collagen, fibrin, and silk) have been successfully spun into fibrous mats and used as cell substrates. The advantages of electrospinning methodology include the ease of production, biomimetic nanofibrous structure, and the wide applicability to a variety of materials. Some of the disadvantages include difficulty in fiber reproducibility from study to study due to many adjustment parameters, as well as difficulties in producing porous 3-D materials [178,179].

Phase separation is another method to create a polymeric nanofibrous scaffolds. Phase separation is based on multi-component systems becoming unstable and separating into multi-phase materials under certain thermodynamic conditions [180]. The phase that contains the majority of the solvent is removed, leaving a porous nanofibrous polymer foam [178]. Both synthetic (PLLA or polyurethane) and bio polymers (collagen) have been used to create such scaffolds [176,178,181]. The process is low cost and does not

require specialized technology. The morphology of this material closely resembles the structure of the native ECM, with nanofiber dimensions controlled to tens of nanometers. The porosity of the material can be controlled with varying parameters such as the choice of solvent, the concentration of polymer solutions, and the incorporation of beads. Disadvantages include the time-consuming process involving several steps such as raw material dissolution, gelation, solvent extraction, freezing, and drying. Also, the orientation of the fibers within the created matrix cannot be controlled with the current phase separation techniques [176,178,179].

Specially designed self-assembling peptide amphiphilic materials have been shown to form nanofibrous materials. The mechanism of self-assembly is based on the design of the peptide unit and depends on the ionic concentration of the solvent, and the hydrophobic-hydrophilic interactions of these units with the solvent, which drive the packing of these amphiphilic molecules into β -sheet type materials [12] or nanofibers [11]. This method has been shown to form highly hydrated and porous hydrogels that can either be used *in vitro* as a cell culture substrate, or can be injected and formed *in vivo* to facilitate tissue repair [11,12]. Additionally, amphiphilic peptide units have been previously designed to carry physiological cell receptor ligands such as RGD or IKVAV. Fibers resulting after the assembly of these units present a very high density of peptides, and have been shown to influence cell differentiation behavior [11]. Some of the disadvantages of this approach include the high cost of the self-assembling units obtained *via* peptide synthesis. Furthermore, the orientation of these materials is hard to manage, and the resulting structures are often susceptible to uncontrolled degradation by enzymes [179].

Hydrogels are a class of highly hydrated polymer materials that are used for tissue engineering scaffolds. They can be composed of hydrophilic synthetic (PLLA or PGA) or natural (collagen, fibrin, or hyaluronic acid) polymer chains. The biological and mechanical properties of hydrogel materials can be controlled by varying the composition of the gel, and the degree as well as the method of its crosslinking [153,182]. As hydrophilic materials, the gels are not inherently adhesive to cells; however, they can be readily decorated with adhesive peptide groups *via* crosslinking, or by the incorporation of peptide sequences within the polymer chain [153,182,183]. Additionally, incorporation of enzyme substrates can allow for the control of gel degradation and sequestering active peptide groups. Another attractive property of these materials as tissue engineering scaffolds is their ability to be injected to the injury site and gel *in vivo*, if this polymerization process is, for example, temperature dependent [153,182]. A disadvantage of this material is the lack of orientation control in the hydrogel structure [179].

As a monodisperse population of filamentous particles, concentrated phage solutions can be aligned through both self-assembly to form liquid-crystalline like structures, and *via* the application of an external force for longer

macroscale orientation. Furthermore, the major and minor coat proteins located on the phage shell can be genetically and chemically modified to create a controlled, spatially dense presentation of biologically active molecules. Due to these reasons, we will investigate the use of the M13 filamentous phage as a macromolecular building block for the creation of directionally oriented and biologically functionalized tissue regeneration scaffolds in the next section.

8.4. PHAGES FOR TISSUE REGENERATION

Tissue engineering scaffolding materials are ultimately designed to imitate ECM, the fibrous protein network that houses cells *in vivo*. This network provides cells with physical support and guidance through the specific topographical and chemical presentation of various adhesive sites and growth factors. Therefore, in order to control cellular behaviors such as adhesion, proliferation, and differentiation within man-made scaffolds, their surface functionalization with bioactive molecules is highly desirable [172,184,185]. Furthermore, control over the density of such bioactive groups [11,174,186] and their geometric patterning [186-188] has been shown to be important in the ability of biomaterials to modulate such behaviors. The majority of current fabrication methods rely on chemical processing to functionalize biomaterials. With this method, the final density of bioactive groups presented on the surface is ultimately dictated by the bulk solution concentration [172,185,189]. The local binding properties of the material surface, such as charge, or the availability of reactive groups or receptors dictate the final spacing of the bioactive groups. Most techniques that allow for a very precise micro and nanoscale chemical patterning of a substrate are lithography based (*i.e.* dip-pen lithography) and are hard to replicate in large scale or in 3-D scaffold materials [184,187]. Recently developed nanofabrication techniques, such as peptide self-assembly, electro-spinning, and polymer phase-separation come closer to mimicking the natural ECM topographically. However, the controlled presentation of single or multiple functional groups is still lacking [11,174,184].

Viruses are some of the best characterized structurally organized large molecules. Their nanoscale size and the inherent monodispersity of their shape and surface chemistry are better than can be achieved with most synthetic nanoparticles to date [190]. Both genetic and chemical pathways have been used to modify either single or multiple virus capsid proteins with functional groups [67,186,190-192]. Moreover, novel binding ligands can be found through evolutionary phage display screening methods [26,52,54]. Such functionalized virus particles have been demonstrated to selectively bind both inorganic and organic particles. Additionally, the templation of virus particles has been utilized for electronic and magnetic materials [193,194], as well as a variety of medical applications [65,66,68]. The M13 bacteriophage is a

filamentous bacterial virus. It has a defined long-rod shape at 880 nm long and 6.7 nm in diameter, with precisely positioned major and minor capsid proteins. These coat proteins can be genetically engineered to express short peptide groups [115,193]. M13 has been previously genetically engineered phage to display cell-adhesive peptides such as RGD and IKVAV on every copy of its pVIII protein [195]. Furthermore, such modified filamentous phages for the construction of aligned 2-D and 3-D materials that are able to support and control the polarization of cells such as fibroblasts and neural progenitor cells was demonstrated [195]. Chimeric displays of binding groups on M13 phage have been demonstrated previously for drug delivery [67], enzyme-linked immunosorbent assay (ELISA) [196], and electronic [193] applications. Additional engineering of the M13 phage to express biotin-like His-Pro-Gln (HPQ) motifs on their capsid proteins will allow for a functional expansion of potential scaffold interactions with the cells, as it will be able to present a variety of immobilized avidin conjugated growth factors and cytokines. The unique biochemical and structural features of genetically engineered phages can be also used in the context of tissue engineering / regeneration in order to control cellular growth or differentiation.

8.4.1. Chemical cue control by engineered phages

Merzlyak *et al.*, for example, have explored the use of genetically modified M13 phages as a novel building block for neural cell engineering materials to make functional biomaterials for tissue regeneration by chemical cue control [35]. This was accomplished by engineering the phage to display specific cell signaling motifs, and then assembling the viral particles into a macroscopic scaffolding material. Many peptide expression systems have previously been demonstrated on the various capsid proteins of the phage through the creation of peptide libraries [26,72]. However, as a biological particle for peptide display, phages possess the inherent limitation of having to be successfully expressed and assembled within the *E. coli* bacterial host, which restricts the type and number of peptides that can be displayed [18,197-199]. These researchers developed a novel cloning approach for the display of an integrin-binding RGD motif on every copy of the pVIII major coat protein [35]. The researchers constructed the phage using a partial library, in which an engineered octamer insert for pVIII included a constrained RGD group that was surrounded by flanking degenerate residues. This allowed for the expression of inserts that retained the desired function of the RGD motif, and yet were biologically compatible with *E. coli* during the intricate phage replication process. After the construction of engineered phages that stably displayed either RGD or IKVAV peptide groups on every copy of the pVIII protein, they constructed aligned 2-D and 3-D scaffolding materials containing phages and tested their applicability for tissue engineering. The biocompatibility of the engineered phage materials was tested by growing NIH-3T3 fibroblasts and neural progenitor cells on phage films and in phage-

-containing media [35,200]. Both cell types showed normal morphology and proliferation when in direct contact with phage materials. Neural progenitor cells either retained their progenitor state or differentiated towards the neural cell phenotype depending on the medium conditions. It was then demonstrated that 3-D phage materials could support the proliferation and differentiation of neural progenitor cells. Both RGD and IKVAV phage matrices facilitated colony formation of neural progenitor cells, which sustained over 85 % viability during the 7 day observation period. In comparison to RGE and wild type phage controls, RGD and IKVAV phages resulted in enhanced binding and spreading of neural progenitor cells with high specificity. Finally, by simple extrusion or spinning of the phage solution, the researchers constructed aligned 3-D phage fiber matrices with embedded neural progenitor cells. The resulting phage fibers encouraged neural cell differentiation and directed cell growth in parallel to the long axis of the fibers [35]. Chung *et al.* showed that mechanical shearing of the phage solution on a glass substrate resulted in 2-D directionally oriented films. These oriented films were shown to direct the alignment and morphology of fibroblasts, osteoblasts, and neural cells [200].

8.4.2. Physical cue control by engineered phages

Studies on the chemical cues and physical cues provided by synthetic phages were performed with RGD and Asp-Gly-Glu-Ala (DGEA) peptides on engineered phage films and fibers. Yoo *et al.* demonstrated the early osteogenic differentiation of mouse preosteoblasts by using a collagen-derived DGEA peptide on nanofibrous phage tissue matrices [39]. They constructed a major coat protein engineered with DGEA, Asp-Gly-Asp-Ala (DGDA) or Glu-Gly-Glu-Ala (EGEA) peptides. By genetic engineering of the phages, they constructed nanofiber-like phages having 2700 copies of the target peptide from the inserted genes with 2 and 2.7 nm spacing laterally and axially, respectively. By constructing the phage-based tissue matrix system, they could investigate the specific effect of biochemical cues, which can be tuned precisely at the single amino acid level with little changes to other physical and chemical properties. They characterized the chemical cue or physical cue effects of DGEA and of RGD peptides on the synthetic M13 phage backbone by applying MC3T3 preosteoblast cells on fabricated phage 2-D film and 3-D fibers. They observed pronounced outgrowth of preosteoblasts on DGEA phage matrices, and the cells spread very well throughout the samples on the DGEA phage matrices. Cells on DGDA, EGEA or RGE phages, which were different by one single amino acid from DGEA or RGD phages, showed that the responses were DGEA peptide-specific, demonstrating that synthetic phage-based chemical cues can be controlled by genetic engineering. A competition assay with the peptide corresponding to the engineered phage confirmed that the peptide-specific chemical cues were controlled by the synthetic phage. The DGEA peptide-specific outgrown morphology of preosteoblasts on the 2-D cultures

phage matrices were also observed in 3-D cultures. In addition, the DGEA-specific morphological responses of preosteoblast cells were linked to early osteogenic differentiation by DGEA peptides.

Virus structure can give more effective and efficient physical cues. The self-assembly capabilities of phages with patterning techniques can enhance phage-specific biochemical and physical cues. Yoo *et al.* developed a facile method of patterning genetically engineered M13 bacteriophage by employing microcontact printing methods to provide human fibroblast cells with specific biochemical and physical cues [36]. They demonstrated that nanofibrous structures, along with the biochemical signals presented by the phage microstructures, were critical to guiding cellular growth and morphology. The enhanced cellular morphological responses to RGD phage topology, rather than the RGD peptide itself, showed that the phage nanofibrous structure contributes to controlling physical cues. Especially rod-like viruses such as M13 and tobacco mosaic virus (TMV) can control their physical cues and mechanical cues, even based on concentration alone. Lin *et al.* reported on the formation of diverse patterns resulting from drying a solution of rod-like TMV particles in a glass capillary tube [201]. The concentration of TMV, the salt concentration in the aqueous solution, and the surface properties of the capillary tube interior were used as three key factors to govern such combined self-assembly behavior. The formation of hierarchical structures, which can be used for guiding directional cellular growth, was determined by the preferred orientation of TMV at the air-liquid interface as well as the pinning-depinning process. By controlling these key factors, they could generate surface roughness together with a patterned structure, which was then used for rat aortic smooth muscle cell (SMC) culture to direct the orientation of cells. These researchers could generate either stress-induced SMC alignment or 2-D patterns by utilizing the TMV patterns.

8.4.3. Multifunctional phage materials

The physiological cellular environment presents a variety of cell signaling motifs simultaneously, including adhesive sites, growth factors, and cytokines that influence cell behavior [153,172,202,203]. Similarly, engineering materials incorporating several signaling motifs simultaneously have shown this synergy to be more effective than a single motif alone [153,172,174,204]. For example, a study by Dr. Jeffrey Hubbell's group demonstrated that the incorporation of several functional peptide groups derived from laminin into a fibrin matrix at the same time resulted in a synergistic effect on cell differentiation. The cell neurites extended further into the combination peptide matrix than was predicted by just an additive effect from each peptide [174]. Immobilization of growth factor molecules to the matrix surface, instead of their untethered encapsulation within it, can decrease the uncontrolled release of these molecules, as well as their internalization and metabolism by cells, and therefore provide cells with a more directed and sustained signal,

further influencing their behavior [172]. Multiple chemical cue controls can be provided by using the M13 engineered phage system. Yoo *et al.* developed a facile growth factor immobilization system by utilizing multiple functionalized M13 synthetic phage-based matrices [37]. The growth factor immobilized by the M13 synthetic phage, together with phage's nanostructure itself, provided a simplified cellular environment which actually consisted of signaling motifs, growth factors, and topological structure effects. The engineered phage-based system showed its advantages in providing multi-functional chemical cues. Multiple signaling and therapeutic peptide motifs can be simultaneously displayed on the pIII, pVIII, and pIX protein coats of the M13 phage through genetic modification [26,72]. These researchers constructed HPQ (His-Pro-Gln) peptides on either the pVIII or pIII phage coat protein. The HPQ motif allows for binding to streptavidin-conjugated molecules, so that streptavidin conjugated growth factors can be immobilized without any size limitation, by decorating the M13 phage coat protein. This facile growth factor immobilization approach by an engineered phage may be useful for studying biochemical cues in cell biology and also in creating tissue engineering materials. Through HPQ sites, they were able to immobilize streptavidin-conjugated bFGF and NGF onto phage matrices. They also modified the RGD peptide, which is well-known to promote cell adhesion and affect the distribution of cells, on major coat proteins. They demonstrated that growth factors immobilized on the multi-functionalized M13 phage matrices with HPQ and RGD peptides were functional and could direct cell growth towards desired cellular morphologies by the RGD peptide and towards cellular fate, *i.e.* bFGF for proliferation and NGF for differentiation (Figure 4).

With the phage particle, modular with the HPQ motif, a variety of factors can be immobilized on the phage matrix, correspondingly influencing different cell behaviors or even different cell types. For example, EGF can be immobilized on the phage to induce the differentiation of progenitor cells into the neuronal phenotype [172]. Similarly, bone morphogenic protein (BMP) and insulin growth factor (IGF) can be immobilized to assist in the differentiation of osteoblasts [204]. Furthermore, VEGF can be immobilized onto the matrix to enhance endothelial cell adhesion for vascular tissue engineering [205]. Several excellent recent reviews have describe the function of many biologically relevant short peptide groups, growth factors, and cytokines [153,172,204,205]. Additionally, as vascular cells are aligned in their native environment, the alignment capabilities of the phage matrices could be further beneficial for their defined directional growth. If needed, even further functionalization of the phage can be accomplished by various chemical conjugation schemes, which have recently been employed in modifying other virus particles [190,192]. After the design and engineering of the individual phage macromolecules, their various ratios can be mixed in a homogenous solution at different concentrations to further explore how molecular concentration gradients can influence cellular behavior *in vitro* [206]. After

such systematic analysis, the design parameters that work best can be incorporated into a final mix solution to be tested on *in vivo* systems.

Figure 4. Multifunctional phage-based tissue engineering materials. Neural progenitor cells cultured on top of synthetic phages responded to the growth factor immobilized by HPQ phages *via* streptavidin. The physical and chemical cues provided by synthetic phages could control cellular behaviors [37].

8.4.4. Immune study of phage materials

As the phage material we discussed is ultimately designed for *in vivo* applications, synthetic phage-based future works will explore both the *in vitro* and *in vivo* immunogenic response to phage matrices. We hypothesize that the phage matrix as a foreign protein mass will be recognized as a “non-self” material *via* the complement system [207]. In the immune privileged environment of the central nervous system, microglia, *i.e.* the specialized immune cells of the brain, will likely mediate the immune response [68,208]. Previous studies have seen no inflammation-related damage at phage targeted tissue sites [68]. However, if a greater concentration of the phage activates

microglia, their recruitment to the site of injury may actually facilitate nerve tissue regeneration by enhancing the clearance of cellular and ECM debris in the glial scar, and expression of the growth factors, and the expression of native extracellular proteins such as laminin [209]. To explore a similar mechanism of action, there is currently a Phase II clinical trial study to test the efficacy of injecting macrophages into the site of spinal injury on stimulating regeneration [187]. *In vitro* immunogenic studies will be conducted to assess the potential of phage materials to induce an immunogenic inflammatory reaction. Similar to a study conducted by Ainslie *et al.* testing the inflammation reaction of the material nPTFE [210], a panel measuring the level of immunostimulatory or inhibitory cytokines can be performed on the supernatant from macrophages grown on phage substrates. Tissue culture polystyrene can serve as a negative control, and macrophages stimulated by lipopolysaccharides as a positive control. The levels of cytokines present can be assessed for their immunostimulatory and immunoinhibitory activity. If very high levels of immunostimulating molecules such as IL-1 or TNF- α are observed, the phage may be modified to express complement inhibiting peptides [211,212]. Furthermore, as was done in a study by Silva *et al.*, *in vivo* studies can be performed by injecting the phage solution into the spinal cord area of rat subjects [11]. Following the injection, the behavior of the animals can be evaluated for changes. After culling the animal, the injection site can be evaluated using histological studies to assess tissue inflammation and fibrosis. A previous study that targeted an engineered phage solution to a β -amyloid plaques in the brain did not observe any adverse tissue reactions in the histological analysis [68].

8.4.5. Mechanical and degradation properties of phage materials

Control of the mechanical and degradation properties of biomaterials are important for tissue engineering applications. In an optimal engineering scenario, the material that is intended to replace or repair a tissue will remain at the site of injury until it is remodeled by cells and replaced by the naturally produced ECM [153,182]. Previous work with hydrogels has demonstrated that both the concentration of the polymer macromolecule units and the degree of their cross-linking can be used to tune the mechanical properties and the rate of degradation of these materials [153,182]. The Lee group encapsulated the phage materials in an agarose gel to keep them stable in the media solution over the course of the experiment [35,39]. A future project that can further improve upon phage scaffolds is to increase their stability in aqueous media environments. Preliminary work conducted on crosslinking chemically biotinylated phages with streptavidin showed much improved stability of the phage fibers, which remained in solution for over a week without degradation [95].

8.4.6. Gene delivery systems

Drug delivery and tissue regeneration materials are often very closely related in both function and architecture. In fact, there is one perspective in the scientific community that tissue engineering scaffolds are just a delivery system of cells into the body [185]. Additionally, the line between the two areas becomes blurred when controlled growth factor or cytokine release is incorporated into the matrix to influence either the contained or the surrounding cells [172,182,185,204]. By the streptavidin crosslinking methods described above, small therapeutic drug molecules may be incorporated into the matrix. Furthermore, the link to the phage can be engineered to be dependent on enzymatic cleavage [153,213], so that the delivered molecules are released only when they are sequestered by cell activity. Therapeutic genetic material to induce tissue regeneration can be incorporated into the phage DNA and carried within the phage capsule for specific delivery to cells *via* receptor uptake [67]. As the M13 phage is non-lytic, they will be continuously produced by bacteria without causing bacterial wall rupture or the resulting debris. By designing peptide expression on the phage capsid, phages can be more locally targeted to cell receptors (*i.e. via* RGD or another ligand). Phage display technology has allowed for the identification of novel homing peptides that target unknown cell surface proteins. The targeting peptides can be incorporated into bacteriophage coat proteins through the genetic engineering techniques described previously [67]. These include peptides (RGD, glioma-binding peptide) [214,215], HER2 receptor targeting antibody [216], growth factors (EGF or FGF2) [217-219] and the penton base of adenovirus [220]. Similar to drug delivery, nucleic acid materials are now being incorporated into scaffolding materials for delivery to cells. Furthermore, it has been shown that DNA materials that are tethered to the matrix, rather than just encapsulated, are more effectively transferred to cells [221]. Phage particles engineered as described above to contain the genetic load for cell delivery, as well as specific cell targeting peptides, can be cross-linked with streptavidin units to produce stable tissue engineering scaffolds. As these scaffolds are taken up and degraded by endocytosis [222], the phage could release its gene cargo and further induce cell behavior.

8.4.7. Diagnosis and therapeutic applications

Thanks to phage display technology, we have considerable useful peptide information, which can be developed further for imaging and the diagnosis of certain diseases, such as cancer [223]. Such applications can be directly assayed by Phage-Chips [17]. Yoo *et al.* demonstrated multifunctional phage matrices that were optically readable for cell proliferation and morphology. The self-assembled nanofibrous network structure enhanced surface plasmon resonance (SPR) monitoring signals, and biochemical cues displayed by phage surfaces controlled cellular proliferation and morphology at the same time. For therapeutic applications, antibody phage display has been developed and

tested for clinical approval [224]. Another application study of utilizing M13 engineered phage properties was also introduced by adopting different useful virus parts. Hajitou *et al.* constructed a hybrid phage with two genes from the phage and the nucleus integrating gene from AAV, called inverted terminal repeats (ITR). Additionally, these phages were engineered to express an integrin binding peptide on minor coat proteins. Therefore, the RGD peptide induced internalization of the phage through integrin-mediated endocytosis and the ITR led to improved transgene expression, which was linked to the function of the delivered gene in the cytoplasm. The resulting AAV/phage system provided superior tumor transduction over the phage alone. Topical delivery of this therapeutic synthetic phage material onto localized disease areas with specific integrating functions might reduce the risk of the side effects and enhance the efficiency of drug delivery.

8.4.8. Tissue engineering and regenerative medicine applications

The aim of tissue engineering is to create desired artificial tissues or organ scaffold structures. Consequently, studies in this field require combining biomaterials and cells to closely mimic *in vivo* tissue environments. Natural tissue microenvironments are composed of networks of various nanofibrous proteins, such as collagen or fibronectin. Therefore, as discussed above, the M13 phage can be the predominant viral type utilized for this purpose because of its ability to display peptides on its coat proteins in various manners, its ability to replicate in large quantities, and its ability to self-assemble into nanofilaments. The multifunctional merits of M13 phage engineering can be further synergized with chemical modification or by conjugating growth factors. As a result of these traits, many studies have investigated using these phages as a platform to enhance the chemical, physical, and mechanical properties of biomimetic matrices (Figure 5). 2-D phage films with nanogroove topography using a facile shearing method or a layer-by-layer method were formed for neural progenitor cell regeneration and differentiation (left top in the Figure 5) [35,95]. In combination with signaling peptides on phage coats, physical properties, such as phage alignment and hierarchical ordering of phages, can help to control cell behavior (left bottom in the Figure 5) [36]. These phages displaying DGEA, a bone-specific peptide, have also been used for the engineering of hard tissues, such as bone (right top in the Figure 5) [39]. Multiple protein coat engineered M13 phages expressing HPQ, a biotin-like peptide, on minor coat proteins and RGD on major and minor coat proteins promoted neural progenitor cell proliferation or differentiation according to the streptavidin-conjugated growth factors, which were specific for neural cells (right bottom in the Figure 5) [37].

Many of the uses of phages in tissue engineering applications are similar to those previously discussed. Although the lack of patentability and the food and drug administration (FDA) guidelines, as well as safety concerns, are still challenging, the success of previous studies using M13 phages for tissue

engineering / regeneration research drives optimism that further clinical applications will emerge from this field. *In vivo* animal studies can help to further characterize the efficacy and safety of phage-based tissue engineering matrices.

Figure 5. Phage based tissue engineering for soft and hard tissues. M13 phage coat proteins can be engineered for various tissue engineering purposes.

8.5. SUMMARY AND FUTURE PERSPECTIVES

In this chapter, the use of M13 bacteriophages (phages) has been explored as a novel building block together with providing specific functions for tissue engineering / regeneration materials. Prior to using M13 as a biomimetic tissue engineering scaffold material, the phage is decorated with cell signaling motifs. An incredible diversity of peptide expression has been previously demonstrated on the various capsid proteins of the phage through the creation of peptide libraries [26,51,52]. A novel cloning approach to display an integrin binding RGD motif on every copy of pVIII was introduced to decorate the major coat protein of the M13 phage. Merzlyak *et al.* did this by using a partial library method, where an engineered octamer insert for pVIII included a constrained RGD group surrounded by a degenerate residue library. This allowed the expression of full inserts that retained the desired RGD motif yet were favorably compatible with all the protein interactions inherent in the phage replication process within *E. coli*. Furthermore, they systematically analyzed

the allowed amino acid sequence space for pVIII inserts by making constrained libraries with chemically variable residues (positive, negative, and hydrophobic) [35]. This approach can be useful for engineering phage particles with a very dense uniform display of short signal peptide motifs that may be beneficial for tissue engineering materials [11,35,174,183].

After demonstrating the phage as a filament particle able to form aligned scaffolds that are both conducive and instructive to cell growth, further phage design improvements can be made by making it multifunctional. The phage was then engineered to incorporate an adhesive peptide motif (RGD) on pVIII, and a constrained biotin-like HPQ motif on the pIII protein [37,67,225]. There are limits in the ability of the phage to display a multivalency of protein molecules based on the size and sequence of the insert [26], and it cannot be altered *via* genetic means to present functional carbohydrate molecules. By exploiting the binding affinity of the biotin-streptavidin bond, with an engineered biotin-like HPQ group, we imparted modular functionality to the phage building block [37]. Any growth factor, cytokine, or otherwise therapeutic molecule conjugated to avidin will be able to bind to our engineered HPQ phage and further functionalize the matrix [37,226].

In summary, we have introduced the utilization of M13 bacteriophage as a functional building block for tissue engineering matrices that can guide the adhesion, polarization, and alignment behavior of cells and promote tissue regeneration. We have also presented a number of avenues that can be used to expand this area of research further to immune / chemokine study and use phages for highly functional and useful biomaterials with potential applications in diagnosis and therapy.

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REFERENCES

1. A. Merzlyak, S.-W. Lee. *Curr. Opin. Chem. Biol.* **10** (2006) 246.
2. V.C. Sundar, A.D. Yablon, J.L. Grazul, M. Ilan, J. Aizenberg. *Nature* **424** (2003) 899.
3. J. Aizenberg, J.C. Weaver, M.S. Thanawala, V.C. Sundar, D.E. Morse, P. Fratzl. *Science* **309** (2005) 275.
4. J.N. Cha, G.D. Stucky, D.E. Morse, T.J. Deming. *Nature* **403** (2000) 289.
5. S. Weiner, W. Traub, H.D. Wagner. *J. Struct. Biol.* **126** (1999) 241.
6. S.R. Whaley, D.S. English, E.L. Hu, P.F. Barbara, A.M. Belcher. *Nature* **405** (2000) 665.
7. S. Zhang. *Nat. Biotechnol.* **21** (2003) 1171.
8. S.-W. Lee, C. Mao, C.E. Flynn, A.M. Belcher. *Science* **296** (2002) 892.
9. J.M. Nam, C.S. Thaxton, C.A. Mirkin. *Science* **301** (2003) 1884.
10. Y. Huang, X. Duan, Y. Cui, L.J. Lauhon, K.H. Kim, C.M. Lieber. *Science* **294** (2001) 1313.
11. G.A. Silva, C. Czeisler, K.L. Niece, E. Beniash, D.A. Harrington, J.A. Kessler, S.I. Stupp. *Science* **303** (2004) 1352.
12. S. Zhang. *Adv. Cancer Res.* **99** (2008) 335.
13. Y.J. Lee, H. Yi, W.J. Kim, K. Kang, D.S. Yun, M.S. Strano, G. Ceder, A.M. Belcher. *Science* **324** (2009) 1051.
14. K.T. Nam, D.-W. Kim, P.J. Yoo, C.-Y. Chiang, N.L. Meethong, P.T. Hammond, Y.-M. Chiang, A.M. Belcher. *Science* **312** (2006) 885.
15. M. Cerruti, J. Jaworski, D. Raorane, C. Zueger, J. Varadarajan, C. Carraro, S.-W. Lee, R. Maboudian, A. Majumdar. *Anal. Chem.* **81** (2009) 4192.
16. J.W. Jaworski, D. Raorane, J.H. Huh, A. Majumdar, S.W. Lee. *Langmuir* **24** (2008) 4938.
17. S.Y. Yoo, J.-W. Oh, S.-W. Lee. *Langmuir* **28** (2012) 2166.
18. D.J. Rodi, S. Mandova, L. Makowski, in *Phage Display in Biotechnology and Drug Discovery*, S.S. Sidhu (Ed.), CRC Press Taylor & Francis Group, Boca Raton, FL, 2005, p. 748.
19. A. Jacobson. *J Virol* **10** (1972) 835.
20. N. Opalka, R. Beckmann, N. Boisset, M.N. Simon, M. Russel, S.A. Darst. *J. Mol. Biol.* **325** (2003) 461.
21. M. Russel, N.A. Linderroth, A. Sali. *Gene* **192** (1997) 23.
22. B. Hohn, H. Lechner, D.A. Marvin. *J. Mol. Biol.* **56** (1971) 143.
23. A. Merzlyak, S.-W. Lee. *Bioconjug. Chem.* **20** (2009) 2300.
24. S.F. Parmley, G.P. Smith. *Gene* **73** (1988) 305.
25. J.K. Scott, G.P. Smith. *Science* **249** (1990) 386.
26. G.P. Smith, V.A. Petrenko. *Chem. Rev.* **97** (1997) 391.
27. Z.M. Carrico, D.W. Romanini, R.A. Mehl, M.B. Francis. *Chem. Commun.* (2008) 1205.
28. R.A. Miller, A.D. Presley, M.B. Francis. *J. Am. Chem. Soc.* **129** (2007) 3104.
29. S. Nicholas, C. Zacharywirt, F. Matthewwofx. *Angew. Chem. Int. Ed.* **48** (2009) 9498.
30. T.L. Schlick, Z. Ding, E.W. Kovacs, M.B. Francis. *J. Am. Chem. Soc.* **127** (2005) 3718.
31. G.P. Smith. *Science* **228** (1985) 1315.

32. C.C. Liu, A.V. Mack, M.L. Tsao, J.H. Mills, H.S. Lee, H. Choe, M. Farzan, P.G. Schultz, V.V. Smider. *Proc. Natl. Acad. Sci. U. S. A.* **105** (2008) 17688.
33. T. Feng, M.L. Tsao, P.G. Schultz. *J. Am. Chem. Soc.* **126** (2004) 15962.
34. S.Y. Yoo, A. Merzlyak, S.-W. Lee. *Mediat. Inflamm.* **2014** (2014) 11.
35. A. Merzlyak, S. Indrakanti, S.-W. Lee. *Nano Lett.* **9** (2009) 846.
36. S.Y. Yoo, W.-J. Chung, T.H. Kim, M. Le, S.-W. Lee. *Soft. Matter.* **7** (2011) 363.
37. S.Y. Yoo, A. Merzlyak, S.-W. Lee. *Soft. Matter.* **7** (2011) 1660.
38. S.R. Bhattarai, S.Y. Yoo, S.-W. Lee, D. Dean. *Biomaterials* **33** (2012) 5166.
39. S.Y. Yoo, M. Kobayashi, P.P. Lee, S.-W. Lee. *Biomacromolecules* **12** (2011) 987.
40. S.Y. Yoo, J.-W. Oh, S.-W. Lee. *Langmuir* **28** (2012) 2166.
41. S.D Yanofsky, D.N. Baldwin, J.H. Butler, F.R. Holden, J.W. Jacobs, P. Balasubramanian, J.P. Chinn, S.E. Cwirla, E. Peters-Bhatt, E.A. Whitehorn, E.H. Tate, A. Akeson, T.L. Bowlin, W.J. Dower, R.W. Barrett. *Proc. Natl. Acad. Sci.* **93** (1996) 7381.
42. A. Yayon, D. Aviezer, M. Safran, J.L. Gross, Y. Heldman, S. Cabilly, D. Givol, E. Katchalski-Katzir. *Proc. Natl. Acad. Sci.* **90** (1993) 10643.
43. W.J. Fairbrother, H.W. Christinger, A.G. Cochran, G. Fuh, C.J. Keenan, C. Quan, S.K. Shriver, J.Y. Tom, J.A. Wells, B.C. Cunningham. *Biochemistry* **37** (1998) 17754.
44. N.C. Wrighton, F.X. Farrell, R. Chang, A.K. Kashyap, F.P. Barbone, L.S. Mulcahy, D.L. Johnson, R.W. Barrett, L.K. Jolliffe, W.J. Dower. *Science* **273** (1996) 458.
45. F.-Y. Wang, T.-Y. Zhang, J.-X. Luo, G.-A. He, Q.-L. Gu, F. Xiao. *Biosci. Biotech. Biochem.* **70** (2006) 2035.
46. B. Li, S.J. Russell, D.M. Compaan, K. Totpal, S.A. Marsters, A. Ashkenazi, A.G. Cochran, S.G. Hymowitz, S.S. Sidhu. *J. Mol. Biol.* **361** (2006) 522.
47. J. Pande, M.M. Szewczyk, A.K. Grover. *Biotechnol. Adv.* **28** (2010) 849.
48. J. Pande, K.K. Mallhi, A.K. Grover. *Eur. J. Pharmacol.* **508** (2005) 1.
49. J. Pande, K.K. Mallhi, A. Sawh, M.M. Szewczyk, F. Simpson, A.K. Grover. *Am. J. Physiol. Cell Physiol.* **290** (2006) C1341.
50. N.C. Gordon, S. Lien, J. Johnson, H.J. Wallweber, T. Tran, B. Currell, M. Mathieu, C. Quan, M.A. Starovasnik, S.G. Hymowitz, R.F. Kelley. *J. Mol. Biol.* **396** (2010) 166.
51. V.A. Petrenko, G.P. Smith, X. Gong, T. Quinn. *Protein Eng.* **9** (1996) 797.
52. V.A. Petrenko, G.P. Smith, M.M. Mazooji, T. Quinn. *Protein Eng.* **15** (2002) 943.
53. G.P. Smith. *Filament. Virol.* **167** (1988) 156.
54. G.A. Kuzmicheva, P.K. Jayanna, I.B. Sorokulova, V.A. Petrenko. *Protein Eng. Des. Sel.* **22** (2009) 9.
55. D.J. Rodi, A.S. Soares, L. Makowski. *J. Mol. Biol.* **322** (2002) 1039.
56. M.L. Scalley-Kim, B.W. Hess, R.L. Kelly, A.R. Krostag, K.H. Lustig, J.S. Marken, P.J. Owendale, A.R. Posey, P.J. Smolak, J.D. Taylor, C.L. Wood, D.L. Bienvenue, P. Probst, R.A. Salmon, D.S. Allison, T.M. Foy, C.J. Raport. *PLoS ONE* **7** (2012) 1.
57. K. Even-Desrumeaux, D. Nevoltris, M.N. Lavaut, K. Alim, J.P. Borg, S. Audebert, B. Kerfelec, D. Baty, P. Chames. *Mol. Cell. Proteomics* **13** (2013) 653.
58. H.-M. Zheng, Y. Jiang, J.-R. Wang, X.-L. Gong, B.-Y. Guo. *Inflamm. Res.* **60** (2011) 759.
59. C. Blanchetot, D. Verzijl, A. Mujić-Delić, L. Bosch, L. Rem, R. Leurs, C.T. Verrips, M. Saunders, H. de Haard, M.J. Smit. *J. Biol. Chem.* **288** (2013) 25173.
60. S.W. Lee, B.M. Wood, A.M. Belcher. *Langmuir* **19** (2003) 1592.
61. A. Merzlyak, S.W. Lee. *Curr. Opin. Chem. Biol.* **10** (2006) 246.

62. R.R. Naik, S.J. Stringer, G. Agarwal, S.E. Jones, M.O. Stone. *Nature Mater.* **1** (2002) 169.
63. K.T. Nam, D.W. Kim, P.J. Yoo, C.Y. Chiang, N. Meethong, P.T. Hammond, Y.M. Chiang, A.M. Belcher. *Science* **312** (2006) 885.
64. H. Bermudez, A.P. Hathorne. *Faraday Discuss.* **139** (2008) 327.
65. T.J. Dickerson, K.D. Janda. *AAPS J* **7** (2005) E579.
66. D.N. Krag, G.S. Shukla, G.P. Shen, S. Pero, T. Ashikaga, S. Fuller, D.L. Weaver, S. Burdette-Radoux, C. Thomas. *Cancer Res.* **66** (2006) 8925.
67. A. Hajitou, M. Trepel, C.E. Lilley, S. Soghomonyan, M.M. Alauddin, F.C. Marini 3rd, B.H. Restel, M.G. Ozawa, C.A. Moya, R. Rangel, Y. Sun, K. Zaoui, M. Schmidt, C. von Kalle, M.D. Weitzman, J.G. Gelovani, R. Pasqualini, W. Arap. *Cell* **125** (2006) 385.
68. D. Frenkel, B. Solomon. *Proc. Natl. Acad. Sci. U S A* **99** (2002) 5675.
69. L.S. Jespers, J.H. Messens, A. De Keyser, D. Eeckhout, I. Van den Brande, Y.G. Gansemans, M.J. Lauwereys, G.P. Vlasuk, P.E. Stanssens. *Nat. Biotech.* **13** (1995) 378.
70. S.M. Changshou Gao, C.-H. L. Lo, P. Wirsching, R.A. Lerner, K.D. Janda. *Proc. Natl. Acad. Sci. U S A* **96** (1999) 6025.
71. C. Gao, S. Mao, G. Kaufmann, P. Wirsching, R.A. Lerner, K.D. Janda. *Proc. Natl. Acad. Sci.* **99** (2002) 12612.
72. V. Petrenko, G. Smith, in *Phage Display In Biotechnology and Drug Discovery*, CRC Press, 2005, p. 63.
73. D.S. Choi, H.-E. Jin, S.Y. Yoo, S.-W. Lee. *Bioconjug. Chem.* **25** (2014) 216.
74. S.W. Lee, A.M. Belcher, *Nano Lett.* **4** (2004) 387.
75. Y.L. Liu, J.D. Adams, K. Turner, F.V. Cochran, S.S. Gambhir, H.T. Soh. *Lab Chip* **9** 3604 (2009) 1033.
76. B. Cao, C. Mao. *Biomacromolecules* **10** (2009) 555.
77. Y. Chen, C. Wiesmann, G. Fuh, B. Li, H.W. Christinger, P. McKay, A.M. de Vos, H.B. Lowman. *J. Mol. Biol.* **293** (1999) 865.
78. J.J. Devlin, L.C. Panganiban, P.E. Devlin. *Science* **249** (1990) 404.
79. X. Cheng, B.K. Kay, R.L. Juliano. *Gene* **171** (1996) 1.
80. E. Ruoslahti. *Annu. Rev. Cell Dev. Biol.* **12** (1996) 697.
81. R.J. Giordano, M. Vila, J.L. Ahdenranta, R. Apasqualini, W. Arap. *Nat. Med.* **7** (2001) 1249.
82. R. Pasqualini, E. Ruoslahti. *Nature* **380** (1996) 364.
83. R. Brissette, N.I. Goldstein, in *Methods in Molecular Biology*, P. Fisher (Ed.), Humana Press, Inc, Totowa, NJ, 2007, p. 203.
84. R. Li, R.H. Hoess, J.S. Bennett, W.F. DeGrado. *Protein Eng.* **16** (2003) 65.
85. J. Lubkowski, F. Hennecke, A. Pluckthun, A. Wlodawer. *Nat. Struct. Mol. Biol.* **5** (1998) 140.
86. S.S. Sidhu, G.A. Weiss, J.A. Wells. *J. Mol. Biol.* **296** (2000) 487.
87. S. Nicholas, C. Zachary, F. Matthew. *Angew. Chem. Int. Ed.* **48** (2009) 9498.
88. G. Whitesides, J. Mathias, C. Seto. *Science* **254** (1991) 1312.
89. W.-J. Chung, D.-Y. Lee, S.Y. Yoo. *Int. J. Nanomedicine* **9** (2014) 5825.
90. R.B. Spruijt, C.J.A.M. Wolfs, J.W.G. Verver, M.A. Hemminga. *Biochemistry* **35** (1996) 10383.
91. M.A. Hemminga, W.L. Vos, P.V. Nazarov, R.B.M. Koehorst, C.J.A.M. Wolfs, R.B. Spruijt, D. Stopar. *Eur. Biophys. J.* **39** (2010) 541.

92. A.R. Khan, K.A. Williams, J.M. Boggs, C.M. Deber. *Biochemistry* **34** (1995) 12388.
93. C. Heinis, T. Rutherford, S. Freund, G. Winter. *Nat. Chem. Biol.* **5** (2009) 502.
94. J. Rong, L.A. Lee, K. Li, B. Harp, C.M. Mello, Z. Niu, Q. Wang. *Chem. Commun.* **41** (2008) 5185.
95. W.-J. Chung, A. Merzlyak, S.-W. Lee. *Soft. Matter.* **6** (2010) 4454.
96. K. Li, Y. Chen, S. Li, H.G. Nguyen, Z. Niu, S. You, C.M. Mello, X. Lu, Q. Wang. *Bioconjug. Chem.* **21** (2010) 1369.
97. K.A. Kelly, P. Waterman, R. Weissleder. *Neoplasia* **8** (2006) 1011.
98. Z. Zhang, J. Buitenhuis, A. Cukkemane, M. Brocker, M. Bott, J.K.G. Dhont. *Langmuir* **26** (2010) 10593.
99. M. Adhikari, S. Dhamane, A.E. Hagström, G. Garvey, W.H. Chen, K. Kourentzi, U. Strych, R.C. Willson. *Analyst.* **138** (2013) 5584.
100. I. Yacoby, M. Shamis, H. Bar, D. Shabat, I. Benhar. *Antimicrob. Agents Chemother.* **50** (2006) 2087.
101. Z.M. Carrico, M.E. Farkas, Y. Zhou, S.C. Hsiao, J.D. Marks, H. Chokhawala, D.S. Clark, M.B. Francis. *ACS Nano* **6** (2012) 6675.
102. J.A. Lamboy, P.Y. Tam, L.S. Lee, P.J. Jackson, S.K. Avrantinis, H.J. Lee, R.M. Corn, G.A. Weiss. *ChemBioChem.* **9** (2008) 2846.
103. S. Ng, M.R. Jafari, W.L. Matochko, R. Derda. *ACS Chem. Biol.* **7** (2012), 1482.
104. M. Murugesan, G. Abbineni, S.L. Nimmo, B. Cao, C. Mao. *Sci. Rep.* **3** (2013) 1820.
105. G.M. Whitesides, B. Grzybowski. *Science* **295** (2002) 2418.
106. R.M. Capito, H.S. Azevedo, Y.S. Velichko, A. Mata, S.I. Stupp. *Science* **319** (2008) 1812.
107. J.-S. Moon, W.-G. Kim, C. Kim, G.-T. Park, J. Heo, S.Y. Yoo, J.-W. Oh. *Mini-Rev. Org. Chem.* **12** (2015) 271.
108. S. Giménez, T. Lana-Villarreal, R. Gómez, S. Agouram, V. Muñoz-Sanjosé, I. Mora-Seró. *J. Appl. Phys.* **108** (2010) 064310.
109. V.M. Karpan, P.A. Khomyakov, A.A. Starikov, G. Giovannetti, M. Zwierzycki, M. Talanana, G. Brocks, J. van den Brink, P.J. Kelly. *Phys. Rev. B* **78** (2008) 195419.
110. H.D. Espinosa, A.L. Juster, F.J. Latourte, O.Y. Loh, D. Gregoire, P.D. Zavattieri. *Nat. Commun.* **2** (2011) 173.
111. J. Aizenberg, J.C. Weaver, M.S. Thanawala, V.C. Sundar, D.E. Morse, P. Fratzl. *Science* **309** (2005) 275.
112. H. Budzikiewicz. *Bacterial Mini Rev. Org. Chem.* **3** (2006) 93.
113. N.S. Malvankar, M. Vargas, K.P. Nevin, A.E. Franks, C. Leang, B.C. Kim, K. Inoue, T. Mester, S.F. Covalla, J.P. Johnson, V.M. Rotello, M.T. Tuominen, D.R. Lovley. *Nat. Nano* **6** (2011) 573.
114. H. Yan, S.H. Park, G. Finkelstein, J.H. Reif, T.H. LaBean. *Science* **301** (2003) 1882.
115. C. Mao, D.J. Solis, B.D. Reiss, S.T. Kottmann, R.Y. Sweeney, A. Hayhurst, G. Georgiou, B. Iverson, A.M. Belcher. *Science* **303** (2004) 213.
116. S.W. Lee, C.B. Mao, C.E. Flynn, A.M. Belcher. *Science* **296** (2002) 892.
117. Y. Lee, J. Kim, D.S. Yun, Y.S. Nam, Y. Shao-Horn, A.M. Belcher. *Energy Environ. Sci.* **5** (2012) 8328.
118. X. Dang, H. Yi, M.H. Ham, J. Qi, D.S. Yun, R. Ladewski, M.S. Strano, P.T. Hammond, A.M. Belcher. *Nat. Nano* **6** (2011) 377.
119. Z. Tang, Y. Wang, P. Podsiadlo, N.A. Kotov. *Adv. Mater.* **18** (2006) 3203.

120. C. Jiang, V.V. Tsukruk. *Adv. Mater.* **18** (2006) 829.
121. A. Javey, Nam, R.S. Friedman, H. Yan, C.M. Lieber. *Nano Lett.* **7** (2007) 773.
122. X. Peng, J. Jin, Y. Nakamura, T. Ohno, I. Ichinose. *Nat. Nano* **4** (2009) 353.
123. S. Karan, S. Samitsu, X. Peng, K. Kurashima, I. Ichinose. *Science* **335** (2012) 444.
124. C.C. Striemer, T.R. Gaborski, J.L. McGrath, P.M. Fauchet. *Nature* **445** (2007) 749.
125. J. He, X.-M. Lin, H. Chan, L. Vuković, P. Král, H.M. Jaeger. *Nano Lett.* **11** (2011) 2430.
126. H.-W. Liang, L. Wang, P.-Y. Chen, H.-T. Lin, L.-F. Chen, D. He, S.-H. Yu. *Adv. Mater.* **22** (2010) 4691.
127. X.W. Zhang, T. Zhang, J. Ng, D.D. Sun, *Adv. Funct. Mater.* **19** (2009) 3731.
128. Y.M. Lee, B. Jung, Y.H. Kim, A.R. Park, S. Han, W.S. Choe, P.J. Yoo. *Adv. Mater.* **26** (2014) 3899.
129. J. Kim, L.J. Cote, F. Kim, W. Yuan, K.R. Shull, J. Huang. *J. Am. Chem. Soc.* **132** (2010) 8180.
130. T. Sawada, T. Serizawa. *J. Mater. Chem. B* **1** (2013) 149.
131. X. Chen, K. Gerasopoulos, J. Guo, A. Brown, C. Wang, R. Ghodssi, J.N. Culver. *ACS Nano* **4** (2010) 5366.
132. B.Y. Lee, J. Zhang, C. Zueger, W.J. Chung, S.Y. Yoo, E. Wang, J. Meyer, R. Ramesh, S.W. Lee. *Nat. Nanotechnol.* **7** (2012) 351.
133. P.Y. Chen, X. Dang, M.T. Klug, J. Qi, N.M. Dorval Courchesne, F.J. Burpo, N. Fang, P.T. Hammond, A.M. Belcher. *ACS Nano* **7** (2013) 6563.
134. P.-Y. Chen, M. Nasim Hyder, D. Mackanic, N.-M. Dorval Courchesne, J. Qi, M.T. Klug, A.M. Belcher, P.T. Hammond. *Adv. Mater.* **26** (2014) 5069.
135. Q. Liu, M.H. Nayfeh, S.-T. Yau. *J. Power Sources* **195** (2010) 7480.
136. J.H. Hur, K.H. Im, S.W. Kim, U.J. Kim, J.H. Lee, S.Y. Hwang, J.J. Song, S.J. Kim, S.W. Hwang, N.Y. Park. *J. Mater. Chem. A* **1** (2013) 14460.
137. V. Gupta, N. Miura. *Electrochim. Acta* **52** (2006) 1721.
138. Y.-K. Zhou, B.-L. He, W.-J. Zhou, H.-L. Li. *J. Electrochem. Soc.* **151** (2004) A1052.
139. T. Abdiryim, A. Ubul, R. Jamal, A. Rahman. *Materials* **5** (2012) 1219.
140. J. Ge, G. Cheng, L. Chen. *Nanoscale* **3** (2011) 3084.
141. J. Liu, J. Sun, L. Gao. *J. Phys. Chem. C* **114** (2010), 19614.
142. Z.Q. Niu, P.S. Luan, Q. Shao, H. Dong, J.Z. Li, J. Chen, D. Zhao, L. Cai, W.Y. Zhou, X.D. Chen, S.S. Xie. *Energy Environ. Sci.* **5** (2012) 8726.
143. K. Wang, P. Zhao, X. Zhou, H. Wu, Z. Wei. *J. Mater. Chem.* **21** (2011) 16373.
144. V. Gupta, N. Miura. *J. Power Sources* **157** (2006) 616.
145. K.A. Kilian, B. Bugarija, B.T. Lahn, M. Mrksich. *P. Natl. Acad. Sci.* **107** (2010) 4872.
146. M.J.P. Biggs, R.G. Richards, N. Gadegaard, R.J. McMurray, S. Affrossman, C.D.W. Wilkinson, R.O.C. Oreffo, M.J. Dalby. *J. Biomed. Mater. Res. Part A* **91A** (2009) 195.
147. M.J.P. Biggs, R.G. Richards, S. McFarlane, C.D.W. Wilkinson, R.O.C. Oreffo, M.J. Dalby. *J. R. Soc. Interface* **5** (2008) 1231.
148. A.J. Engler, S. Sen, H.L. Sweeney, D.E. Discher. *Cell* **126** (2006) 677.
149. J. Wang, M. Yang, Y. Zhu, L. Wang, A.P. Tomsia, C. Mao. *Adv. Mater.* **26** (2014) 4961.
150. J. Street, M. Bao, L. deGuzman, S. Bunting, F.V. Peale Jr, N. Ferrara, H. Steinmetz, J. Hoeffel, J.L. Cleland, A. Daugherty, N. van Bruggen, H.P. Redmond, R.A. Carano, E.H. Filvaroff. *Proc. Natl. Acad. Sci. U. S. A.* **99** (2002) 9656.

151. M.J. Lysaght, A. Jaklenec, E. Deweerdt. *Tissue Eng. Part A* **14** (2008) 305.
152. R.G. Flemming, C.J. Murphy, G.A. Abrams, S.L. Goodman, P.F. Nealey. *Biomaterials* **20** (1999) 573.
153. J.F. Pollock, K. Healy, in *Strategies in Regenerative Medicine*, M. Santin (Ed.), Springer New York, 2009, p. 97.
154. R. Raghov. *FASEB J* **8** (1994) 823.
155. T. Zigova, E.Y. Snyder, P.R. Sanberg (Eds.), *Neural Stem Cells for Brain and Spinal Cord Repair*, Humana Press, Totowa, NJ, 2003.
156. M.M. Stevens, J.H. George. *Science* **310** (2005) 1135.
157. M.J. Dalby, M.O. Riehle, H. Johnstone, S. Affrossman, A.S. Curtis. *Cell. Biol. Int.* **28** (2004) 229.
158. M.J. Dalby, N. Gadegaard, R. Tare, A. Andar, M.O. Riehle, P. Herzyk, C.D.W. Wilkinson, R.O.C. Oreffo. *Nat. Mater.* **6** (2007) 997.
159. F. Yang, R. Murugan, S. Wang, S. Ramakrishna. *Biomaterials* **26** (2005) 2603.
160. D. Ceballos, X. Navarro, N. Dubey, G. Wendelschafer-Crabb, W.R. Kennedy, R.T. Tranquillo. *Exp. Neurol.* **158** (1999) 290.
161. P. Clark, P. Connolly, A.S. Curtis, J.A. Dow, C.D. Wilkinson. *J. Cell. Sci.* **99** (Pt 1) (1991) 73.
162. A.S. Crouch, D. Miller, K.J. Luebke, W. Hu. *Biomaterials* **30** (2009) 1560.
163. P. Weiss. *J. Exp. Zool.* **100** (1945) 353.
164. M.J. Dalby. *Med. Eng. Phys.* **27** (2005) 730.
165. N. Dubey, P.C. Letourneau, R.T. Tranquillo. *Exp. Neurol.* **158** (1999) 338.
166. G.A. Dunn, J.P. Heath. *Exp. Cell. Res.* **101** (1976) 1.
167. A.B.J. Alberts, J. Lewis, M. Raff, K. Roberts, P. Walter (Eds.), *Molecular Biology of the Cell*, Garland Science, New York and London, 4th Ed., 2002.
168. H.G. Hentschel, A. van Ooyen. *Proc. Biol. Sci.* **266** (1999) 2231.
169. M.E. Fini. *Prog. Retin. Eye Res.* **18** (1999) 529.
170. R. Milner, I.L. Campbell. *J. Neurosci. Res.* **69** (2002) 286.
171. R.C. Burrows, D. Wancio, P. Levitt, L. Lillien. *Neuron* **19** (1997) 251.
172. Y. Ito. *Soft. Matter.* **4** (2008) 46.
173. G. Maheshwari, G. Brown, D.A. Lauffenburger, A. Wells, L.G. Griffith. *J. Cell. Sci.* **113** (Pt 10) (2000) 1677.
174. J.C. Schense, J. Bloch, P. Aebischer, J.A. Hubbell. *Nat. Biotechnol.* **18** (2000) 415.
175. E. Engel, A. Michiardi, M. Navarro, D. Lacroix, J.A. Planell. *Trends Biotechnol.* **26** (2008) 39.
176. J.J. Norman, T.A. Desai. *Ann. Biomed. Eng.* **34** (2006) 89.
177. A. Curtis, C. Wilkinson. *Biomaterials* **18** (1997) 1573.
178. P.X. Ma. *Adv. Drug. Deliv. Rev.* **60** (2008) 184.
179. M. Goldberg, R. Langer, X. Jia. *J. Biomater. Sci. Polym. Ed.* **18** (2007) 241.
180. P.X. Ma, in *Encyclopedia of Polymer Science and Technology*, J.I. Kroschwitz (Ed.), JohnWiley & Sons Inc, Hoboken, NJ, 2005, vol. 12, p. 261.
181. J. Guan, K.L. Fujimoto, M.S. Sacks, W.R. Wagner. *Biomaterials* **26** (2005) 3961.
182. J. L. Drury, D. J. Mooney, *Biomaterials* **24** (2003), 4337.
183. K. Saha, E.F. Irwin, J. Kozhukh, D.V. Schaffer, K.E. Healy. *J. Biomed. Mater. Res. A* **81** (2007) 240.
184. E. Engel, A. Michiardi, M. Navarro, D. Lacroix, J. A. Planell. *Trends Biotechnol.* **26** (2008) 39.
185. M. Goldberg, R. Langer, X.Q. Jia. *J. Biomat. Sci. Polym. E* **18** (2007) 241.

186. J.H. Huang, S.V. Gräter, F. Corbellini, S. Rinck-Jahnke, E. Bock, R. Kemkemer, H. Kessler, J.D. Ding, J.P. Spatz. *Nano Lett.* **9** (2009) 1111.
187. M. Arnold, M. Schwieder, J. Blümmel, E.A. Cavalcanti-Adam, M. López-Garcia, H. Kessler, B. Geiger, J.P. Spatz. *Soft. Matter.* **5** (2009) 72.
188. N. Gomez, S.C. Chen, C.E. Schmidt, *J. R. Soc. Interface* **4** (2007) 223.
189. J.M. Goddard, J.H. Hotchkiss. *Prog. Polym. Sci.* **32** (2007) 698.
190. E. Strable, M.G. Finn, in *Viruses and Nanotechnology*, M. Manchester, N.F. Steinmetz (Eds.), Springer-Verlag, Berlin, 2009, vol. 327, pp. 1–21.
191. S.S. Gupta, J. Kuzelka, P. Singh, W.G. Lewis, M. Manchester, M.G. Finn. *Bioconjug. Chem.* **16** (2005) 1572.
192. E.W. Kovacs, J.M. Hooker, D.W. Romanini, P.G. Holder, K.E. Berry, M.B. Francis. *Bioconjug. Chem.* **18** (2007) 1140.
193. Y. Huang, C.-Y. Chiang, S.K. Lee, Y. Gao, E.L. Hu, J.D. Yoreo, A.M. Belcher. *Nano Lett.* **5** (2005) 1429.
194. S.K. Lee, D.S. Yun, A.M. Belcher. *Biomacromolecules* **7** (2006) 14.
195. A. Merzlyak, S. Indrakanti, S.W. Lee. *Nano Lett.* **9** (2009) 846.
196. J. Light, R.A. Lerner. *Bioorg. Med. Chem. Lett.* **2** (1992) 1073.
197. G. Iannolo, O. Minenkova, R. Petruzzelli, G. Cesareni. *J. Mol. Biol.* **248** (1995) 835.
198. G.A. Kuzmicheva, P.K. Jayanna, I.B. Sorokulova, V.A. Petrenko. *Protein Eng. Des. Sel.* **22** (2009) 9.
199. L. Makowski. *Gene* **128** (1993) 5.
200. W.-J. Chung, A. Merzlyak, S.Y. Yoo, S.-W. Lee. *Langmuir* **26**(2010) 9885.
201. Y. Lin, E. Balizan, L.A. Lee, Z. Niu, Q. Wang. *Angew. Chem. Int. Ed.* **49** (2010) 868.
202. B. Grimpe, J. Silver. *Prog. Brain. Res.* **137** (2002) 333.
203. C. H. Streuli, N. Akhtar. *Biochem. J.* **418** (2009) 491.
204. T. Boontheekul, D.J. Mooney. *Curr. Opin. Biotechnol.* **14** (2003) 559.
205. A. de Mel, G. Jell, M.M. Stevens, A.M. Seifalian. *Biomacromolecules* **9** (2008) 2969.
206. L.G. Griffith, M.A. Swartz. *Nat. Rev. Mol. Cell. Biol.* **7** (2006) 211.
207. K. Elward, P. Gasque. *Mol. Immunol.* **40** (2003) 85.
208. G.W. Kreutzberg. *Trends Neurosci.* **19** (1996) 312.
209. W.J. Streit, *Microglia in the Regenerating and Degenerating Central Nervous System*, W.J. Streit (Ed.), Springer, New York, 2002, p. 315.
210. K.M. Ainslie, E.M. Bachelder, S. Borkar, A.S. Zahr, A. Sen, J.V. Badding, M.V. Pishko. *Langmuir* **23** (2007) 747.
211. R.J. Johnson, S.A. Maves, in *USPTO C. Baxter Healthcare* (Ed.), Innate Biotech Inc., USA, 2004.
212. A. Kapil, B. Kundu, S.K. Khare. *Protein Pept. Lett.* **4** (1997) 405.
213. H. Bar, I. Yacoby, I. Benhar. *BMC Biotechnol.* **8** (2008) 14.
214. S.L. Hart, A.M. Knight, R.P. Harbottle, A. Mistry, H.D. Hunger, D.F. Cutler, R. Williamson, C. Coutelle. *J.Biol. Chem.* **269** (1994) 12468.
215. J.D. Mount, T.I. Samoylova, N.E. Morrison, N.R. Cox, H.J. Baker, V.A. Petrenko. *Gene* **341**(2004) 59.
216. M.-A. Poul, J.D. Marks. *J. Mol. Biol.* **288** (1999) 203.
217. M.A. Bur, K. Jensen-Pergakes, A.M. Gonzalez, P. Ravey, A. Baird, D. Larocca. *Cancer Res.* **62** (2002) 977.
218. D. Larocca, A. Witte, W. Johnso, G.F. Pierce, A. Baird. *Hum. Gene Ther.* **9** (1998) 2393.

- 219. Y. Seow, M.J. Wood. *Mol. Ther.* **17** (2009) 767.
- 220. S. Piersanti, G. Cherubini, Y. Martina, B. Salone, Da. Avitabile, F. Grosso, E. Cundari, G. Di Zenzo, I. Saggio. *J. Mol. Med.* **82** (2004) 467.
- 221. T. Segura, L.D. Shea. *Bioconjug. Chem.* **13** (2002) 621.
- 222. V.V. Ivanenkov, F. Felici, A.G. Menon. *Biochim. Biophys. Acta – Molecular Cell Research* **1448** (1999) 450.
- 223. S.L. Deutscher. *Chem. Rev.* **110** (2010) 3196.
- 224. T.M. Holger Thie, T. Schirrmann, M. Hust, S. Dubel. *Curr. Pharm. Biotechnol.* **9** (2008) 439.
- 225. L. B. Giebel, R.T. Cass, D.L. Milligan, D.C. Young, R. Arze, C.R. Johnson. *Biochemistry* **34** (1995) 15430.
- 226. S. Y. Yoo, S. M. Kwon. *Mediat. Inflamm.* **2013** (2013) 11.

NEOTISSUE REMODELING OF TISSUE-ENGINEERED ARTERIAL GRAFT

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9.1. INTRODUCTION

Atherosclerotic cardiovascular diseases (CVDs), a systemic narrowing and hardening of arteries, include conditions such as coronary arterial disease, carotid artery stenosis, and peripheral arterial disease. CVDs are a leading cause of death or impaired quality of life for millions of individuals in developed nations. The most common corrective procedure for CVDs is surgical intervention using autologous arterial and/or venous grafts. However, the use of autologous grafts leads to prolonged operative times and increased risk of peri-operative infection. Additionally, many patients lack suitable donor tissue, either as a result of their underlying vascular disease or previous surgery. Synthetic materials such as expanded poly(tetrafluoroethylene) (ePTFE, Goretex®) and poly(ethylene terephthalate) (PET, Dacron®) provide an alternative strategy, but when applied to small-diameter (<6 mm) arterial grafts, this approach has limited efficacy due to various complications such as progressive stenosis, thrombosis, calcium deposition, host rejection, increased risk of infection, and the persistent need for anticoagulation therapy.

The purpose of tissue engineering is to develop alternative materials that integrate with the patient's native tissue to restore physiologic function [1]. This often involves the use of biodegradable synthetic materials, termed scaffolds, to provide a three-dimensional surface for cellular proliferation and neotissue formation during neovessel remodeling. In addition, the traditional concept of tissue engineering consists of the isolation and use of cells or cell substitutes, and the integration of the isolated cells and scaffold *via* a seeding technique [2]. In 1986, Weinberg and Bell produced what was widely regarded as the first tissue-engineered blood vessel substitute. It consisted of bovine endothelial cell (EC), smooth muscle cell (SMC) and fibroblast cell cultures embedded in a collagen gel [3]. However, this tissue-engineered construct lacked adequate strength and required reinforcement with a Dacron® mesh. In following years, vascular grafts were improved, refined, and ultimately reached clinical application in 1999 [4]. The first human clinical trial investigating tissue engineered vascular graft (TEVG) implantation in children with congenital heart defects began in 2001. The highly porous biodegradable polymeric scaffold was comprised of poly(L-lactide-co- ϵ -caprolactone) (PLCL) reinforced by poly(glycolic acid) mesh [5] and seeded with bone marrow derived mononuclear cells (BM-MNCs). The application of TEVGs in a low-pressure environment for patients with congenital heart disease has several advantages such as growth potential, favorable biocompatibility, and low risk of infection or rejection. Clinical evidence has now established that TEVGs are safe and effective to use in pediatric patients undergoing extracardiac total cavopulmonary connection procedures [5-7]. However, to apply TEVGs to an arterial system, a scaffold must be able to withstand mechanical loadings akin to the native artery such as arterial pressure, shear stresses, durability, and

compliance. With this in mind, the efficacy of various TEVG types, with or without cell seeding, for small diameter arterial grafts has been demonstrated in animal models [8-11].

A biodegradable vascular graft is completely reconstituted by host-derived cells over the course of its inflammation-mediated process [12]. It was previously believed that the progenitor cells in BM-MNCs differentiated into mature vascular cells in the developing TEVG. In fact, small populations of hematopoietic and vascular progenitor cells were identified within seeded BM-MNC populations. However, the number of seeded cells in the graft decreased rapidly within the first few days after implantation, ultimately resulting in the absence of all BM-MNCs within one-week, leaving many questions as to the mechanism of TEVG neotissue formation [12]. Even though the complete mechanism of neotissue formation during graft-material absorption remains to be fully elucidated, multiple TEVG remodeling processes have been proposed. In this chapter, we introduce the remodeling process and putative mechanisms of neotissue formation in arterial TEVGs, and lastly feature the differences in scaffold materials and constructions to fabricate an ideal arterial TEVG.

9.1.1. Neoartery components as a basis for TEVG remodeling

9.1.1.1. Endothelial cells (ECs)

ECs possess various physiologic functions *in vivo*, such as the transport of blood constituents, thromboresistance, angiogenesis induction, and blood pressure control *via* vasoconstriction/vasodilation. Additionally, ECs are able to prevent neointimal hyperplasia through the inhibition of bioactive substances responsible for extracellular matrix (ECM) production, and smooth muscle cell (SMC) migration and proliferation.

The development of a confluent endothelial monolayer is a vital and primary step for well-organized TEVG neotissue formation. However, ECs have limited capacity for regeneration and can no longer divide after about 70 cell cycles. In humans, currently available graft implants show limited EC ingrowth, typically not extending 1–2 cm beyond the anastomoses [13]. After implantation for peripheral artery disease, the number of ECs observed on the luminal surface of synthetic grafts was less than 10 % of that seen in native vessels [14]. To improve graft surface endothelialization, EC seeding has been applied to synthetic arterial grafts. However the clinical efficacy of EC seeding to produce patent synthetic grafts remains a controversial topic [15]. As a result, the safekeeping of ECs from damaging factors, such as oxidative and sheer stresses, remains a major hurdle for the clinical application of arterial TEVGs.

9.1.1.2. Smooth muscle cells (SMCs)

SMCs regulate vasocontraction and are an integral component of an efficient and stable blood vessel. SMCs are the predominant component of the ECM complex that ultimately defines blood vessel mechanical properties [3]. Yue *et al.* investigated the seeding of cultured SMCs onto biodegradable scaffolds implanted in rat aortas, and in comparison to unseeded controls, the implanted constructs demonstrated rapid neointima formation [16]. Similarly, Niklason *et al.* developed a tissue-engineered graft by seeding SMCs onto a poly(glycolic acid) (PGA) scaffold that was then cultured *in vitro* in a pulsatile radial stress environment for 8 weeks [17]. The SMC-seeded PGA grafts demonstrated physiologic and mechanical functions comparable to native human vessels and are being considered for clinical application [18].

SMCs are complex cells capable of existing in heterogeneous populations and switching phenotypes when exposed to various stimuli (*i.e.* contractile to synthetic) [19]. A synthetic dedifferentiated SMC phenotype is characterized by its migration, proliferation, and ECM synthesis in the vascular wall. These dedifferentiated SMCs can be identified early on in the developing vasculature through α -SMA expression detection [20]. In contrast, the contractile apparatus of differentiated SMCs have less proliferation potential and are distinguished by their expression of differentiated SMC markers such as smooth muscle-myosin heavy chain (SM-MHC) [20]. Although synthetic SMCs are necessary to obtain favorable ECM formation in neovessels during the neotissue remodeling process, excessive synthetic SMCs may also induce TEVG stenosis causing neotissue hyperplasia.

9.1.1.3. Extracellular matrix (ECM)

As the scaffold degrades, the ECM undergoes continuous remodeling. The ECM is predominantly comprised of elastin, proteoglycans, and collagen type I and III. ECM mechanical properties that are relevant to arterial vessel function includes tensile stiffness, compressibility, and elasticity. Collagen provides the ECM with the tensile strength to resist rupture and proteoglycans contribute to ECM compressibility [21,22].

Elastin is the most important component of the ECM as it confers elasticity to arteries. It prevents dynamic tissue creep by stretching under load and recoils back to its original conformation after the load is released. Elastin is also a potent autocrine regulator of vascular SMC activity. Accordingly, elastin's regulatory effects are significant in preventing fibrocellular pathology [23]. Upon insult however, elastic matrices are difficult to repair due to: 1) poor elastin precursor (tropoelastin) synthesis by adult cells, 2) inefficient recruitment and crosslinking of tropoelastin into an elastic matrix, and 3) further organization into elastic fibers [23,24]. Hence, the resourceful utilization of elastic materials and tropoelastin coatings on scaffolds, may be helpful to augment arterial TEVG elastin functions.

9.1.2. Tissue remodeling process in arterial TEVG

A better understanding of the tissue remodeling process is critical for the improvement, continued development, and rational design of arterial TEVGs. Neovessel formation is a dynamic process characterized by progressive scaffold degradation due to hydrolysis, cellular infiltration into scaffold, and ECM deposition. As the remodeling time course and process can be altered through the use of different materials and scaffold structures, the trade-off between scaffold degradation and neotissue formation must be considered when TEVGs are applied to the high pressures of arterial circulation.

During the acute phase of tissue remodeling (within 6 weeks post implantation), vascular neotissue of arterial TEVGs constructed with poly(L-lactic acid) (PLA) fibers coated with PLCL are characterized by: 1) collagen type I and III deposition, 2) a lack of elastin, 3) SMC proliferation in the medial layer, and 4) partial endothelialization in the internal scaffold lumen [8]. Within 4 months post-implantation, favorable endothelialization is achieved (Figure 1) [25], along with gradual increases in collagen deposition, SMC proliferation, and elastin deposition (Figure 2) [25].

Figure 1. Endothelial cell identification with CD 31 immunohistochemical staining. Grafts at 4, 8, 12 months after implantation and native abdominal aortas of mice were explanted and analyzed [25].

Figure 2. Representative histologic image of Masson's Trichrome staining for collagen deposition, Elastica van Gieson (EVG) staining for elastin formation, and immunohistochemical smooth muscle actin (SMA) staining for smooth muscle cells within the neointimal at 4, 8, 12 months after implantation into mouse abdominal aorta [25].

The primary cellular component during the early stages of arterial TEVG remodeling are matrix metalloproteinase 9 (MMP-9) promoted macrophages and monocytes [26]. MMP-9 is the most prominent MMP type present during foreign body inflammatory responses [27] and is shown in an acute phase of neotissue formation, peaking 1 week after TEVG implantation, and decreasing thereafter. Moreover, SMC migration is dependent on both MMP-9 and -2. MMP-2 is primarily produced by inflammatory cell stimulated mesenchymal cells and peaks during the late phase of neotissue formation. However, MMP activity in the arterial TEVG model, though similar to the venous model, is prolonged because of remaining fiber remnants.

TGF- β is a multifunctional cytokine that regulates cell adhesion, migration, differentiation, proliferation and apoptosis. TGF- β signaling plays an essential role in vascular remodeling and disruptions to its signaling pathway is known to cause vascular dysfunctions such as aortic aneurysms. After short-term

(6 week) follow-up, PLA-PLCL aortic grafts possessed adequate mechanical properties and strength to function as arterial grafts and displayed no signs of aneurysmal change [8]. However, many grafts formed aneurysmal changes or ruptured later (12 months) with TGF- β elevation [25]. Thus, continuous TGF- β elevation may induce vascular dysfunction of arterial TEVGs followed by subsequent aneurysmal changes. Of further interest, angiotensin receptor blockers are known to inhibit TGF- β signaling and prevent aortic aneurysm in Marfan syndrome [28,29]. Consequently, angiotensin receptor blockers have the therapeutic potential to prevent aneurysmal changes in arterial TEVGs.

9.1.3. Calcific deposition

Implants are susceptible to calcification during neovessel remodeling, a potentially fatal longer term complication. However, the role that the physical scaffold structure has on long-term calcific deposition in arterial TEVG neotissue remains a field yet to be studied.

Chondroid metaplasia takes place in intimal hyperplasia layers and contributes to arterial TEVG calcification [30]. Although the precise mechanisms underlying osteogenic differentiation of vascular cells during atherosclerosis remain undefined, vascular SMCs can undergo osteogenic differentiation and calcification [31,32]. However, normal vascular SMCs have the potential to prevent calcium deposition as they express transcription factors of both osteogenesis and osteoclastogenesis. Well organized neointima, abundant with SMCs, better prevents calcific deposition in comparison to thin neointima on small-pore nanofiber grafts (Figure 3) [33]. Therefore, a degree of neointimal SMCs is required to eliminate calcium deposition and sustain the balance of osteogenesis and osteoclastogenesis.

Figure 3. Large-pore PLA-PLCL grafts created a well-organized neointima and prevented calcific deposition compared to small-pore, electrospun PLA-nanofiber grafts [33].

The scaffold degradation period is another key aspect to consider calcification in tissue remodeling for biodegradable vascular grafts [10]. At one year follow up, in a rapid remodeling process, fast degrading elastomers developed into well-organized neotissue without calcification [34]. In contrast, high density (small pore size) nanofiber fabric have longer polymer degradation periods and lack transmural cellular migration onto the scaffold [33]. Consequently, the combined effects of small pore size and slow degradation, results in calcified deposition in neotissue remodeling and prolonged foreign body reactions.

9.2. PUTATIVE MECHANISMS OF NEOTISSUE FORMATION IN TEVG REMODELING

Although, the precise mechanism of neotissue formation in TEVG remodeling remains to be fully elucidated, molecular and cellular mechanisms underlying TEVG remodeling and vascular neotissue formation have been proposed.

9.2.1. Inflammatory mediated process

Monocyte and macrophage mediated inflammation plays a crucial role in vascular neotissue formation (Figure 4), as well as the development of stenosis in a scaffold [12]. Clodronate is known to deplete monocytes and macrophages

in vivo. Liposomal clodronate was administered into a mouse model to evaluate the function of monocytes and macrophages, and the study demonstrated the importance of monocytes/macrophages in TEVG tissue remodeling as clodronate administration effectively suppressed neotissue formation and vascular repair [35]. On the other hand, excessive macrophage infiltration produces neotissue hyperplasia, which leads to stenotic/occluded grafts.

Figure 4. Proposed mechanism of neovessel formation after implantation of a cell-seeded biodegradable scaffold. Early pulse of monocyte chemoattractant protein-1 (MCP-1) and related cytokines from seeded bone marrow-derived mononuclear cells (BM-MNCs) enhances early monocyte recruitment to the scaffold. Infiltrating monocytes release multiple angiogenic cytokines and growth factors (*e.g.*, VEGF), which recruit smooth muscle cells and endothelial cells [12].

Macrophages in the scaffold release a variety of chemokines, cytokines, and growth factors. Macrophage-derived signaling molecules induce migration of adjacent host SMCs and ECs into the scaffold. As scaffold degradation begins, macrophages, fibroblasts, and SMCs start to deposit ECM, which continues to undergo remodeling throughout the formation of a viable TEVG. Given sufficient time, the biomechanical properties of the neovessel are no longer determined by scaffold characteristics, but are instead characterized by its collagen and elastin content [36]. As a final result, the TEVG has completely transformed into a neovessel, with cellular, biomechanical, and physiologic characteristics similar to that of a native vessel.

9.2.2. Endothelial-to-mesenchymal transition

The classical mechanism for cell differentiation is for progenitor cells to transform from immature to mature cell types *via* a well-ordered pathway. On the other hand, the endothelial-to-mesenchymal transition (EndMT) is a complex process, where endothelial cells lose their cellular polarity and adhesion abilities, while gaining migratory and invasive properties as they develop into mesenchymal cells, including SMC-like and fibroblast-like cells [37]. The EndMT has been shown to occur in wound healing, organ fibrosis, and also contributes to postnatal processes, such as promoting vein graft remodeling [38]. The EndMT contribution to vascular neotissue and stenosis formation have been demonstrated during TEVG remodeling [39]. TGF- β is thought to drive EndMT in both a small mothers against decapentaplegic (SMAD)-dependent and independent manner [40,41]. To this end, anti-TGF- β therapy has been pointed to for its potential utility to prevent TEVG stenosis.

9.2.3. Cell source for TEVG remodeling

In contrast to previous suggestions, neotissue cells are not derived from seeded cells [42]. Instead, several sources have been proposed for the spontaneous *in vivo* endothelialization and SMC proliferation in TEVGs. Such proposals include: 1) the transanastomotic outgrowth from the adjacent native blood vessel, 2) transmural ingrowth of capillaries from the adventitial side of the vascular graft, and 3) the migration of circulating endothelial progenitor cells from the luminal surface of vascular grafts (Figure 5).

Figure 5. Schematic image of cell source for TEVG remodeling. Three different sources have been proposed for the spontaneous *in vivo* endothelialization and SMC proliferation in TEVGs.

9.2.3.1. Adjacent blood vessel (*Transanastomotic outgrowth*)

Transanastomotic outgrowth from the adjacent blood vessel is the principal source of SMCs and ECs in implanted TEVGs [43]. After implantation however, transanastomotic outgrowth slows down gradually [44], and typically does not extend 1–2 cm beyond the anastomoses [13]. Additionally, transanastomotic intimal hyperplasia is a major contributor to graft failure. Transanastomotic outgrowth provides sufficient endothelial coverage for short vascular grafts in small animal models, however other cells sources will be necessary to obtain favorable SMC proliferation and endothelialization in longer grafts.

9.2.3.2. *Transmural ingrowth*

Transmural *endothelium* ingrowth was clearly distinguished experimentally from transanastomotic outgrowth by using a unique looped interposition graft model and provides another realistic mechanism of spontaneous *in vivo* endothelialization [44]. Capillaries can penetrate the graft wall and provide sources of SMCs and luminal *endothelium*, where the aforementioned cells can adopt large vessel phenotypes. Moreover, capillaries can provide rapid coverage of porous synthetic graft surfaces that are in contact with the arterial circulation [14]. Of note however, is that tightly knit, electrospun nanofiber by itself, or used as a scaffold sheath to provide mechanical strength, may restrict transmural capillary ingrowth and/or cell migration from outside the graft.

9.2.3.3. *Migration from circulating blood stream*

CD34⁺ endothelial progenitor cells (EPCs) exist in circulating blood and can differentiate into mature ECs [45]. After mobilization from bone marrow, CD34⁺ EPCs participate in postnatal neovascularization [46]. After seeding onto decellularized xenogeneic arterial grafts, *ex vivo* expanded EPCs isolated

from peripheral sheep blood, effectively achieved luminal coverage. This indicates EPCs possess the ability to endothelialize TEVGs [47]. Additionally, bone marrow cells may have potential to give rise to other vascular progenitor cells that contribute to vascular remodeling [48]. To increase EPC and smooth muscle progenitor cell migration from the peripheral blood onto the luminal surface, a stromal cell-derived factor-1 α /heparin coating was applied to a TEVG. The coated TEVG resulted in accelerated endothelialization and SMC differentiation [49].

9.3. BIODEGRADABLE POLYMERS FOR ARTERIAL TEVGs

The role of a scaffold is to provide a temporary three-dimensional structure for cellular attachment, infiltration, and proliferation. Scaffold materials should be highly porous and possess biomimetic properties, thereby facilitating cellular infiltration, neotissue formation, and integration with native tissue [50]. Biodegradable polymers utilized in arterial scaffolds are well suited to fulfill these requirements (Figure 6).

Figure 6. Flowchart for arterial scaffold. Both synthetic and natural materials are applied to arterial TEVGs as a biodegradable polymer.

Biodegradable polymers serve as temporary blood vessel scaffolds before being replaced by ingrowing tissue. Scaffold degradation first exhibits loss of mechanical properties, followed by a decrease in mass/volume. The degradation rate of biodegradable polymers depends on its initial molecular weight, exposed surface area, and crystallinity. The ideal scaffold for vascular applications meet the following criteria: 1) provides a suitable surface for cell adhesion and proliferation, 2) is highly porous to allow for neotissue growth and the exchange of nutrients and metabolic waste, and 3) is biodegradable, ultimately restoring physiologic function without reinforcement with

non-degradable synthetic materials such as Goretex® and Dacron®. The crucial first step in vascular engineering construct design is the selection of appropriate biodegradable scaffold materials and this is dependent on various factors, such as biocompatibility, biodegradability, and mechanical properties (Table 1).

Table 1. Biodegradable polymers for TEVGs. T_m : melting temperature; T_g : glass-forming temperature; Mpa: mega pascal; GPa: giga pascal; P(LA/CL): copolymer of L-lactide and ϵ -caprolactone; PCL: poly(ϵ -caprolactone); PHB: poly(3-hydroxybutyrate).

Polymer	T_m	T_g	Initial tensile strength (Mpa)	Elastic modulus (GPa)	Elongation at break (%)	Degradation period
PGA	230	36	890	8.4	30	2–3 weeks
PLA	170	56	900	8.5	25	6–12 months
P(LA/CL) (75 : 25)	140	22	500	4.8	70	8–10 weeks
P(LA/CL) (50 : 50)	105	17	12	0.9	600	4–6 weeks
PCL	60	60	50	0.3	70	12 weeks
PHB	177	4	43	–	5	> 6 weeks

Several biodegradable polymers have been investigated for their suitability in arterial tissue engineering applications. PLA and PCL are commonly used materials for constructing arterial scaffolds due to their successful clinical history [51]. Both PLA and PCL have hydrophobic properties and are maintained within the body for prolonged periods of time. Combining PCL and PLA forms new copolymers called PLCL. Further fine tuning of PLCL scaffold mechanical properties and degradation rates is accomplished by changing molecular weights through composition ratio adjustments. The feasibility of PLA-PLCL scaffolds as small diameter arterial conduits in high pressure environments *in vivo* has been confirmed [8]. Natural proteins, such as silk fibroin and chitosan, have also been utilized as biodegradable materials for small diameter arterial grafts, and have shown favorable vessel remodeling with long-term patency (Figure 6) [52,53].

9.3.1. Structural characteristics of arterial TEVGs

Ideal small diameter arterial TEVGs are; readily available (“off-the-shelf”), biocompatible, easily implanted, capable of transforming into neotissue comparable to that of native arteries, and is resistant to thrombosis,

aneurysmal dilatation, and ectopic calcification [54]. Biodegradable scaffolds with large pore sizes, fast degradability, and high elasticity are thought to be crucial to obtain the aforementioned TEVG characteristics, as well as promote better and more rapid neotissue formation in small-diameter arterial grafts.

9.3.1.1. *Sponge type scaffold*

Highly porous, PLCL sponge-type scaffolds, reinforced with PGA mesh, have been successfully applied clinically in low pressure environments [5]. PLCL co-polymer, prepared from 50 % L-lactide and 50 % ϵ -caprolactone, has high elasticity that is suitable for tissue-engineering applications [55,56]. Even though outer layer reinforcement is required to withstand high arterial pressure, PLCL sponge type scaffolds show great potential and promise as arterial scaffolds due to their elastic properties [57].

While the slow degradation of certain polymers aids in mechanical property retention, allowing grafts to endure high pressures for longer periods of time, it consequently results in undesirable delayed tissue remodeling. Wu *et al.* were able to demonstrate rapid tissue remodeling using a sponge type scaffold composed of the fast degrading poly(glycerol sebacate) (PGS) elastomer [10], and in a rat arterial implantation model, the PGS scaffold displayed favorable vessel remodeling, in addition to good patency [34].

9.3.1.2. *Electrospinning technique*

Electrospinning technologies have enabled the production of nanofiber-based scaffolds, and because of their ability to improve cellular infiltration and endothelialization in comparison to standard synthetic grafts [58], have shown promise with regards to arterial scaffold fabrication (Figure 7).

Figure 7. Representative electronic microscope images of poly(lactic acid) nanofiber-based scaffold for aortic graft. Electrospinning technologies have enabled the production of nanofiber-based scaffolds, which have shown promise with regard to arterial scaffold fabrication [54].

Small-diameter electrospun scaffolds composed of PCL [59], and PLCL [60], have shown good surgical and mechanical properties with high patency rates in an arterial implantation model. However, small-fiber diameter and tightly knit electrospun scaffolds displayed poor cellular migration into the scaffold, causing prolonged neotissue remodeling and foreign body reactions. On the other hand, electrospun thick-PCL-fiber, with large pore scaffolds enhanced the remodeling process and vascular regeneration by mediating M2 phenotype macrophage polarization [61]. Furthermore, electrospun nanofibers show more promise with their encapsulation and controlled drug release potentials [60,62] that may one day lead to cell-free TEVGs.

9.4. CONCLUSION

Arterial TEVGs must be able to withstand high arterial pressure until they are completely reconstituted by host derived cells. Well-designed arterial TEVGs require the formation of well-organized neotissue, characterized by: confluent endothelialization surrounded by SMCs, favorable elastin deposition, and the absence of calcification and stenosis. For the next generation of arterial TEVGs, scaffold improvements will focus on: facilitating cellular infiltration through high porosity and large pore sizes; and fast scaffold degradation in order to enable rapid remodeling, thereby reducing calcification caused by foreign body reactions.

REFERENCES

1. J.P. Vacanti, R. Langer. *Lancet* **354** (Suppl 1) (1999) SI32–SI34.
2. R. Langer, J.P. Vacanti. *Science*. **260** (1993) 920–926.
3. C.B. Weinberg, E. Bell. *Science* **231** (1986) 397–400.
4. T. Shin'oka, Y. Imai, Y. Ikada. *N. Engl. J. Med.* **344** (2001) 532–533.
5. G. Matsumura, N. Hibino, Y. Ikada, H. Kurosawa, T. Shin'oka. *Biomaterials* **24** (2003) 2303–2308.
6. T. Shin'oka, G. Matsumura, N. Hibino, Y. Naito, M. Watanabe, T. Konuma, T. Sakamoto, M. Nagatsu, H. Kurosawa. *J. Thorac. Cardiovasc. Surg.* **129** (2005) 1330–1338.
7. N. Hibino, E. McGillicuddy, G. Matsumura, Y. Ichihara, Y. Naito, C. Breuer, T. Shinoka. *J. Thorac. Cardiovasc. Surg.* **139** (2010) 431–6, 436 e1–2.
8. J.D. Roh, G.N. Nelson, M.P. Brennan, T.L. Mirensky, T. Yi, T.F. Hazlett, G. Tellides, A.J. Sinusas, J.S. Pober, W.M. Saltzman, T.R. Kyriakides, C.K. Breuer. *Biomaterials* **29** (2008) 1454–1463.
9. T.L. Mirensky, G.N. Nelson, M.P. Brennan, J.D. Roh, N. Hibino, T. Yi, T. Shinoka, C.K. Breuer. *J. Pediatr. Surg.* **44** (2009) 1127–1132; discussion 1132–1133.
10. W. Wu, R.A. Allen, Y. Wang. *Nat. Med.* **18** (2012) 1148–1153.
11. F. Kuwabara, Y. Narita, A. Yamawaki-Ogata, M. Satake, H. Kaneko, H. Oshima, A. Usui, Y. Ueda. *J. Artif. Organs* **15** (2012) 399–405.

12. J.D. Roh, R. Sawh-Martinez, M.P. Brennan, S.M. Jay, L. Devine, D.A. Rao, T. Yi, T.L. Mirensky, A. Nalbandian, B. Udelsman, N. Hibino, T. Shinoka, W.M. Saltzman, E. Snyder, T.R. Kyriakides, J.S. Pober, C.K. Breuer. *Proc. Natl. Acad. Sci. U.S.A.* **107** (2010) 4669–4674.
13. Y. Naito, T. Shinoka, D. Duncan, N. Hibino, D. Solomon, M. Cleary, A. Rathore, C. Fein, S. Church, C. Breuer. *Adv. Drug. Deliv. Rev.* **63** (2011) 312–323.
14. A.W. Clowes, T.R. Kirkman, M.A. Reidy. *Am. J. Pathol.* **123** (1986) 220–230.
15. J.G. Meinhart, M. Deutsch, T. Fischlein, N. Howanietz, A. Froschl, P. Zilla. *Ann. Thorac. Surg.* **71** (2001) S327–S331.
16. X. Yue, B. van der Lei, J.M. Schakenraad, G.H. van Oene, J.H. Kuit, J. Feijen, C.R. Wildevuur. *Surgery* **103** (1988) 206–212.
17. L.E. Niklason, J. Gao, W.M. Abbott, K.K. Hirschi, S. Houser, R. Marini, R. Langer. *Science* **284** (1999) 489–493.
18. M. Peck, D. Gebhart, N. Dusserre, T.N. McAllister, N. L'Heureux. *Cells Tissues Organs.* **195** (2012) 144–158.
19. A. Muto, T.N. Fitzgerald, J.M. Pimiento, S.P. Maloney, D. Teso, J.J. Paszkowiak, T.S. Westvik, F.A. Kudo, T. Nishibe, A. Dardik. *J. Vasc. Surg.* **45** (Suppl A) (2007) A15–A24.
20. G.K. Owens. *Physiol. Rev.* **75** (1995) 487–517.
21. A. Ratcliffe. *Matrix Biol.* **19** (2000) 353–357.
22. L. Buttafoco, N.G. Kolkman, P. Engbers-Buijtenhuijs, A.A. Poot, P.J. Dijkstra, I. Vermes, J. Feijen. *Biomaterials* **27** (2006) 724–734.
23. A. Patel, B. Fine, M. Sandig, K. Mequanint. *Cardiovasc Res.* **71** (2006) 40–49.
24. L. Venkataraman, A. Ramamurthi. *Tissue Eng. Part A* **17** (2011) 2879–2889.
25. S. Tara, H. Kurobe, M.W. Maxfield, K.A. Rocco, T. Yi, Y. Naito, C.K. Breuer, T. Shinoka. *J. Vasc. Surg.* **62** (2015) 734–743.
26. J. Hu, P.E. Van den Steen, Q.X. Sang, G. Opdenakker. *Nat. Rev. Drug. Discov.* **6** (2007) 480–498.
27. J.A. Jones, A.K. McNall, D.T. Chang, L.A. Qin, H. Meyerson, E. Colton, I.L. Kwon, T. Matsuda, J.M. Anderson. *J. Biomed. Mater. Res. A* **84** (2008) 158–166.
28. J.P. Habashi, D.P. Judge, T.M. Holm, R.D. Cohn, B.L. Loeys, T.K. Cooper, L. Myer, E.C. Klein, G. Liu, C. Calvi, M. Podowski, E.R. Neptune, M.K. Halushka, D. Bedja, K. Gabrielson, D.B. Rifkin, L. Carta, F. Ramirez, D.L. Huso, H.C. Dietz. *Science* **312** (2006) 117–121.
29. B.S. Brooke, J.P. Habashi, D.P. Judge, N. Patel, B. Loeys, H.C. Dietz. *N. Engl. J. Med.* **358** (2008) 2787–2795.
30. S. de Valence, J.C. Tille, D. Mugnai, W. Mrowczynski, R. Gurny, M. Moller, B.H. Walpoth. *Biomaterials* **33** (2012) 38–47.
31. M.Y. Speer, H.Y. Yang, T. Brabb, E. Leaf, A. Look, W.L. Lin, A. Frutkin, D. Dichek, C.M. Giachelli. *Circ. Res.* **104** (2009) 733–741.
32. Y. Sun, C.H. Byon, K. Yuan, J. Chen, X. Mao, J.M. Heath, A. Javed, K. Zhang, P.G. Anderson, Y. Chen. *Circ. Res.* **111** (2012) 543–552.
33. S. Tara, H. Kurobe, K.A. Rocco, M.W. Maxfield, C.A. Best, T. Yi, Y. Naito, C.K. Breuer, T. Shinoka. *Atherosclerosis* **237** (2014) 684–691.
34. R.A. Allen, W. Wu, M. Yao, D. Dutta, X. Duan, T.N. Bachman, H.C. Champion, D.B. Stolz, A.M. Robertson, K. Kim, J.S. Isenberg, Y. Wang. *Biomaterials* **35** (2014) 165–173.

35. N. Hibino, T. Yi, D.R. Duncan, A. Rathore, E. Dean, Y. Naito, A. Dardik, T. Kyriakides, J. Madri, J.S. Pober, T. Shinoka, C.K. Breuer. *FASEB J.* **25** (2011) 4253–4263.
36. Y. Naito, M. Williams-Fritze, D.R. Duncan, S.N. Church, N. Hibino, J.A. Madri, J.D. Humphrey, T. Shinoka, C.K. Breuer. *Cells Tissues Organs.* **195** (2012) 60–72.
37. P.Y. Chen, L. Qin, C. Barnes, K. Charisse, T. Yi, X. Zhang, R. Ali, P.P. Medina, J. Yu, F.J. Slack, D.G. Anderson, V. Kotelianski, F. Wang, G. Tellides, M. Simons. *Cell Rep.* **2** (2012) 1684–1696.
38. B.C. Cooley, J. Nevado, J. Mellad, D. Yang, C. St Hilaire, A. Negro, F. Fang, G. Chen, H. San, A.D. Walts, R.L. Schwartzbeck, B. Taylor, J.D. Lanzer, A. Wragg, A. Elagha, L.E. Beltran, C. Berry, R. Feil, R. Virmani, E. Ladich, J.C. Kovacic, M. Boehm. *Sci. Transl. Med.* **6** (2014) 227ra34.
39. D.R. Duncan, P.Y. Chen, J.T. Patterson, Y.U. Lee, N. Hibino, M. Cleary, Y. Naito, T. Yi, T. Gilliland, H. Kurobe, S.N. Church, T. Shinoka, T.M. Fahmy, M. Simons, C.K. Breuer. *J. Am. Coll. Cardiol.* **65** (2015) 512–514.
40. A. Kitao, Y. Sato, S. Sawada-Kitamura, K. Harada, M. Sasaki, H. Morikawa, S. Shiomi, M. Honda, O. Matsui, Y. Nakanuma. *Am. J. Pathol.* **175** (2009) 616–626.
41. D. Medici, S. Potenta, R. Kalluri. *Biochem. J.* **437** (2011) 515–520.
42. G. Matsumura, S. Miyagawa-Tomita, T. Shin'oka, Y. Ikada, H. Kurosawa. *Circulation* **108** (2003) 1729–1734.
43. N. Hibino, G. Villalona, N. Pietris, D.R. Duncan, A. Schoffner, J.D. Roh, T. Yi, L.W. Dobrucki, D. Mejias, R. Sawh-Martinez, J.K. Harrington, A. Sinusas, D.S. Krause, T. Kyriakides, W.M. Saltzman, J.S. Pober, T. Shin'oka, C.K. Breuer. *FASEB J.* **25** (2011) 2731–2739.
44. T. Pennel, P. Zilla, D. Bezuidenhout. *J. Vasc. Surg.* **58** (2013) 1053–1061.
45. T. Asahara, T. Murohara, A. Sullivan, M. Silver, R. van der Zee, T. Li, B. Witzenbichler, G. Schatteman, J.M. Isner. *Science* **275** (1997) 964–967.
46. T. Asahara, H. Masuda, T. Takahashi, C. Kalka, C. Pastore, M. Silver, M. Kearne, M. Wagner, J.M. Isner. *Circ. Res.* **85** (1999) 221–228.
47. S. Kaushal, G.E. Amiel, K.J. Guleserian, O.M. Shapira, T. Perry, F.W. Sutherland, E. Rabkin, A.M. Moran, F.J. Schoen, A. Atala, S. Soker, J. Bischoff, J.E. Mayer, Jr. *Nat. Med.* **7** (2001) 1035–40.
48. M. Sata. *Trends Cardiovasc. Med.* **13** (2003) 249–253.
49. J. Yu, A. Wang, Z. Tang, J. Henry, B. Li-Ping Lee, Y. Zhu, F. Yuan, F. Huang, S. Li. *Biomaterials* **33** (2012) 8062–8074.
50. K.C. Rustad, M. Sorkin, B. Levi, M.T. Longaker, G.C. Gurtner. *Organogenesis* **6** (2010) 151–157.
51. K.A. Athanasiou, G.G. Niederauer, C.M. Agrawal. *Biomaterials* **17** (1996) 93–102.
52. S. Enomoto, M. Sumi, K. Kajimoto, Y. Nakazawa, R. Takahashi, C. Takabayashi, A. Asakura, M. Sata. *J. Vasc. Surg.* **51** (2010) 155–164.
53. M. Zhou, W. Qiao, Z. Liu, T. Shang, T. Qiao, C. Mao, C. Liu. *Tissue Eng. Part A* **20**(1-2) (2013) 79–91.
54. S. Tara, K.A. Rocco, N. Hibino, T. Sugiura, H. Kurobe, C.K. Breuer, T. Shinoka. *Circ J.* **78** (2014) 12–19.
55. A.G. Mikos, Y. Bao, L.G. Cima, D.E. Ingber, J.P. Vacanti, R. Langer. *J. Biomed. Mater. Res.* **27** (1993) 183–189.

56. S.I. Jeong, B.S. Kim, Y.M. Lee, K.J. Ihn, S.H. Kim, Y.H. Kim. *Biomacromolecules* **5** (2004) 1303–1309.
57. S.H. Kim, E. Chung, S.H. Kim, Y. Jung, Y.H. Kim, S.H. Kim. *J. Biomater. Sci. Polym. Ed.* **21** (2010) 289–302.
58. H. Wu, J. Fan, C.C. Chu, J. Wu. *J. Mater. Sci. Mater. Med.* **21** (2010) 3207–3215.
59. E. Pektok, B. Nottelet, J.C. Tille, R. Gurny, A. Kalangos, M. Moeller, B.H. Walpoth. *Circulation* **118** (2008) 2563–2570.
60. S. Wang, X.M. Mo, B.J. Jiang, C.J. Gao, H.S. Wang, Y.G. Zhuang, L.J. Qiu. *Int. J. Nanomedicine* **8** (2013) 2131–2139.
61. Z. Wang, Y. Cui, J. Wang, X. Yang, Y. Wu, K. Wang, X. Gao, D. Li, Y. Li, X.L. Zheng, Y. Zhu, D. Kong, Q. Zhao. *Biomaterials* **35** (2014) 5700–5710.
62. Y. Su, Q. Su, W. Liu, G. Jin, X. Mo, S. Ramakrishna. *J. Biomater. Sci. Polym. Ed.* **23**(7) (2012) 861–871.

POLYMER- AND NANOPARTICLE-BASED SURFACE MODIFICATION OF ARTIFICIAL VASCULAR GRAFTS

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10.1. INTRODUCTION

Vascular disease, or atherosclerosis, is a widespread disease of civilization and the number of patients with this disease is constantly increasing. Atherosclerosis is a degenerative disease of the vascular wall. Its development is a long process that involves solidification of the artery wall and narrowing of its lumen to form so-called atherosclerotic plaques. As a result of this narrowing, there is insufficient blood circulation to the organ supplied by a vessel. The main cause of atherosclerosis is an increased level of cholesterol in the blood. A higher risk of its occurrence is associated with diabetes, high blood pressure, obesity and cigarette smoking [1,2].

In most developed countries, atherosclerosis is the most common cause of death since it is a contributing factor to most heart attacks and strokes, yet a hundred years ago it belonged to the rare and unstudied diseases. Diet and lifestyle change (to consume more fruits, vegetables and fish; to reduce excess weight; to eliminate smoking and alcohol intake and to increase the frequency of physical activity) can provide possible protection [1].

Vascular surgery undoubtedly belongs among the highly specialized branches of medicine. Close cooperation between angiologists and diabetologists plays an important role in the diagnosis and in solving of both acute and chronic vascular diseases. The area of vascular surgery involves venous issues (varicose veins, oedema and venous insufficiency) and arterial issues (signs of limb ischemia, narrowing of the arteries or their closures).

The use of autologous *vena saphena magna* as a patch or bypass is always a priority for the reconstruction of peripheral vessels. In cases where it is impossible to perform autologous reconstruction, alternative vascular grafts (graft knitted from polyester yarn, graft made from expanded poly(tetrafluoroethylene) (PTFE) and others) are used. Implantation of artificial vascular grafts is associated with a higher risk of infection [1].

Infection is the most serious complication of vascular surgery, which significantly increases the morbidity and mortality of patients in this field. Infection is closely related to the increased use of artificial vascular grafts, which started during the second half of the 20th century. According to literature sources, infection affects 2–3 % of patients undergoing vascular surgery [1,2,8,9].

10.2. VASCULAR GRAFTS

The implanted vascular graft must heal well into the arterial system and should fully take over the function of the original arteries. The vascular graft healing process is quite specific for the system and should be completed within all layers of the arterial wall. Vascular graft should ensure a permanent mechanical support and its properties must not negatively affect the formation of a new blood vessel wall [1,2,10].

10.2.1. Properties of vascular grafts

The main criterion for introduction of implants into the practice is their biosecurity. There are strictly defined tests that evaluate the safety of the materials that come into contact with a patient's tissues and blood. Most of the tests are performed *in vitro*, i.e., in cell culture, but tests using laboratory animals are irreplaceable. It is impossible to create conditions in tissue culture that imitate the behaviour within the organism. However, the development of technologies for *in vitro* testing is advancing, and therefore, the number of laboratory animals needed is decreasing. However, vascular grafts must still meet a number of criteria, which can be divided into biological, surgical and technical properties [1,2,10].

10.3. TYPES OF VASCULAR GRAFTS

Vascular grafts are divided into three groups. The first is the biological substitutes: biological origin, regardless of further modification and adaptation. Another kind of graft is artificial: they are made from non-biological material. A third group of grafts are designated as special types. In these grafts, basic characteristics are amended by modifications, the parameters of which are not possible to define as in the previous two basic groups [1]. Individual groups of artificial vascular grafts are further subdivided according to the manufacturing technologies used (Figure 1).

Figure 1. Basic sorting of commercially produced types of vascular grafts. Dividing them into separate subgroups is according to natural or artificial origin of recovered material and according to techniques of vascular grafts industrial production.

10.3.1. Biological vascular grafts

Biological, autologous grafts are selected in cases where a low flow rate or an increased risk of infection is expected. However, the limitation of their use depends on the availability and also on the dimensions (length, lumen). Biological grafts can be further subdivided into the following categories:

- vascular autograft – graft is removed and implanted in the same organism
- vascular allograft – graft is removed from one organism and implanted into another organism of the same species
- vascular xenograft – graft removed from one organism and implanted into another organism of a different species

It should be noted that even autologous transplantation has its drawbacks and obstacles. The main challenge is obtaining a graft with the required dimensions. Every large artery is critical for supplying an organ and cannot be removed without sequelae. Atherosclerosis does not only affect particular parts of the body but also the disease overall, and therefore, the same implanted tissue will tend to develop the same pathological changes [1,2].

10.3.2. Artificial vascular grafts

Currently, there are three basic types of artificial vascular grafts. They differ in their properties, which are given by production technology. The first group consists of the so-called knitted grafts. These grafts are prepared by knitting

synthetic fibres, typically polyester fibres. The walls of these grafts are porous. Blood loss is minimized by impregnation with collagen or gelatin. The second group includes woven grafts. These grafts have walls with very low porosity. Their disadvantage is insufficient anchorage of the internal fibrin layer. Grafts of expanded poly(tetrafluoroethylene) (ePTFE) are so-called casted artificial grafts and form the third group. They are non-porous and their water-repellent walls are suitable for the smaller flow rates. Predominantly grafts with small diameter are used due to their higher price. Textile grafts can be used in the chest, abdomen and pelvic areas. PTFE grafts cannot be used in peripheral reconstructions, but can be used for arteriovenous connections, *e.g.*, locations on the forearms, arms and legs. The selection of materials for vascular grafts is nowadays subject to rigorous quality control, frequently regarding biocompatibility. Polyester fibres, PTFE and most polyurethanes fulfil these requirements. The synthetic materials dacron and teflon are currently most commonly used for the production of fibres [1,2].

10.4. INFECTIONS OF VASCULAR RECONSTRUCTIONS

Infection complicates approximately 2–3 % of angiosurgical operations [10,11]. Complications that follow a surgical operation may occur immediately after surgery or even several days or weeks later (Table 1). The emergence of infection is influenced by many factors. Some of them can be eliminated, while others cannot be affected. Often, the infection is associated with a risk of loss of a limb or even death. Complications are associated with high morbidity and mortality due to the accompanying sepsis [12]. Infection may occur because of nonspecific complications. Timely and correct anamnesis and clinical examination of the patient is important. For these reasons, it is important to transfer vascular surgery patients with incidence of infection to specialist centres, which have excellent diagnostic and therapeutic options and particularly experienced, professional staff [1,8,11,13].

10.4.1. Classification of infection

The following modified classification by Szilagyi is the most commonly used in practice [14]. A first-degree infection skin involves only the skin, a second-degree infection extends into the subcutaneous tissue above the reconstruction and a third-degree infection affects vascular reconstruction. Within 30 days after the primary vascular operation, the infection is referred to as an early, and 1 month after implantation, a late infection. Infection of reconstruction can even occur 10 or more years after the primary operation [14].

10.4.2. Possible risks of infection

The presence of defective limbs during the vascular reconstruction increases the risk of late infection and the necessity of reoperation. This risk also increases in the case where a patient suffers from multiple diseases, such as diabetes mellitus, renal insufficiency, nicotine abuse or infectious fungal aneurysm, *etc.* Before the operation, it is important to eliminate an eventual chronic infection [15,16].

The incidence of infection increases with reoperation in the same area. In the case of artery operation, the duration of the reconstruction plays a role (artery exposure time). In order to prevent colonization of the reconstruction immediately after implantation, it is necessary to operate in surroundings protected by coagulum [1,17,18].

New procedures and technologies have been introduced into clinical practice for the reduction of infections by implanted vascular grafts. These are laparoscopic and robot-assisted surgery.

Table 1. List of early and late complications during operations in transplant surgery and percentage risk of emergence of complications. Adapted from [19].

Early complications	Risk (%)	Late complications	Risk (%)
Infections of the skin and subcutaneous tissue	15	Infections of the skin and subcutaneous tissue	2
Other reoperation	13	Prosthetic infection	3
Vascular reoperation	2	Pseudoaneurysms	4
Thrombosis	2	Vascular reoperation	18
Embolism	1	Thrombosis	11
Haemorrhage	1	Embolism	0
Amputation	2	Amputation	5

10.4.3. Bacteria causing postoperative infection

The causative agents of infection are usually *Staphylococcus aureus*, *Staphylococcus epidermidis*, and Gram-negative bacteria, especially *Escherichia coli*. Information about infection by MRSA (methicillin-resistant *Staphylococcus aureus*) can be also found in the literature. In most cases, results of cultivation

are negative, despite a macroscopically obvious infection of reconstruction [1,12,14,15,17,18].

10.4.4. Treatment

There are many recommended practices for the treatment of infections after vascular reconstruction. Some papers report options for infection treatment in the affected area by the long-term lavage (by disinfectant or antibiotics). In most cases, experts are inclined to agree that it is impossible to get rid of the infection in the already infected graft. The infection is only suppressed by the use of antibiotics or disinfectant. Usually, reoperation is necessary [1,20]. Despite the efforts of manufacturers of vascular grafts, none of them are 100 % resistant to infection.

10.5. SURFACE MODIFICATION OF VASCULAR GRAFTS

Vascular grafts are an essential part of contemporary medicine and find their application in clinical practice. They are prepared from fabric, the nanostructuralized network of which resembles the extracellular matrix in natural blood vessels and improves the biocompatibility of artificial blood vessels [21]. Vascular grafts have to be further modified to increase their biocompatibility, leading to acceptance by an immune system. The graft must also be modified to maintain flow and anticlotting properties.

Bacterial infections (especially those caused by *Staphylococcus aureus*) belong to the most serious complications of operations associated with the use of vascular implants [22-24]. Vascular grafts may therefore be modified by antibiotics that are bound to collagen or gelatin [22,25]. The drawback of antibiotic use is a rapid development of bacteria that are resistant to these drugs. Another possibility for antibacterial modification is the use of silver ions and the more effective silver nanoparticles [26-29]. Additionally, silver nanoparticles can be used in combination with palladium nanoparticles and other antibacterial agents [30,31]. Antimicrobial biopolymers, such as hyaluronic acid and chitosan, are also known. Both substances are highly biodegradable and biocompatible with the human organism [32,33].

The most common technique for the assessment of antimicrobial activity of the test component is a disc dilution method, which uses impregnation of discs (from woven vascular grafts with a diameter of 1 cm) by the test substance. After their attachment to the agar in Petri dishes coated by bacterial culture, the formation of inhibition zones is monitored, indicating the degree of antimicrobial activity (Figure 2).

Figure 2. Testing the antimicrobial activity of vascular grafts by the dilution method.

Squares from the knitted vascular graft (made by VUP Medical, a.s., Brno, Czech Republic) are soaked with the antimicrobial compound and placed into a Petri dish covered with the bacterial culture diluted to an absorbance of 0.1 AU. After 24 h of incubation (37 °C) the resulting inhibition zones are measured. An increasing size of the inhibition zones indicates increasing antimicrobial activity of the test substance.

10.5.1. Modification by metal or semimetal nanoparticles

Nanotechnologies are one of the most advanced scientific disciplines today. The aim is to form nanoparticles that are in compliance with the size, shape, structure and distribution in the organism. Metal nanoparticles with a size of 1–200 nm may be modified by layers of different compounds, such as biopolymers [34]. Nanoparticles prepared by this method show altered physical and chemical properties compared with nanoparticles based only on metals [35,36].

Due to specific properties of the nanoparticles, they are used as catalysts and important components of sensors based on optical or electrochemical methods. They have also found wide use in biomedical applications that require bacterial sterility [37]. A high catalytic ability, given by the large surface of the nanoparticles, and the ability to generate reactive oxygen species causes a high reactivity of nanoparticles, which thus become more toxic to bacteria [38].

Using modern techniques, metal nanoparticles can be functionalized, and thus it is possible to increase their antimicrobial activity [39]. Due to their properties, nanoparticles exhibit greater retention in the body, so it is easier to achieve the desired distribution effect [40].

One potential solution that can reduce the number of infections in operations is the application of silver nanoparticles (micrograph depicted in Figure 3) [4,5]. Silver nanoparticles are commonly used in the textile industry and in medicine [41]. Until recently, silver nanoparticles were commonly used for implant materials that reduced the incidence of postoperative complications. However, over time these applications were associated with a potential risk of toxicity [42].

Figure 3. AgNPs characterized by scanning electron microscope (SEM). Micrographs: a) SEM HV: 15 kV, view field: 1.445 μm , WD: 3.761 mm, det: InBeam, b) SEM HV: 15 kV, view field: 8.668 μm , WD: 2.984 mm, det: InBeam.

Silver ions affect the process of cell proliferation and silver ions and nanoparticles have thus found the use in dermatology for facilitating wound healing and in a wide spectrum of therapeutic use [43].

The mechanism of silver nanoparticle action is still unclear. However, the possible effects of silver nanoparticles are proposed on the basis of enzyme inhibition, alteration of membrane integrity, penetration into the bacterial cytoplasm and accumulation in the periplasmic space or in the formation of reactive oxygen species. It was confirmed that the composition of the cell wall and plasma membrane plays an important role in the penetration of

nanoparticles into cells. In G⁺ bacteria, transmittance is significantly slower than in case of G⁻ bacteria [44].

Another, newer option in terms of nanotechnology and bacterial infections, is selenium nanoparticles. Selenium nanoparticles were investigated for various medical applications and as a potential material for orthopaedic implants [45]. Studies that point out the ability of the selenium compounds to inhibit the growth of bacteria and the formation of bacterial biofilms are now available [45]. In a number of selenium compounds (such as 2,4,6-tri-*para*-methoxyphenylselenopyrylium chloride, 9-*para*-chlorophenyloctahydro-selenoxanthen, and perhydroselenoxanthene) *in vitro* antibacterial activity, particularly against *Staphylococcus aureus* has been demonstrated. However, the effects of elemental selenium nanoparticles remain largely unknown [46,47].

10.5.2. Modification by nonpolymeric or polymeric substances

Application of metal ions or nanoparticles in combination with other substances having antimicrobial properties is another way to increase antimicrobial activity [30,31]. The combination of nanoparticles with certain polymeric substances was confirmed to be very effective at minimizing the risk of bacterial infection [6,7]. Complexes of polymer materials with silver nanoparticles are widely used as growth inhibitors of G⁺ and G⁻ bacteria [48] and also as the substances used for the treatment of post-traumatic and postoperative tissues [49,50].

The first of the most commonly used polymeric substances, chitosan, possessed a number of useful properties [7]. Chitosan is a linear polysaccharide composed of 2-amino-2-deoxy-D-glucopyranose and 2-acetamido-2-deoxy-D-glucopyranose units [51]. It is synthesized either in an animal body or artificially by partial deacetylation of chitin, which is the main component of crustacean shells [52]. It is the most widespread biopolymer material [53]. Due to its nontoxic properties, biocompatibility and biodegradability, chitosan is used in targeted drug delivery [54] and also stands out for its antimicrobial properties. For this reason, it is commonly used as a substance to maintain the sterility of artificial implants [55,56]. Due to the polymeric structure of chitosan, it easily forms complexes with metals [56-58].

Another equally important polymeric material that is utilized in the formation of complexes with metals is hyaluronic acid. Hyaluronic acid, or its salt, is a linear polysaccharide of disaccharide units of D-glucuronic acid and N-acetylglucosamine [59]. It is naturally present in mammals, extracellular matrix, muscle and nerve tissues in the vitreous humor and on the skin [60]. It is compatible with the human organism and biodegradable [61-64]. Hyaluronic acid synthesis occurs in the plasma membrane of fibroblasts [65]. Hyaluronic acid in the intracellular matrix binds water to proteins, is involved in the construction of extracellular matrix and also plays an important role in the

organization of proteoglycan complexes in soft connective tissues [32,33]. The greatest incidence of this polymer in humans is in areas with proliferating cells. Due to its organization, hyaluronic acid is used in eye surgery, tissue engineering and cosmetics, which are used to accelerate tissue regeneration [66-70].

Interactions of biopolymer materials with metals are based on the binding of metal ions to the amino groups of chitosan *via* chelating mechanisms [71]. Similar mechanisms have also been described in connection with the binding of metal ions or nanoparticles to hyaluronic acid [72].

One of the other biopolymer materials is adiponectin. Adiponectin is a protein produced mainly by mature adipocytes and is present in a surprisingly large amount in human plasma [73]. It was found that adiponectin significantly regulates the metabolism of carbohydrates and lipids, and increases the utilization and transport of glucose and free fatty acids into muscle, liver and fat cells [74]. Adiponectin in human plasma occurs in the form of several polymeric isoforms. Adiponectin accumulates subendothelially in damaged areas of vessel walls and increases in adiponectin significantly reduce the progression of atherosclerotic lesions in animal models of atherosclerosis. Hypoadiponectinemia was found to be an independent predictor of cardiovascular disease in patients with advanced stage renal insufficiency. In further work, high plasma levels of adiponectin was associated with a low risk of myocardial infarction in men [75].

10.5.3. *In vivo* application of surface modified vascular grafts

Finally, the application of modified vascular grafts in animal cell cultures was used. In our experiments *in vivo*, three different cell lines (quail and human fibroblasts and endothelial cells) were studied. All lines were stabilized at the beginning of experiment in two independent passages, while ensuring optimal conditions of cultivated media. Cultivation was carried out at 37 °C and 5 % CO₂. In addition, we designed a simple simulation of blood flow through oscillation. For the assessment of biological properties, we found that the cells exhibited very good morphological properties. The cell lines were further characterized based on other indicators, such as the structure of the nucleus and nucleolus, the activity of mitochondria and RNA. During each experiment, there were no significant differences in the input morphological indicators (Figure 4).

Figure 4. Morphological indicators of used cell cultures

Cell cultures were grown on various types of cultivation surfaces (various sizes of specialized Petri dishes, cultivation bottles or cultivation tubes). We did not observe any major differences between cultivation on various types of surfaces. Therefore, it was possible to interchange the individual cultivation methods as necessary.

Figure 4 shows a massive growth of fibroblasts in the central portion of the prosthesis after the application of 25 ng mL^{-1} of adiponectin. Figure 5 shows the cultivation of fibroblast cells in a culture tube, which was subsequently placed on a thermomixer. To these tubes, it was possible to place the vascular prosthesis and subsequently add a fibroblast cell culture. Moreover, it was possible to apply the adiponectin to the different parts of the graft.

Cultivation of cells

Visualization of metabolic activity on vascular graft with adiponectin

Figure 5. Possible methods of cell cultivation

If the vascular prostheses were placed on a cycler (simulation of blood flow), it was possible to observe a better growth of the cell culture on the surface of the graft. The amount of cells captured on the vascular graft has increased about 30 % compared with the variant without oscillation. However, the intensity of oscillation could not exceed 20 Hz or else the coming cells would grow less rapidly. Figure 6 shows photographs of the cultivation of vascular grafts in a cultivation bottle. There the cells also grow and colonize the graft.

Figure 6. Cultivation of fibroblasts on grafts in a cultivation bottle

Cell lines derived from endothelium showed similar behaviour to that of fibroblast cells. If the cell lines were cultivated on the graft in the presence of adiponectin, a gradual increase in cell amount was observed (Figure 7). The amount of cells increased by an average of 20–30 % in comparison with vascular grafts without the application of adiponectin. In the experiments, the adiponectin concentrations in the range of 5–15 ng mL⁻¹ were monitored. However, the increase in cell amount in these experimental variants was similar.

Figure 7. Cultivation of endothelial culture on the fabric after application of adiponectin

The aim of this prospective study was to verify the properties of the new species of vascular grafts with adiponectin in the experiment. Before conducting clinical trials of a new kind of vascular graft on humans, it is necessary to evaluate the rheological and immunological response of this vascular graft in live animals *in vivo*. The experiment cannot be replaced by alternative methods *in vitro*. The number of animals is chosen so that the study is informative, while being feasible under the given conditions.

A Sheep model was selected as an experimental animal due to the similar behaviour of the arterial vessels in sheep to those in humans. According to literal data, for the transplantation of vascular grafts of the required size, only sheep are suitable for experimentation with vascular grafts in the carotid artery, due to the size of the carotid artery and the long and easily accessible neck [76–80]. Another advantage is that in the postoperative period, sheep require minimal care and monitoring is not so expensive.

Nine sheep were observed from 30–100 days (3 sheep every 30 days). All implanted grafts (vascular prostheses in the control group and vascular prostheses with adiponectin) remained feasible during the experiment. Favourable macroscopic images of the implanted grafts with adiponectin in the sheep body have demonstrated excellent healing of grafts due to their good rheological properties. This image was more favourable than those of the grafts in the control groups, both in the terms of the reaction of the environment (the control group had a prosthesis with a more pronounced reaction) and in terms of the subtleties of the inner surface of the graft (the control group had a rough inner surface). Histological findings demonstrated the high quality and speed of healing of the prostheses with adiponectin.

Dissection of carotid artery in sheep

Implantation of vascular graft with adiponectin

Figure 8. Transplantation of artificial vascular graft with adiponectin into the sheep body as a replacement for carotid artery

10.6. CONCLUSION

The material used, preoperative preparation, the actual surgical procedure and postoperative care play important roles in vascular surgery during vascular reconstructions.

The selection of vascular grafts is usually determined by surgeons. Traditionally, they used to favour the use of artificial vascular grafts knitted or woven, which were coated with a compact layer of either collagen or collagen with subsequent treatment. This is a modification of the antibacterial effect.

Nowadays, the selection of artificial vascular grafts is very diverse. But in spite of such a wide range of materials, surgeons struggle with the emergence of sepsis at the reconstruction site. There are many different opinions on how to proceed in the treatment of infections: whether to treat the infection or to reoperate. Time plays a particularly important role in this case. Early diagnosis

and detection of advancing infection can rescue the patient's limb, or even their life.

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REFERENCES

1. M. Krajicek, J.H. Peregrin, M. Rocek, P. Sebesta, et. al., *Chirurgická a intervenční léčba cévních onemocnění (Surgical and interventional treatment of vascular diseases)*, Grada Publishing a.s., Prague 2007 p. 436.
2. Z. Krska, *Techniky a technologie v chirurgických oborech (Techniques and technologies in surgery)*, Grada Publishing a.s., Prague, 2011.
3. R. Rasool, S. Hasnain, N. Nishat. *Des. Monomers Polym.* **17** (2014) 217–226.
4. J.B.D. Green, T. Fulghum, M.A. Nordhaus. *Biointerphases* **6** (2011) MR13–MR28.
5. M. Osinska-Jaroszuk, G. Ginalska, A. Belcarz, A. Uryniak. *Eur. J. Vasc. Endovasc. Surg.* **38** (2009) 697–706.
6. A. de Mel, K. Chaloupka, Y. Malam, A. Darbyshire, B. Cousins, A.M. Seifalian. *Mater. Res. A. Part A* **100A**(9) (2012) 2348–2357.
7. M. Fujita, M. Kinoshita, M. Ishihara, Y. Kanatani, Y. Morimoto, M. Simizu, T. Ishizuka, Y. Saito, H. Yura, T. Matsui, B. Takase, H. Hattori, M. Kikuchi, T. Maehara. *J. Surg. Res.* **121** (2004) 135–140.
8. S. Franke, R. Voit. *Ann. Vasc. Surg.* **11** (1997) 406–412.
9. J.K. Chang, K.D. Calligaro, S. Ryan, D. Runyan, M.J. Dougherty, J.J. Stern. *Ann. Vasc. Surg.* **17** (2003) 91–96.
10. W.L. Xu, F. Zhou, C.X. Ouyang, W.X. Ye, M. Yao, B.G. Xu. *J. Biomed. Mater. Res. Part A* **92A** (2010) 1–8.
11. L.L. Gordon, R.T. Hagino, M.R. Jackson, J.G. Modrall, R.J. Valentine, G.P. Clagett. *Arch. Surg.* **134** (1999) 615–620.
12. C.L. Bell, A.T. Ali, J.G. Brawley, V.J. D'Addio, J.G. Modrall, R.J. Valentine, G.P. Clagett. *J. Am. Coll. Surg.* **200** (2005) 831–836.
13. A. Skarpa, I. Drescik, A. Sepic, D. Curuvija, D. Vukas, S. Clanak. *Acta. Fac. Med. Flum.* **16** (1991) 65–70.
14. D.E. Szilagyi, M.P. Vrandecic, R.F. Smith, J.P. Elliott. *Ann. Surg.* **176** (1972) 321–333.
15. J.W. Babich, A.J. Fischman. *Adv. Drug. Deliv. Rev.* **37** (1999) 237–252.
16. D.F. Bandyk, M.L. Novotney, B.L. Johnson, M.R. Back, S.R. Roth. *J. Surg. Res.* **95** (2001) 44–49.
17. J.P. Bagger, D. Zindrou, K.M. Taylor. *Lancet* **363** (2004) 706–708.
18. E.J. Cohn, W.R. Flinn, M. E. Benjamin, M.P. Lilly, W.A. Purtill, D.J. Hanna. *J. Vasc. Surg.* **30** (1999) 1004–1015.
19. R. Vlachovsky, Z. Gregor, Z. Kriz, R. Staffa, M. Dvorak, in *X Slovak congress of vascular surgery with international participation*, Jasna, Slovakia, 2006.

20. J.M. Seeger, H.A. Pretus, M.B. Welborn, C.K. Ozaki, T.C. Flynn, T.S. Huber, *J. Vasc. Surg.* **32** (2000) 451–459.
21. J.P. Stegemann, S.N. Kaszuba, S.L. Rowe. *Tissue Eng.* **13** (2007) 2601–2613.
22. T.M. Perl, J.J. Cullen, R.P. Wenzel, M.B. Zimmerman, M.A. Pfaller, D. Sheppard, J. Twombly, P.P. French, L.A. Herwaldt, A. Mupirocin. *N. Engl. J. Med.* **346** (2002) 1871–1877.
23. D.J. Anderson, D.J. Sexton, Z.A. Kanafani, G. Auten, K.S. Kaye. *Infect. Control Hosp. Epidemiol.* **28** (2007) 1047–1053.
24. J.J. Earnshaw. *Eur. J. Vasc. Endovasc. Surg.* **24** (2002) 283–286.
25. W. Lew, W. Moore. *Semin. Vasc. Surg.* **24** (2011) 211–219.
26. M.K. Rai, S.D. Deshmukh, A.P. Ingle, A.K. Gade. *J. Appl. Microbiol.* **112** (2012) 841–852.
27. C. Unger, C. Luck. *J. Appl. Microbiol.* **112** (2012) 1212–1219.
28. W.R. Li, X.B. Xie, Q.S. Shi, S.S. Duan, Y.S. Ouyang, Y.B. Chen. *Biomaterials* **24** (2011) 135–141.
29. O. Choi, K.K. Deng, N.J. Kim, L. Ross, R.Y. Surampalli, Z.Q. Hu. *Water Res.* **42** (2008) 3066–3074.
30. D.P. Dowling, A.J. Betts, C. Pope, M.L. McConnell, R. Eloy, M.N. Arnaud. *Surf. Coat. Technol.* **163** (2003) 637–640.
31. S. Ruden, K. Hilpert, M. Berditsch, P. Wadhwani, A.S. Ulrich. *Antimicrob. Agents Chemother.* **53** (2009) 3538–3540.
32. S.P. Evanko, T.N. Wight. *J. Histochem. Cytochem.* **47** (1999) 1331–1341.
33. G. Kogan, L. Soltes, R. Stern, P. Gemeiner. *Biotechnol. Lett.* **29** (2007) 17–25.
34. M.A.M. Khan, S. Kumar, M. Ahamed, S.A. Alrokayan, M.S. Alsalhi, M. Alhoshan, A.S. Aldwayyan. *Appl. Surf. Sci.* **257** (2011) 10607–10612.
35. G. Banfi, V. Degiorgio, D. Ricard. *Adv. Phys.* **47** (1998) 447–510.
36. L. Banyai, Y.Z. Hu, M. Lindberg, S.W. Koch. *J. De Phys.* **49** (1988) 225–228.
37. N.G. Khlebtsov, L.A. Dykman. *J. Quant. Spectrosc. Radiat. Transf.* **111** (2010) 1–35.
38. L.K. Limbach, P. Wick, P. Manser, R.N. Grass, A. Bruinink, W.J. Stark. *Environ. Sci. Technol.* **41** (2007) 4158–4163.
39. Z. Magdolenova, A. Collins, A. Kumar, A. Dhawan, V. Stone, M. Dusinska, *Nanotoxicology* **8** (2014) 233–278.
40. P. Kanmani, J.W. Rhim. *Food Chem.* **148** (2014) 162–169.
41. B.K. Sharma, A.K. Gupta, N. Khare, S.K. Dhawan, H.C. Gupta. *Synth. Met.* **159** (2009) 391–395.
42. S. Liu, J.W. Zhao, H.J. Ruan, W. Wang, T.Y. Wu, W.G. Cui, C.Y. Fan. *Mater. Sci. Eng. C-Mater. Biol. Appl.* **33** (2013) 1176–1182.
43. M.N. Shahzad, N. Ahmed. *J. Pak. Med. Assoc.* **63** (2013) 225–230.
44. A. Grigor'eva, I. Saranina, N. Tikunova, A. Safonov, N. Timoshenko, A. Rebrov, E. Ryabchikova. *Biomaterials*. **26** (2013) 479–488.
45. Q. Wang, T.J. Webster. *J. Biomed. Mater. Res. Part A* **100A** (2012) 3205–3210.
46. P.A. Tran, T.J. Webster. *Int. J. Nanomedicine* **6** (2011) 1553–1558.
47. J.F. Ramos, P.A. Tran, T.J. Webster, *IEEE, Selenium Nanoparticles for the Prevention of PVC-related Medical Infections*, 2012, p. 185–186.
48. J.W. Rhim, L.F. Wang, S.I. Hong. *Food Hydrocoll.* **33** (2013) 327–335.
49. F. Pishbin, V. Mourino, J.B. Gilchrist, D.W. McComb, S. Kreppel, V. Salih, M.P. Ryan, A.R. Boccaccini. *Acta Biomater.* **9** (2013) 7469–7479.

50. G. Sandri, M.C. Bonferoni, F.D'Autilia, S. Rossi, F. Ferrari, P. Grisoli, M. Sorrenti, L. Catenacci, C. Del Fante, C. Perotti, C. Caramella. *Eur. J. Pharm. Biopharm.* **84** (2013) 84–90.
51. A. Ghosh, M.A. Ali. *J. Mater. Sci.* **47** (2012) 1196–1204.
52. C. Mohamed, K.A. Clementine, M. Didier, L. Gerard, D.C.M. Noelle. *Food Hydrocoll.* **30** (2013) 576–580.
53. H.W. Chang, Y.S. Lin, Y.D. Tsai, M.L. Tsai. *J. Appl. Polym. Sci.* **127** (2013) 169–176.
54. R. Jayakumar, D. Menon, K. Manzoor, S.V. Nair, H. Tamura. *Carbohydr. Polym.* **82** (2010) 227–232.
55. N.R. Pires, P.L.R. Cunha, J.S. Maciel, A.L. Angelim, V.M.M. Melo, R.C.M. de Paula, J.P.A. Feitosa. *Carbohydr. Polym.* **91** (2013) 92–99.
56. K. Madhumathi, P.T.S. Kumar, S. Abhilash, V. Sreeja, H. Tamura, K. Manzoor, S.V. Nair, R. Jayakumar. *J. Mater. Sci.-Mater. Med.* **21** (2010) 807–813.
57. G.Q. Huang, Y.T. Sun, J.X. Xiao, J. Yang. *Food Chem.* **135** (2012) 534–539.
58. S.B. Lee, Y.H. Kim, M.S. Chong, Y.M. Lee. *Biomaterials* **25** (2004) 2309–2317.
59. E. Hrabarova, J. Rychly, V. Sasinkova, K. Valachova, I. Janigova, K. Csomorova, I. Juranek, L. Soltes. *Cellulose* **19** (2012) 2093–2104.
60. L. Kenne, S. Gohil, E.M. Nilsson, A. Karlsson, D. Ericsson, A.H. Kenne, L.I. Nord. *Carbohydr. Polym.* **91** (2013) 410–418.
61. B.M. Teh, Y. Shen, P.L. Friedland, M.D. Atlas, R.J. Marano. *Expert Opin. Biol. Ther.* **12** (2012) 23–36.
62. E. Hrabarova, K. Valachova, J. Rychly, P. Raptá, V. Sasinkova, M. Malikova, L. Soltes. *Polym. Degrad. Stabil.* **94** (2009) 1867–1875.
63. M.B. Sintzel, S.F. Bernatchez, C. Tabatabay, R. Gurny. *Eur. J. Pharm. Biopharm.* **42** (1996) 358–374.
64. R. Stern. *Glycobiology* **13** (2003) 105R–115R.
65. E.R. Bastow, S. Byers, S.B. Golub, C.E. Clarkin, A.A. Pitsillides, A.J. Fosang, *Cell. Mol. Life Sci.* **65** (2008) 395–413.
66. K.L. Goa, P. Benfield. *Drugs* **47** (1994) 536–566.
67. L.C. Becker, W.F. Bergfeld, D.V. Belsito, C.D. Klaassen, J.G. Marks, R.C. Shank, T.J. Slaga, P.W. Snyder, F.A. Andersen, *Int. J. Toxicol.* **28** (2009) 5–67.
68. J. Kim, Y. Park, G. Tae, K.B. Lee, C.M. Hwang, S.J. Hwang, I.S. Kim, I. Noh, K. Sun. *J. Biomed. Mater. Res. Part A* **88A** (2009) 967–975.
69. P.W. Noble. *Matrix Biol.* **21** (2002) 25–29.
70. J.P. Ortonne. *J. Dermatol. Treat.* **7** (1996) 75–81.
71. E. Guibal. *Sep. Purif. Technol.* **38** (2004) 43–74.
72. A.M. Abdel-Mohsen, R. Hrdina, L. Burgert, G. Krylova, R.M. Abdel-Rahman, A. Krejcova, M. Steinhart, L. Benes. *Carbohydr. Polym.* **89** (2012) 411–422.
73. Y. Nakano, A. Shoji, A. Arakawa, Y. Iizuka, Y. Kikuchi, M. Kobayashi, T. Tobe. *J. Lipid Res.* **51** (2010) 210–215.
74. N. Stefan, B. Vozarova, T. Funahashi, Y. Matsuzawa, C. Weyer, R.S. Lindsay, J.F. Youngren, P.J. Havel, R.E. Pratley, C. Bogardus, P.A. Tataranni. *Diabetes* **51** (2002) 1884–1888.
75. M.M. Hossain, A. Mukheem, T. Kamarul. *Life. Sci.* **135** (2015) 55–67.
76. M. Ahmed, G. Hamilton, A.M. Seifalian. *Biomaterials* **35** (2014) 9033–9040.
77. P.B. de Albuquerque, B.D. Barni, R.B. Silva, T.M. Pinto, G.C. Gianotti, L.F.D. Moura, M.Z. Boss, M.M. Alievi. *Cienc. Rural.* **45** (2015) 718–723.

78. F. Wolf, S.M. Koch, M.E. Mertens, V. Gesche, P. Schuster, J. Frese, T. Lammers, F. Kiessling, P. Mela, S. Jockenhovel. *J. Tissue Eng. Regen. Med.* **8** (2014) 394–394.
79. Z.H. Syedain, L.A. Meier, M.T. Lahti, S.L. Johnson, R.T. Tranquillo. *Tissue Eng. Part A* **20** (2014) 1726–1734.
80. L.A. Meier, Z.H. Syedain, M.T. Lahti, S.S. Johnson, M.H. Chen, R.P. Hebbel, R.T. Tranquillo. *J. Cardiovasc. Transl. Res.* **7** (2014) 242–249.

**TEMPOROMANDIBULAR DISORDERS AND
BIO-IPNS: *IN VITRO* APPROACH TO FIND
MOLECULAR SOLUTION TO BIOLOGICAL
PROBLEM**

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11.1. INTRODUCTION

In the last few decades, tissue engineering has emerged as a promising multidisciplinary approach for the repair and regeneration of damaged bone tissue.

The molecular events that underline the degenerative temporomandibular joint (TMJ) diseases are poorly understood. Mechanical stresses are generated during functional or para-functional movements of the jaw, adaptive mechanism of the TMJ may be exceeded by free radical accumulation leading to a dysfunctional state (*i.e.* disease state). Biologically relevant free radicals are very reactive and unstable molecular entities that have an unpaired electron and they can produce participate in the propagation chain reaction to form a new radical. Although oxygen free radicals participate in many physiological processes, they can be harmful to tissue when either their action or their generation have been left uncontrolled. The most common source of free radicals in biological systems is oxygen. The elevation of reactive oxygen species (ROS) lead to oxidative stress that causes molecular damage to the vital structures and functions.

This chapter addresses the principal intrinsic and extrinsic factors that impede integration and describe how manipulation of these factors using a host of strategies can positively influence cartilage integration based on the designer biomaterial scaffolds and combinations designed by others and us *in vitro* development.

11.1.1. Introduction to temporomandibular disorders: anatomy and physiology summary

Signs and symptoms of temporomandibular disorders (TMDs) may include pain, impaired jaw function, malocclusion, deviation or deflection, limited range of motion, joint noise, and locking. Headache, tinnitus, visual changes, and other neurologic complaints may also accompany TMDs. Because of many etiologic factors, the diagnosis and treatment of patients with TMDs is complex. TMDs can be subdivided into muscular and articular categories. Differentiation between the two is sometimes difficult because muscle disorders may mimic articular disorders, and they may coexist. Myogenic disorders include myalgia (myofascial pain (MFP), fibromyalgia), myospasm, splinting, and fibrosis/contracture. Articular disorders include synovitis/capsulitis, joint effusion, trauma/fracture, internal derangement, arthritis, and neoplasm.

TMJ is a compound articulation formed from the articular surfaces of the temporal bone and the mandibular condyle. Both surfaces are covered by dense articular fibrocartilage. Each condyle articulates with a large surface

area of temporal bone consisting of the articular fossa, articular eminence, and preglenoid plane. The TMJ functions uniquely in that the condyle both rotates within the fossa and translates anteriorly along the articular eminence. Because of the condyle's ability to translate, the mandible can have a much higher maximal incisal opening than would be possible with rotation alone. The joint is thus referred to as "gynghimodiartrodial": a combination of the terms gynghymoid (rotation) and arthrodial (translation) [1].

A cartilaginous disc resides between the articular surfaces of the temporal bone and mandibular condyle. Although other articular cartilages are composed of hyaline cartilage, this disc is composed of fibrocartilage; thus, the disc contains a much higher percentage of collagen, increasing its stiffness and durability. The disc does not have any direct vascularization or innervation; however, the posterior attachment of the disc (also known as retrodiscal tissue) is both highly vascularized and highly innervated and, therefore, pertinent to the discussion of joint pain. The superior lamina of the retrodiscal tissue limits extreme translation, whereas the inferior lamina limits extreme rotation. The lateral pterygoid muscle controls the opening of the mandible. The superior segment of this muscle attaches to the anterior portion of the disc, and the inferior segment attaches inferior to the condyle. As both segments contract the condyle translates anteriorly along the articular eminence, and the disc remains interposed between the condyle and the temporal bone at all points of translation.

The joint is stabilized by three ligaments: collateral (discal), capsular, and temporomandibular. These attach to the disc at the medial and lateral poles of the mandibular condyle, as well as to the temporal fossa. These ligaments limit extreme condylar movement. The capsular ligament surrounds the joint space and disc and acts to contain the synovial fluid within the joint space.

The capsule is lined by a synovial membrane. Synovial tissue covers all intra-articular surfaces except for the pressure-bearing fibrocartilage (*i.e.* disc, condyle, eminence). The synovial tissue is highly innervated and vascularized and has regulatory, phagocytic, and secretory functions. The synovial fluid has metabolic and nutritional functions and is essential to joint surface lubrication [2].

The masseter, medial pterygoid, lateral pterygoid, and temporalis muscles are the muscles of mastication. The masseter, medial pterygoid, and temporalis are primarily responsible for mandibular closure and bite force, whereas the lateral pterygoid and infrahyoid muscles are responsible for mandibular opening. Mandibular movement is also influenced by the digastric, geniohyoid, mylohyoid, stylohyoid, sternohyoid, omohyoid, sternothyroid, and thyrohyoid muscles, which as a group coordinate complex mandibular movements including opening, protrusion, retrusion, lateral excursion, and closure.

At rest, the condyle is seated passively in the temporal fossa with the fibrocartilage disc interposed at the most superior and anterior position of the

condyle. Mandibular opening commences with contraction of the lateral pterygoid and infrahyoid muscles, which rotates the condyle. Mandibular opening proceeds with lateral pterygoid contraction pulling the condyle forward along the articular eminence (translation). The superior segment of the lateral pterygoid muscle coordinates the translation of the disc with the condyle. During jaw closing the ligaments and retrodiscal lamina pull the condyle and disc back into resting position.

The TMJ receives its vascular supply from the superficial temporal, maxillary, and masseteric arteries. Innervation of the joint is provided mainly by the auriculotemporal nerve and, to a lesser extent, the masseteric and posterior deep temporal nerves. The production of synovial fluid is also under a certain amount of neuronal control.

11.1.1.1. Temporomandibular disorders: general introduction

The term “TMJ pain” varies greatly in meaning among clinicians, patients, and the general population. Historically, symptom-based classification of the disorder has been problematic. As stated by Laskin [3,4], the difficulty began with the introduction of a “TMJ syndrome.” Then clinicians erroneously grouped a “variety of etiologically unrelated conditions into one diagnostic category based on the fact that they produced similar signs and symptoms,” and this led to “one diagnosis equals one treatment.” Only later was it recognized that many of these patients suffered from muscle-related conditions. The terms MFP and myofascial pain and dysfunction (MPD) evolved [5], and “TMJ disorders” became “TMDs.” were differentiation of the TMDs into articular (joint) and nonarticular (myogenic) disorders can be made and will be further discussed.

11.1.1.2. Myogenic disorders

Traditionally, it was thought that structural abnormalities (*i.e.* dental malocclusion, condylar malposition) led to muscular dysfunction and pain [6,7]. Muscles were thought to be under an increased burden in the presence of these skeletal and/or dental misalignments. As such, a “vicious cycle” model was proposed: Structural → abnormality → muscle hyperactivity ↔ pain ↔ mandibular dysfunction where pain and muscle hyperactivity potentiate each other and emotional stress is thought to have an additive effect [6,7]. Over time, there has been a lack of scientific evidence to support this theory.

MFP of the masticatory muscles is more frequently induced by stress-related parafunctional habits (*i.e.* clenching and grinding) and rarely by mechanical causes such as occlusal prematurities or high dental restorations. MFP and MPD, although considered to be muscular disorders, are thought to possibly play a causative role in degenerative disease of the TMJ.

11.1.1.3. Articular disorders

The etiology of articular disorders may be degenerative, traumatic, infectious, immunologic, metabolic, neoplastic, congenital, or developmental.

Articular disc displacement (internal derangement) Anterior disc displacement (ADD) is the most frequently encountered articular disorder. Disc displacement (also known as internal derangement) is defined as “a disturbance in the normal anatomic relationship between the disc and condyle that interferes with smooth movement of the joint and causes momentary catching, clicking, pop-ping, or locking” [8-11].

The ability of the joint to adapt to biomechanical stress and disc derangement has been a subject of debate. In his classification system, Wilkes [12] promotes the theory that internal derangement logically progresses to degenerative joint disease (DJD). Historically, surgical and nonsurgical approaches have been used to reposition the displaced disc, with the goal of arresting this progression [13]. In an opposing view, Milam [9] states that “the adaptive capacity of the TMJ is not infinite ...some individuals are... capable of mounting an adaptive response to an articular disc displacement; other individuals may not adapt to these structural derangements, and a progressive DJD may result.” Factors considered to compromise the adaptive response include age, sex, stress, and illness [9,14]. He concludes that disc derangement may exist variably as cause or effect, but does not always progress to disease.

11.2. FREE RADICALS AND TEMPOROMANDIBULAR JOINT IN ACTION

Mechanical stresses are generated during functional or para-functional movements of the jaw, adaptive mechanism of the TMJ may be exceeded by free radical accumulation leading to a dysfunctional state (*i.e.* disease state). Biologically relevant free radicals are very reactive and unstable molecular entities that have an unpaired electron and they can produce participate in the propagation chain reaction to form a new radical. Although oxygen free radicals participate in many physiological processes, they can be harmful to tissue when either their action or their generation have been left uncontrolled. The most common source of free radicals in biological systems is oxygen. The elevation of ROS lead to oxidative stress that causes molecular damage to the vital structures and functions. ROS are generated on a regular basis in biological pathways as a product or as a signal transducer.

However, excessive production or ineffective scavenging of ROS can cause over accumulation, which can injure or kill cells. All basic molecules in living organisms can be attacked by ROS, *e.g.*, lipids, carbohydrates, proteins and nucleic acids [15-17].

The unsaturated fatty acids of cell membrane lipid are susceptible to peroxidative reaction. Lipid peroxidation of cell membranes has been implicated in the wide range of tissue injuries and diseases. Accumulation of lipid hydroperoxides in a membrane disrupts its function and causes it to collapse and have range of cytotoxic radicals. The most series of which are aldehydes. They may also react with transition metals like iron or copper to form stable aldehyde such as malodialdehyde for example, which will damage the cell membrane. Because hemoglobin constitutes the largest iron store in the body, it is speculated to be a potential source of redox activity iron, which can catalyze the formation of free radicals that might be damaging to the joint [18].

Antioxidant defense mechanisms involve both enzymatic and non-enzymatic strategies. Common antioxidants include the vitamins A, C and E, glutathione, and the enzymes superoxide dismutase, catalyase, glutathione peroxidase and glutathione reductase. They work in synergy with each other and against different types of free radicals. Antioxidant enzymes in living organisms have evolved as very sophisticated and effective scavengers of ROS. Superoxide dismutase (SOD) is an essential antioxidant enzyme protecting many cellular components by converting two superoxide anions into a molecule of hydrogen peroxide and one molecule of oxygen. SODs are found in many organisms including all oxygen-consuming organisms [19].

11.3. AETIOLOGY OF TEMPOROMANDIBULAR JOINT IN A NUTSHEL

Most of the scientists explain that osteoarthritis (OA) as an inflammatory process, TMJ disorder being the most frequent one, is characterised by proliferative changes in the synovial and primary degeneration of the cartilage and surrounding tissues [20,21]. It is found that 28 % of the adult population have symptoms and clinical signs of TMD [22,23]. The aetiological factors of TMJ disorders as follows: systemic diseases (rheumatoid arthritis (RA), psoriasis, pseudogout, ankylosing spondylitis, etc.), secondary inflammatory component from the neighbouring regions (otitis, maxillary sinusitis, tonsillitis), trauma (chronical), prevalence of dental arch defects e.g. missing of molar teeth [24], malocclusion, endocrinological disturbances, odontogenic infections (impacted third molars). Presence of specific bacterial organisms such as *Staphylococcus aureus*, *Streptococcus mitis*, *Mycoplasma fermentas*, *Actinobacillus actinomycetemcomitans* in the synovial fluid have been found [25]. Serum antibodies against *Chlamydia* species in patients with monoarthritis of the TMJ have also been reported [26].

Although patients without internal derangement may develop OA [27], a complex two-way relationship exists. Controversy continues as to whether disc

derangement is a cause or a result of DJD; however, scientific evidence strongly supports the latter conclusion [27-31].

11.4. PATHOGENESIS OF TEMPOROMANDIBULAR JOINT UP TO DATE

Inflammation mainly affects the posterior disc attachment [20,27]. Several inflammatory mediators play an important role in the pathogenesis of TMJ disorders like tumor necrosis factor alpha (TNF- α), interleukin-1beta (IL-1 β), prostaglandin E2 (PGE2), leukotriene B4 (LkB4), matrix metalloproteinases (MMPs), serotonin or 5-hydroxytryptamine (5-HT) [22,28]. MMPs are the early marker or detector to determine TMJ arthritis [27]. Serotonin is the mediator of pain and inflammation is produced in enterocromaffin cells of the gastrointestinal mucosa and absorbed by platelets. It is also produced in the synovial membrane and in the synovial fluid which causes TMJ pain in cases of systemic inflammatory joint diseases [28-31]. Inflammation results in tissue response as: vasodilatation, extra vasation, release of mediators, activation of nociceptors, release of neuro peptides as substance P (SP), neuropeptide Y (NPY), which stimulate release of inflammatory mediators like histamine and serotonin and hyperalgesia.

11.4.1. Capsulitis and synovitis

Inflammation of the capsular ligament may manifest with swelling and continuous pain localized to the joint. Movements that stretch the capsular ligament cause pain with resultant limitation of such movement. Significant inflammation may increase joint fluid volume. When this occurs, one may see an ipsilateral posterior open bite (lack of contact between maxillary and mandibular teeth) secondary to inferior displacement of the condyle [7]. Similarly, inflammation due to trauma or abnormal function may affect the retrodiscal tissue. Edema in this area may cause anterior displacement of the condyle and an acute malocclusion with painful limitation of mandibular movements.

The highly innervated and vascularized synovial membrane digests debris and pain mediators released from cartilage degradation. When this ability is overwhelmed, inflammation (acute synovitis) results. Inflammation of the synovial membrane is an early sign of DJD [32]. Inflammatory and pain mediators have been identified in TMJ synovial fluid [33,34]. Chemical breakdown of degenerative byproducts is thought to stimulate the production of inflammatory and pain mediators (PGE2 and LkB4, among others) through the arachidonic acid cascade. PGE2 is a powerful vasodilator and LkB4 attracts inflammatory cells. Their presence creates acute synovitis pain and stimulates further damage from cytokines and proteases. For this reason arthrocentesis and arthroscopy for joint lavage and lysis of adhesions are believed to have a

therapeutic effect [35-37]. These procedures remove particulate debris and pain mediators, aiding reduction of joint inflammation and pain. Results are similar with and without disc repositioning [35]. Lysis of adhesions may improve range of motion. Steroid injections are also used to reduce synovial inflammation and pain. Recent investigations have looked at intra-articular morphine for sustained pain relief in patients [37]. Research is now focusing on the role of biochemical mediators in the development and progression of TMJ pain and dysfunction [19,33] and the identification of biochemical “markers” of TMJ disease [14].

11.4.2. The artritides

Arthritis of the TMJ has many etiologies: frequently OA and RA and less often infectious, metabolic (gout), or immunologic (ankylosing spondylitis, lupus). DJD, also known as OA, has a multifactorial pathogenesis including biomechanical, biochemical, inflammatory, and immunologic insults. Excessive and repetitive mechanical stress has been implicated [19]. Inflammatory mediators and waste products may play a role in DJD [34-49]. Inflammatory states cause changes in the viscosity of synovial fluid, which changes its ability to nourish the articular cartilage, thus changing cartilage metabolism.

11.5. POTENTIALS SOLUTIONS TO THE TEMPOROMANDIBULAR JOINT PROBLEM THROUGH BIOMATERIALS

11.5.1. Temporomandibular joint and regeneration scaffolds

The craniofacial structure consists of bone, cartilage, soft tissue, nerves, and blood vessels. Acquired defects after cancer surgeries, trauma as well as congenital or developmental deformities require a reconstructive procedure as the bones of the craniofacial region support the rest of the elements. The procedures used today for temporomandibular reconstruction are mostly autologous, allogenic, or alloplastic, with variable clinical outcomes and morbidities [50]. Distraction histogenesis has emerged as a possible alternative to regenerate ramus condyle unit [51].

Regeneration using osteoconductive scaffolds, osteoinductive growth factors, committed progenitor cells and stem cells is being investigated by researchers and surgeons alike [52]. Osteoconduction and osteoinduction are very important features for bone tissue scaffolds. Osteoinduction implies the process of the conversion of non-osseous cells into bone forming cells, whereas osteoconduction is the process by which implanted scaffold supports the bone growth [53].

The ideal bone construct for repair should be able to replicate the lost structure, restore function, be harmless, reliable and biodegradable *i.e.* should degrade during the process of tissue regeneration and replaced with fully functional tissue.

11.5.2. Scaffolds: general introduction

A crucial requirement for joint repair is that the scaffolding should be attached to the cartilage lesions and should integrate with the tissue. Not only this but also the attachment must balance temporary mechanical function with mass transport to aid biological delivery and tissue engineering. In addition to being patient specific, the scaffolds should facilitate cell attachment and regulate cell differentiation. Also they must be biodegradable, with nontoxic by-products, and exhibit favourable resorption kinetics to maintain initial stability.

The materials can be divided into natural or synthetic, based on the sources. Natural scaffolds may be subdivided into [54] protein based matrices such as collagen and fibrin [55], mineral-based matrices such as autogenous, allogenic and xenogenic bone grafts, and [56] carbohydrate-based matrices such as alginate, agarose, chitosan (CTS), and hyaluronan. Synthetic materials have been used extensively both *in vitro* and *in vivo*. They include poly(lactic acid) (PLA), poly(glycolic acid) (PGA), polycaprolactone (PCL) and their derivatives, for example poly(lactic-co-glycolic acid) (PLGA). The synthetic materials have been popular because of their easy moulding characteristics, relatively easy production, and the ability to control dissolution and degradation. However, their major weakness is biocompatibility. They are degraded by a hydrolytic reaction, thereby high concentrations of acidic by-products and particulates can be released, causing inflammation, giant cell reaction and chondrocyte death owing to a reduction in pH 50.

11.5.3. Biomaterial for scaffolds

The major materials used in craniofacial tissue engineering are natural and synthetic polymers, ceramics, composite materials, and electrospun nanofibers [57]. Biomaterial to be used as a scaffold must possess sufficient mechanical strength, large pore volumes and pore interconnectivity to allow continuous tissue in growth, and transport properties to allow the influx of nutrients and elimination of waste products [58]. Randomly positioned pores contribute to better cell seeding and better cell aggregation in the designed scaffolds [59]. Natural scaffolds like collagen type I, CTS, calcium alginate, hyaluronic acid, and composites have been shown to be osteoconductive, but with problems like lack of mechanical strength when implanted, risk of infection, immunogenicity, and rapid degradation rate [60-65].

Bone contains 85 % calcium phosphate, hence ceramics such as hydroxyapatite (HAp), tricalcium phosphate (TCP), and composites such as biphasic calcium phosphate (BCP), have been widely investigated for bone scaffolds. The HA

ceramics are well suited as biomaterials because of their biocompatibility, not eliciting an inflammatory response, lack of immune reaction, and easy radiographic assessment. TCP demonstrates a too fast degradation rate *in vivo*, whereas HA degrades too slowly, is not resorbed, and resides in the defect for several years after callus formation. BCP has more favorable degradation rates compared with TCP and HA. However, the problem with use of ceramics is their brittleness which makes them mechanically inadequate for load bearing [65-67].

Polymers include poly(ethylene glycol) (PEG), PGA, poly-L-lactic acid and poly(D-lactic acid) (PLA), PLGA, PCL, poly-urethanes, and composites. Polymers are flexible and biodegradable through their hydrolysis or by means of cellular or enzymatic pathways when implanted. Polymers have low mechanical strength and hence are often combined with high-modulus micro or nanoscale ceramic constituents like HA.

11.6. INTELLIGENT FUNCTIONAL BIOMATERIALS

11.6.1. Hydrogels as carrier molecules

The idea of developing a drug that selectively destroys diseased cells without damaging healthy cells was proposed by the Nobel Prize winner Paul Ehrlich, almost a century ago. He called this hypothetical drug the “magic bullet” [68]. Today, several decades later, many scientists have focused their attention on the development of ideal drugs that specifically target the site of action. Such a targeted drug-delivery system needs three components: a therapeutic agent, a targeting moiety, and a carrier system. The choice of the carrier molecule is of high importance, since it can significantly affect the pharmacokinetics and pharmacodynamics of the drugs [69,70]. Hydrogels are natural polymers that have been widely used as carrier systems into which; the drug can be incorporated by passive absorption or chemical conjugation. Hydrogels are biocompatible hydrophilic three-dimensional matrices that can act as drug carriers and protectors, especially for peptide and protein-based drugs. Because of their hydrophilic characteristics, hydrogels have improved bioadhesive properties, which make them suitable carrier materials for sustained topical drug-delivery systems for targeted treatment of some of the oral diseases [71,72].

11.6.2. Interpenetrating polymeric network hydrogels as a topical drug-delivery system in the oral environment

Multicomponent drug-delivery systems have found several potential diagnostic and therapeutic applications. Among these, the interpenetrating polymeric network (IPN) has emerged as one of the most useful functional biomaterial. IPNs are entanglement of polymers with at least one network

synthesized and / or cross-linked in the presence of the other. They are ideally held together only by a permanent topological interactions [73], presenting a three-dimensional network structure that provides free volume space for an easy encapsulation of drugs.

Many researchers suggest that specific IPN characteristics, such as, non-separable network, adhesive property, high-tensile strength and biocompatibility enabled the enhancement of the implementation of these hydrogels. Furthermore, IPNs are distinguishable from blends, block copolymers, and graft copolymers for two important reasons; firstly, an IPN swells but does not dissolve in solvents, and secondly, the creep and flow is suppressed. Because of its distinguishable features, these biocompatible, nontoxic and biodegradable polymers are acquiring a unique place in various biomedical applications, such as, cartilage scaffolds, biological tissue graft, tissue engineering, wound dressing and for drug delivery [74-76].

IPNs can be prepared for different applications and by using various matrices such as poly-urethane, polybutadiene, methacrylic acid, L-lysine, glutamic acid, poly(vinyl alcohol), carboxymethyl cellulose, poly(acrylic acid), gelatin, poly(vinylpyrrolidone), alginate, dextran, xanthane, guar-gum, CTS, PEG, HAp, etc. [77-83].

The newly designed hydrogel system, bioactive-functionalized interpenetrating network (BIOF-IPN) hydrogel, presented in this chapter represents a novel interplay between bio-molecular scaffold designs with functionality. The system optimizes the use of the host : guest science playing an important role as a “build-in” free radical defense mechanism, and acting as a “proof of concept” for the functional multi-dimensional restorative repair materials. This chapter reports the preliminary investigations of the BIOF-IPN utilizing a combination of materials such as CTS in the restorative and regenerative dental field specifically aimed at TMJ *in vitro* evaluations [84-93].

11.6.3. Chitosan

CTS is a natural, biodegradable, non-toxic, mucoadhesive and biocompatible polymer that has garnered immense attention in the pharmaceutical field, inclusive as a vehicle for oral drug-delivery. The suitability of CTS based IPN hydrogels stems from their capability of imbibing large amount of body fluid without solubilisation, great potential for encapsulation of a large amount of drugs, and adaptability to be combined with specific responsive polymer(s).

Furthermore, it presents good biocompatibility, low toxicity and unique biological and physical-chemical characteristics [94]. The primary reactive functional groups (hydroxyl and amine groups) located on the backbone of CTS allow for chemical modification to control its mechanical and solubility properties. When a hydrophobic moiety is conjugated to a CTS molecule, a chemically cross-linked three-dimensional nanoparticle is formed that can physically (entanglement) or chemically, encapsulate a number of drugs. When

applied to a specific site of action, the immobilized active agents or biomolecules are released in a well-defined and precise manner [95].

CTS is metabolized by certain human enzymes, especially lysozyme, and is considered biodegradable [96]. It is also a non-immunogenic, non-carcinogenic and mucoadhesive polymer, which makes it a suitable candidate for biomedical applications, such as wound management, tissue engineering and drug delivery vehicle [97-101]. CTS as well shows permeation enhancer properties, and it can improve the paracellular route of absorption, which is important for the transport of hydrophilic compounds such as therapeutic peptides and antisense oligonucleotides across the membrane [102-104].

CTS has generated considerable interest as a bioadhesive material. The interactions are strong at acidic and slightly acidic pH levels in oral environment, at which the charge density of CTS is high, and an increase in the molecular weight of CTS results in a stronger adhesion [105]. CTS also displays additional biological properties *viz.*, regenerative effect on connective gingival tissue, accelerates formation of osteoblast, is haemostatic, fungistatic, spermicidal, antitumor, anticholesteremic and immune adjuvant [106].

11.6.4. Temporomandibular joint bioengineering: general introduction

Ideal engineered constructs for mandibular condyle regeneration must have integrated bone and cartilage layers in a single osteochondral construct to meet the demands for anatomic, structural, and functional regeneration. The challenge in TMJ bioengineering is to promote matrix synthesis and tissue maturation of stem-cell-derived chondrogenic and osteogenic cells in biocompatible and bioactive scaffolds, which may be possible by incorporating an array of growth factors and/or transcription factors separately for chondrogenesis and osteogenesis. The mechanical properties of the tissue-engineered mandibular condyle must match with that of an anatomic condyle for *in situ* implantation into the human TMJ. Also, the tissue-engineered mandibular condyle must have a remodeling potential [107].

11.6.5. Temporomandibular joint disc bioengineering up to date summary

The earliest such study was performed in rabbit disc where cultured cells were used in collagen I meshes [108]. Later hyaline cartilage was engineered in the shape of a human TMJ disc [109]. Four years later, Girdler harvested hyaline cartilage cells along with chondroprogenitor cells and cultured them to form disc [110]. Recently, human and porcine disc cells have been cultured in 2 dimensions on expanded poly(tetrafluorethylene) monofilaments, PLA monofilaments, polyamide mono-filaments, and natural bone mineral blocks [111].

Recent studies have identified that a scaffold of non-woven PGA mesh in combination with cell seeding technologies, could provide an engineered disc [112]. Three growth factors: insulin-like growth factor-I, basic fibroblast growth factor and transforming growth factor- β 1 have been assessed in maintaining disc-like tissue in culture [113,114].

In another study, a scaffold material composed of porcine-derived extracellular matrix, configured to mimic the shape and size of the TMJ, was implanted in a canine model of bilateral TMJ discectomy. The results showed the formation of site-appropriate, functional host tissue resembling native TMJ disk [115].

Poly(glycerol sebacate) (PGS), a biocompatible, biodegradable elastomer, was used as a porous scaffold material for the TMJ disc, where goat fibrochondrocytes were seeded at three seeding densities (25, 50, 100 million cells mL⁻¹ scaffold), respectively, and cultured. The results showed that cell seeding density and culture time, both effect the biochemical and biomechanical properties of PGS scaffolds. The findings demonstrated PGS as a favorable scaffold material for TMJ disc engineering [116].

11.6.6. Chitosan/gelatin/hydroxyapatite scaffolds as potential biomaterials for hard tissue regeneration: *in vitro* approach

Bones are rigid organs that support and protect various organs of the body. Repair techniques for bones with defects or loss due to disease, trauma or tumor resections are the subject of intensive research [117]. Bone grafts – a well recognized and standard treatment method for reconstructive orthopedic surgery [118] – employ three types of bone or tissue substitution: allograft, autograft and xenograft. Allografts transplant bone or soft tissue from one individual to another in the same species. Allografts have many advantages, including osteoinduction and strong mechanical properties, but carry the risk of disease transmission from the donor, such as HIV, hepatitis or cancer. Autografts, or autologous bone transplants, transplant bone tissue from one site to another in the same individual. This graft offers excellent biocompatibility and does not stimulate host inflammatory response. However, the procedure may cause long lasting pain and discomfort for the patient. Furthermore, there is additional risk of wound infection at the surgery sites [119]. Xenografts remove cells or sections of tissue from one species and graft them on or into a different species. Bovine bone [120] and mollusk shell [121] are common materials used in xenografts. However, the bioactive properties of xenografts are weaker than allografts and autografts. To improve xenografts, a new treatment technique has been introduced for bone or tissue repair called bone tissue engineering – a procedure to regenerate damaged bone by implanting cells, proteins and scaffold to provide mechanical support for gap areas [122]. Bone substitute morphology has many forms, such as a compact and porous structure. As bone replacements, a compact structure provides good mechanical strength while a porous structure is suitable for cell

attachment and blood supply [123]. Bone tissue engineering creates a biological material that provides the option for implantation and/or prosthesis. Bone tissue engineering has three main requirements: osteoconductive biomaterial scaffolds, osteogenic cells and osteoinductive molecules [124]. Materials widely used in bone tissue engineering include CTS, gelatin and HA. CTS is a polysaccharide that can be synthesized from crustacean shell and squid pen. CTS's structure is similar to glycosaminoglycans, the major component of the extracellular matrix of bone and cartilage [125,126]. Gelatin can be obtained by thermal denaturation and chemical degradation of collagen [127], and is known to benefit cell viability [128]. In the meantime, HA has a chemical composition similar to human mineral tissue and can be synthesized from many natural sources with calcium-based structures, such as bovine bone, mollusk shell and coral [129-131]. In combination, these three biomaterials offer potential synergies between physical properties and bioactivity for use as bone substitutes in bone grafts, benefiting a range of surgical applications.

11.6.7. Chitosan/hydroxyapatite for bone/hard tissue engineering

Research on biomaterials for bone implantation and replacement has expanded considerably over the last four decades. In recent years, significant progress has been made in organ transplantation, surgical reconstruction and the use of artificial prostheses to treat the loss or failure of an organ or bone tissue. The establishment of a load bearing biomaterial must be incorporated with natural bone. The implanted biomaterial should possess the following criteria: biocompatibility, osteoconductivity, high porosity and biomechanical compatibility [132]. For this requirement, autografts and allografts are used extensively for bone grafts. In the autograft technique, bone from another part will be harvested within the body, and this material fills the gap and provides optimal osteoinductivity, osteoconductivity and osteogenic properties. However, it has its own disadvantages: autografting often leads to complications in wound healing, additional surgery, donor pain and an inadequate supply of bone to fill the gap [133]. The main problem of the allograft technique has been linked with rejection problems and therefore use of immunogenic natural 572 polymer composite materials are becoming increasingly important as scaffolds 573 for bone tissue engineering. Next generation biomaterials should combine bioactive and bioresorbable materials, which mimic the natural function of bone and activate *in vivo* mechanisms of tissue regeneration. Composite materials based on combinations of biodegradable polymers and bioactive ceramics, including CTS and HAp, are discussed as suitable materials for scaffold fabrication. These composites exhibit tailored physical, biological and mechanical properties as well as predictable degradation behavior. The appropriate selection of a particular composite for a given application requires a detailed understanding of relevant cells and/or tissue response. An overview of these findings is

presented and discussed in this review, highlighting the influence of material preparation methods, scaffold mechanical strength, *in vitro* activity of scaffold materials and chemical interaction with CTS polymer matrixes. The chapter also emphasizes future artificial bone materials, suggesting the utility of polymer composites in this field of biomaterials science. The limitations and concerns, such as adverse rejection reactions and the risk of acquiring transmissible diseases (AIDS and hepatitis) from tissues and fluids, have created substantial interest in the development of artificial materials as bone graft substitutes [134]. Very few compounds are classified as bioactive, biodegradable and osteoconductive. CTS and HAp are among the best bioactive biomaterials in bone tissue engineering and renowned for their excellent biocompatibility with the human body environment [135].

Various marine sources polysaccharides have been used for treatment of bone diseases like osteoporosis [136], arthritis [137], and so on. In order to create a moist environment for rapid wound healing, a hydrogel sheet composed of a blended powder of alginate, chitin/CTS and fucoidan has been developed as a functional wound dressing [138].

11.6.8. Composite materials for bone tissue engineering

Composite materials are now playing predominant role as scaffolds in bone tissue engineering. CTS has numerous advantageous properties for orthopedic applications, as described above and elsewhere [139], which make it ideal as a bone graft substituent. CTS scaffolds are flexible and their mechanical properties are inferior to those of normal bone, as it is unable to support load bearing bone implants. Moreover, CTS itself is not osteoconductive, although addition of ceramic materials improves its osteoconductivity and mechanical strength. CTS scaffolds alone cannot imitate all the properties of natural bone. The substantial development of composite materials with CTS mimics all the properties of bone. As proven, calcium phosphate materials are osteoconductive to mimic the inorganic portion of natural bone, while CTS / HAp composite materials show promise in mimicking the organic portion as well as the inorganic portion of natural bone (Figure 1). Several studies have been conducted with CTS/HAp composite materials for bone tissue engineering [140-145]. Calcium phosphate compounds are of great interest in the field of bone tissue engineering. HAp $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$ is one of the most stable forms of calcium phosphate and it occurs in the bone as a major component (60–65 %) [146]. HAp also possesses a variety of uses, including orthopedic, dental and maxillofacial applications. Therefore, HAp has recently emerged as an important compound for artificial bone preparation. It stimulates osteoconduction being gradually replaced by the host bone after implantation. It is being used for orthopedic replacements, especially in bone regeneration and dental implant treatment. The mechanical properties of HAp are poor, though, so it cannot be used for load bearing bone tissues. Polymers have been used to improve the mechanical properties of HAp (compressive

strength, Young's modulus, fracture toughness) [147]. When CTS is combined with HAp, it might be able to mimic the function of natural bone.

Figure 1. Chemical interaction between HAp and CTS

11.6.9. Carbon nano tubes for bone tissue engineering

Carbon nanotubes (CNTs) are allotropes of carbon with a cylindrical nanostructure and constructed with length-to-diameter ratio of up to 28,000,000 : 1. These cylindrical carbon molecules have novel properties, which make them potentially useful in many applications in nanotechnology, electronics, optics and materials sciences. CNTs have a high Young's modulus (1.0–1.8 TPa), high tensile strength (30–200 GPa) and high elongation at break (10–30 %). In addition, they have extremely small size (about 1–10 nm in diameter), high aspect ratio (> 1,000), high structural and chemical stability, and stiffness, as well as remarkable electrical, thermal, optical and bioactive properties [148,149]. All these properties make CNTs especially promising candidates as reinforcement fillers in the development of nano composites. It has been observed that the combination of CNT with CTS leads to an enormous increase in the mechanical strength of the composite [149].

CNTs hold great interest with respect to biomaterials, particularly those to be positioned in contact with bone such as prostheses for arthroplasty, plates or screws for fracture fixation, drug delivery systems, and scaffolding for bone regeneration. The most important concerns for the use of CNTs as a biomaterial are tissue safety, but only few reports have addressed the toxicity of CNTs. In particular, bone tissue compatibility is extremely important for using CNTs in biomaterials. Usui *et al.*, who first developed CNT, found that these tubes have good bone tissue compatibility and are capable of permitting bone repair and becoming closely integrated with bone tissue and accelerate bone formation stimulated by recombinant human bone morphogenetic protein-2 [150].

A big challenge in the bone tissue engineering is mechanical strength improvement of the scaffold materials. One of the main purposes of creating CTS composites is to improve the mechanical strength of the material. CNT is a promising material to fulfill that gap due to its strong mechanical properties. Several authors have developed many composites as well as scaffold materials with CNT. It has been observed that cell adhesion on multi-walled CNT (MWCNT) coated dish is much higher than that on the collagen coated dish [151].

Our approach to the complexity of the developing suitable model for the *in vitro* TMJ prototype model to investigate excessive free radical generation and harnessing of the imbalance comes from a different angle: developing functional CTS based hydrogel system [84-94]. CTS is a natural cationic polysaccharide derived from chitin, with the structural composition being heavily influenced by the pH factors. The unique property of the CTS lies in its ability to form a controlled biofunctional interface with the dentin, enamel, restorative materials, as well as to the skin and other oral tissues. This process occurs through a favorable chemical and structural compatibility of the bioactive material (Figure 1). The biointeractive tissue influenced properties of CTS and its derivatives highlight an opportunity for development of dual functional restorative materials such as BIOF-INPs. The important points to address in developing the *in vitro* model to evaluate the amount of TMJ damaged caused by the oxidative stress as well as promote the repair of the fibrous cartilage and provide specific antimicrobial defense as well as pain management "*in situ*" and assess the cytotoxicity of the new materials lead us to design and investigate specific class of hydrogels and the preliminary results are summarized in the Figure 2.

Figure 2. Summary of the performance of designer bioactive materials investigated in our group. a. SEM images of the prepared biomaterials. b. SEM images of the active hydroxyapatite surface exposed to artificial saliva. c. Microbiological evaluation of the prepared biomaterials. d. Cytotoxicity investigation summary of functionalized biomaterials. e. and f. Drug delivery capacity of the biomaterials evaluated in the study and evaluation of dentin regeneration capability of the materials *in vitro*.

In summary the Figure 2 represents the scanning electron microscope (SEM) of the typical BIOF-INPs material designed and used in the study. The SEM images of the reactive surface of the dentin (as model of fibrous hard tissue) structure after application of the hydrogen (Figure 2b immediately after application. Sample is kept in the artificial saliva for the entire time. The undamaged mouse 3T3 fibroblast cell(s) after application of the BIOF-INPs in the Figure 2a and damaged mouse 3T3 fibroblast cell(s) after application of the standard material in the Figure 2c. The example of *Staphylococcus aureus* inhibition zones and application of the therapeutic agent containing BIOF-INPs. Cumulative release of the Naproxen as a model therapeutic component of BIOF-IPN as summarized in the Figure 2e. The SEM images of the reactive surface of the dentin structure after application of the hydrogen (Figure 2d immediately after application. Sample is kept in the artificial saliva for the entire time).

Present results demonstrate the capability of the BIOF-INPs to play an important role in the functional multi-dimensional therapeutic restorative repair materials.

11.7. CONCLUSION AND FUTURE DIRECTIONS

This chapter brings together the advances in basic material science, biotechnology and designer functional materials with the specific aim of exploiting molecular mechanistic understanding of TMJ disorders with particular emphasis on the harnessing of excess of free radicals *via* functional biopolymers. The advantage of the intelligent designer functional materials represents amalgamation of molecular design, mechanism, bioanalytical advancement, molecular biology and ability to easily refocus the aim of the problem at hand make these materials only limited by imagination, creativity and multidimensional science linking scientist, health professionals and will only continue to progress when the recent advances will cross successfully the barrier from *in vitro* molecular design to *in vivo* application.

REFERENCES

1. M.C. Fletcher, J.F. Piecuch, S.E. Liebllich, in *Peterson's Principles of Oral and Maxillofacial Surgery*, 2nd Ed., M.M. Hamilton (Ed.), BC Decker, Ontario, USA, 2004, pp. 933–947.
2. D.W. Howerton, M. Zysset. *Oral Maxillofac. Surg. Clin. North Am.* **1** (1989) 229–247.
3. D.M. Laskin. *J. Oral Maxillofac. Surg.* **56** (1998) 121.
4. D.M. Laskin. *Oral Maxillofac. Surg. Clin. North Am.* **7** (1995) 73–78.
5. S.F. Dworkin, L. LeResche. *J. Craniomandib. Disord.* **6** (1992) 301–355.
6. O.E. Ogle, M.B. Hertz. *Oral Maxillofac. Surg. Clin. North Am.* **12** (2000) 217–231.

7. *Fundamentals of Occlusion and Temporo-mandibular Disorders*, J.P. Okeson (Ed.), C.V. Mosby, St. Louis, USA; 1985.
8. C.S. Stohler, in *Oral and Maxillofacial Surgery. Temporomandibular Disorders*, R.J. Fonseca et al. (Eds.), WB Saunders, Philadelphia, USA, 2000, pp. 38–45.
9. S.B. Milam, in *Oral and Maxillofacial Surgery. Temporomandibular Disorders*, R.J. Fonseca et al. (Eds.), WB Saunders, Philadelphia, USA, 2000, pp. 46–72.
10. D.M. Laskin. *Oral Maxillofac. Surg. Clin. North Am.* **5** (1994) 217–222.
11. P.L. Westesson, L. Eriksson, K. Kurita. *Oral Surg. Oral Med. Oral Pathol.* **68** (1989) 551–554.
12. C.H. Wilkes. *Arch. Otolaryngol. Head Neck Surg.* **115** (1989) 469–467.
13. C.S. Greene, D.M. Laskin. *J. Am. Dent. Assoc.* **117** (1988) 461–465.
14. S.B. Milam, J.P. Schmitz. *J. Oral Maxillofac. Surg.* **53** (1995) 1448–1454.
15. P.H. Chan. *J. Cereb. Blood Flow Metab.* **21** (2001) 2–14.
16. M.K. Irmak, E. Fadillioglu, M. Güleç. *Cell Biochem. Funct.* **20** (2002) 279–283.
17. M.M. Yaser, M.M. Randa, A. Belacy. *J. Pharm. Biomed. Anal.* **26** (2001) 605–608.
18. A. Pompella. *Int. J. Vitam. Nutr. Res.* **67** (1997) 289–297.
19. H. Sumii, H. Inoue, J. Onoue, A. Mori, T. Oda, T. Tsubokura. *Hiroshima J. Med. Sci.* **45** (1996) 51–55.
20. A.B. Holmlund, S. Axelsson. *Int.J.Oral Maxillofac. Surg.* **25**(3) (1996) 266–271.
21. R. Emshoff. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol. Endod.* **100**(4) (2005) 409–414.
22. U. Voog, P. Alstergren, S. Eliasson, E. Leibur, R. Kallikorm, S. Kopp. *Acta Odontol. Scand.* **61**(1) (2003) 57–64.
23. U. Voog, P. Alstergren, S. Eliasson, E. Leibur, R. Kallikorm, S. Kopp. *Acta Odontol. Scand.* **62**(1) (2004) 7–13.
24. R.H. Tallents, D.J. Macher, S. Kyrkanides, R.W. Katzberg, M.E. Moss. *J. Prosthet. Dent.* **87**(1) (2002).
25. S.J. Kim, Y.H. Park, S.P. Hong, B.O. Cho, J.W. Park, S.G. Kim. *J. Oral Maxillofac. Surg.* **61**(10) (2003) 1156–1161.
26. D.I. Paegle, A.B. Holmlund, M.R. Östlund, L. Grillner. *J. Oral Maxillofac. Surg.* **62**(4) (2004) 435–439.
27. E. Leibur, O. Jagur, P. Mürsepp, L. Veede, Ü. Voog-Oras. *J. Cranio-Maxillofac. Surg.* **38**(8) (2010) 615–620.
28. P. Alstergren, S. Kopp, E. Theordosson. *Acta Odontol. Scand.* **57**(1) (2003) 278–282.
29. A. Kamada, K. Kakudo, T. Arika, J. Okazaki, M. Kano, T. Sakaki. *J. Cranio-MaxilloFac. Surg.* **28**(3) (2000) 247–248.
30. P. Alstergren, S. Kopp. *Pain* **72**(1-2) (1997).
31. U. Voog, P. Alstergren, E. Leibur, R. Kallikorm, S. Kopp. *Life Sci.* **68**(5) (2000) 591–602.
32. L.G.M. de Bont, G. Boering, R.S.B. Liem, F. Eulderink, P.-L. Westesson. *J. Oral Maxillofac. Surg.* **44** (1986) 634–643.
33. B. Stegenga, L.G.M. de Bont, G. Boering. *J. Oral Maxillofac. Surg.* **47** (1989) 249–256.
34. J.W. Nickerson, G. Boering. *Oral Maxillofac. Surg. Clin. North Am.* **1** (1989) 27–43.
35. B. Stegenga, L.G.M. de Bont, G. Boering, J.D. Van Willigen. *J. Oral Maxillofac. Surg.* **49** (1991) 1079–1088.

36. H.A. Israel, C.J. Langevin, M.D. Singer, D.A. Behrman. *J. Oral Maxillofac. Surg.* **64** (2006) 1066–1074.
37. J.H. Quinn. *Oral Maxillofac. Surg. Clin. North Am.* **1** (1989) 47–57.
38. S. Kopp. *J. Oral Maxillofac. Surg.* **56** (1998) 189–191.
39. S.B. Milam. *Oral Maxillofac. Surg. Clin. North Am.* **12** (2000) 5–26.
40. J.J. Moses, D. Sartorius, R. Glass, T. Tanaka, I. Poker. *J. Oral Maxillofac. Surg.* **47** (1989) 674–678.
41. D.W. Nitzan, M.F. Dolwick, M.W. Heft. *J. Oral Maxillofac. Surg.* **48** (1990) 798–801.
42. J.P. McCain, B. Sanders, M.G. Koslin, J.D. Quinn, P.B. Peters, A. Thomas Indresano. *J. Oral Maxillofac. Surg.* **50** (1992) 926–930.
43. P.A. Brennan, V. Ilankovan. *J. Oral Maxillofac. Surg.* **64** (2006) 949–951.
44. S.B. Milam, G. Zardeneta, J.P. Schmitz. *J. Oral Maxillofac. Surg.* **56** (1998) 214–223.
45. T. Shibata, K.I. Murakami, E. Kubota, H. Maeda. *J. Oral Maxillofac. Surg.* **56** (1998) 209–213.
46. L.C. Dijkgraaf, G. Zardeneta, F.W. Cordewener, R.S.B. Liem, J.P. Schmitz, L.G.M. de Bont, S.B. Milam. *J. Oral Maxillofac. Surg.* **61** (2003) 101–111.
47. J.H. Quinn, in *Modern Practice in Orthognathic and Reconstructive Surgery*, W.H. Bell (Ed.), WB Saunders, Philadelphia, USA, 1992, pp. 471–481.
48. L.C. Dijkgraaf, L.G.M. de Bont, G. Boering, R.S.B. Liem. *J. Oral Maxillofac. Surg.* **53** (1995) 1182–1192.
49. L.C. Dijkgraaf, S.B. Milam, in *Oral Maxillofacial Surgery Knowledge Update, vol 3*, J.F. Piecuch (Ed.). Rosemont, IL: American Association of Oral and Maxillofacial Surgeons, 2001, pp. 5–28.
50. L.B. Kaban, C. Bouchard, M.J. Trellis. *J. Oral Maxillofac. Surg.* **67** (2009) 1966–1978.
51. D. Mehrotra, A.L. Chellappa, C. Gupta, D. Passi, S. Kumar. *J. Oral Biol. Craniofac. Res.* **2**(3) (2012) 144–148.
52. V. Petrovic, P. Zivkovic, D. Petrovic, V. Stefanovic. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol.* **114** (2012) e1–e9.
53. J. Glowacki, J.B. Mulliken. *Plast. Reconstr. Surg.* **92** (1993) 601.
54. M.I. Gray, A.M. Pizzanelli, A.J. Grodzinsky, R.C. Lee. *J. Orthop. Res.* **6** (1988) 777–792.
55. H.A. Israel. *Oral Maxillofac. Surg. Clin. North Am.* **1** (1989) 85–92.
56. W.Y. Yih. *Oral Maxillofac. Surg. Clin. North Am.* **1** (1989) 93–109.
57. H.K. Kleinman, D. Philp, M.P. Hoffman. *Curr. Opin. Biotechnol.* **14** (2003) 526–532.
58. B.B. Ward, S.E. Brown, P.H. Krebsbach. *Oral Dis.* **16** (2010) 709–716.
59. A.C. Jones, C.H. Arns, D.W. Hutmacher, B.K. Milthorpe, A.P. Sheppard, M.A. Knackstedt. *Biomaterials.* **30** (2009) 1440–1451.
60. J. Malda, T.B. Woodfield, F. van der Vloodt, F.K. Kooy, D.E. Martens, J.C. Tramper, C.A. van Blitterswijk, J. Riesle. *Biomaterials* **25** (2004) 5773–5780.
61. X. Li, Q. Feng, X. Liu, W. Dong, F. Cui. *Biomaterials* **27** (2006) 1917–1923.
62. D.M. Cruz, M. Gomes, R.L. Reis, D. Moratal, M. Salmerón-Sánchez, J.L. Gómez Ribelles, J.F. Mano. *J. Biomed. Mater. Res. A* **95** (2010) 1182–1193.
63. K. Tanaka, T. Goto, T. Miyazaki, Y. Morita, S. Kobayashi, T. Takahashi. *J. Dent. Res.* **90** (2011) 906e911.

64. T. Fujii, T. Anada, Y. Honda, Y. Shiwaku, H. Koike, S. Kamakura, K. Sasaki, O. Suzuki. *Tissue Eng. Part A*. **15** (2009) 3525–3535.
65. J.R. Porter, T.T. Ruckh, K.C. Popat. *Biotechnol. Prog.* **25** (2009) 1539–1560.
66. H. Yoshimoto, Y.M. Shin, H. Terai, J.P. Vacanti. *Biomaterials* **24** (2003) 2077–2082.
67. L. Ciocca, D. Donati, M. Fantini, E. Landi, A. Piattelli, G. Iezzi, A. Tampieri, A. Spadari, N. Romagnoli, R. Scotti. *J. Biomater. Appl.* **28**(2) (2012) 207–218.
68. K. Strebhardt, A. Ullrich. *Nat. Rev. Cancer*. **8**(6) (2008) 473–480.
69. S.L. Kitson, D.J. Quinn, T.S. Moody, D. Speed, W. Watters, D. Rozzell. *Chim. Oggi*. **31**(4) (2013) 30–36.
70. Y. Miao, M. Hylarides, D.R. Fisher, T. Shelton, H. Moore, D.W. Wester, A.R. Fritzberg, C.T. Winkelmann, T. Hoffman, T.P. Quinn. *Clin. Cancer Res.* **11**(15) (2005) 5616–5621.
71. H.R. Maecke, J.C. Reubi. *J. Nucl. Med.* **52**(6) (2011) 841–844.
72. R. Hooda, M. Tripathi, K. Kapoor. *The Pharma Innovation* **1**(1) (2011) 14–21.
73. J.D. Smart. *Adv. Drug Deliv. Rev.* (1993) 11253–11270.
74. J. Hao, P.W. Heng. *Drug Dev. Ind. Pharm.* **29**(8) (2003) 821–832.
75. F. Winau, O. Westphal, R. Winau. *Microbes Infect.* **6**(8) (2004) 786–789.
76. V. Prajapati, M. Bansal, P. Sharma. *Int. J. Pharmtech. Res.* **4**(2) (2012) 582–589.
77. R. Mohammad, in *Biopolymers*, M. Elnasahar (Ed.), Intech, Sciyo, Rijeka, Croatia, 2010, pp. 575–596.
78. V. Bansal, P. Sharma, N. Sharma, O. Pal, R. Malviya. *Adv. Biol. Res.* **5**(1) (2011) 28–37.
79. J.H. Park, G. Saravanakumar, K. Kim, I.C. Kwon. *Adv. Drug Deliv. Rev.* **62**(1) (2010) 28–41.
80. J. Balcerzak, M. Mucha. *PCACD* (2010) 15117–15125.
81. V.T. Perchyonok, S. Zhang, T.G. Oberholzer. *Open Nanosci. J.* (2013) 71–77.
82. V.T. Perchyonok, I. Lykakis, A. Postigo, *Streamlining free radical green chemistry*, The Royal Society of Chemistry, Cambridge, UK, 2012.
83. V.T. Perchyonok, S. Zhang, T.G. Oberholzer. *Curr. Nanosci.* **8**(4) (2012) 541–547.
84. V.T. Perchyonok, S. Zhang, T.G. Oberholzer. *Curr. Org. Chem.* (2011) 162437–162439.
85. V.T. Perchyonok, S. Zhang, T.G. Oberholzer. *Curr. Drug Deliv.* **10**(1) (2013) 144–150.
86. V.T. Perchyonok, V. Reher, S. Zhang, S.R. Grobler, T.G. Oberholzer, W. Massey. *OJST* **4**(2) (2014) 73–83.
87. V.T. Perchyonok, S. Zhang, T.G. Oberholzer. *Curr. Org. Chem.* (2012) 162430–162436.
88. V.T. Perchyonok, S. Zhang, S.R. Grobler, T.G. Oberholzer, W. Massey. *Eur. J. Gen. Dent.* **3**(1) (2014) 57–65.
89. V.T. Perchyonok, S. Zhang, N.J. Basson, S.R. Grobler, T.G. Oberholzer, W. Massey. *Adv. Tech. Biol. Med.* **2**(1) (2014).
90. S.R. Grobler, A. Olivier, V.T. Perchyonok, D. Moodley. *Int. J. Dent. Oral Sci.* (2014) (*In press*).
91. V.T. Perchyonok, S. Zhang, N.J. Basson, S.R. Grobler, T.G. Oberholzer, W. Massey. *OJST* **4** (2014) 99–108.
92. V.T. Perchyonok, S. Zhang, S.R. Grobler, T.G. Oberholzer, W. Massey. *Eur. J. Gen. Dent.* **3**(1) (2014) 57–65.

93. V.T. Perchyonok, S. Zhang, S.R. Grobler, T.G. Oberholzer. *Eur. J. Dent.* **7**(4) 2013 412–418.
94. C.K. Sen, G.M. Gordillo, S. Roy, R. Kirsner, L. Lambert, T.K. Hunt, F. Gottrup, G.C. Gurtner, M.T. Longaker. *Wound Repair Regen.* **17** (2009) 763–771.
95. M.N. Kumar, R.A. Muzzarelli, C. Muzzarelli, H. Sashiwa, A.J. Domb. *Chem. Rev.* **104** (2004) 6017–6084.
96. M.J.C. Fresno, A.D. Ramírez, M.M. Jiménez. *Eur. J. Pharm. Biopharm.* **54** (2002) 329–335.
97. V.M. Ndesendo, V. Pillay, Y.E. Choonara, R.A. Khan, L. Meyer, E. Buchmann, U. Rosin. *Int. J. Pharm.* **370** (2009) 151–159.
98. D.S. Jones, A.D. Woolfson, A.F. Brown, W.A. Coulter, C. McClelland, C.R. Irwin. *J. Control. Release* **67** (2000) 357–368.
99. H. Blanco-Fuente, S. Anguiano-Igea, F.J. Otero-Espinar, J. Blanco-Méndez. *Int. J. Pharm.* **142** (1996) 169–174.
100. X. Qi, R.F. Tester. *J. Pharm. Pharm. Sci.* **14** (2011) 60–66.
101. R.G. Riley, J.D. Smart, J. Tsibouklis, P.W. Dettmar, F. Hampson, J.A. Davis, G. Kelly, R.W. Wilber. *Int. J. Pharm.* **217** (2001) 87–100.
102. K.R. Kamath, K. Park, in *Encyclopedia of Pharmaceutical Technology*, vol. 10, J. Warbrick, J.C. Boylan (Eds.), Marcel Dekker, New York, USA, 1994, pp. 133–163.
103. H.S. Ch'ng, K. Park, P. Kelly, J.R. Robinson. *J. Pharm. Sci.* **74** (1985) 399–404.
104. E.E. Hassan, J.M. Gallo. *Pharm. Res.* **7** (1990) 491–498.
105. A. Maltese, A. Borzacchiello, L. Mayol, C. Bucolo, F. Maugeri, L. Nicolais, L. Ambrosio. *Biomaterials* **27** (2006) 5134–5142.
106. M.J. Colthurst, R.L. Williams, P.S. Hiscott, I. Grierson. *Biomaterials* **21** (2000) 649–665.
107. J.J. Mao, W.V. Giannobile, J.A. Helms, S.J. Hollister, P.H. Krebsbach, M.T. Longaker, S. Shi. *J. Dent. Res.* **85**(11) (2006) 966–979.
108. H. Abukawa, H. Terai, D. Hannouche, J.P. Vacanti, L.B. Kaban, M.J. Troulis. *J. Oral Maxillofac. Surg.* **61** (2003) 94–100.
109. Y. Weng, Y. Cao, C. Arevalo, M.P. Vacanti, C.A. Vacanti. *J. Oral Maxillofac. Surg.* **59** (2001) 185–190.
110. M.M. Bailey, L. Wand, C.J. Bode, K.E. Mitchell, M.S. Detamore. *Tissue Eng.* **13** (2007) 2003–2010.
111. S. Zhu, B. Zhang, C. Man, Y. Ma, J. Hu. *Osteoarthr. Cartil.* **19** (2011) 743–750.
112. H. Kang, Y.D. Bi, Z.Q. Li, M.Y. Qi, E.M. Peng. *Chinese J. Stomatol.* **46** (2011) 541–546.
113. W.C. Puelacher, J. Wisser, C.A. Vacanti, N.F. Ferraro, D. Jaramillo, J.P. Vacanti. *J. Oral Maxillofac. Surg.* **52** (1994) 1172.
114. N.M. Girdler. *Scand. J. Rheumatol.* **27** (1998) 446.
115. I.N. Springer, B. Fleiner, S. Jepsen, Y. Açil. *Biomaterials* **22** (2001) 2569.
116. A.J. Almarza, K.A. Athanasiou. *Tissue Eng.* **10** (2004) 1787–1795.
117. O. Gauthier, R. Müller, D.V. Stechow, B. Lamy, P. Weiss, J.M. Boulter, E. Aguado, G. Daculsi. *Biomaterials* **26**(27) (2005) 5444–5453.
118. M. Mehta, K. Schmidt-Bleek, G.N. Duda, D.J. Mooney. *Adv. Drug Deliv. Rev.* **64** (2012) 1257–1276.
119. J. Venkatesan, Z.J. Qian, B. Ryu, K.N. Ashok, S.K. Kim. *Carbohydr. Polym.* **83**(2) (2011) 569–577.

120. N.A.M. Barakat, M.S. Khil, A.M. Omran, F.A. Sheikh, H.Y. Kim. *J. Mater. Process. Technol.* **209**(7) (2009) 3408–3415.
121. K.S. Vecchio, X. Zhang, J.B. Massie, M. Wang, C.W. Kim. *Acta Biomater.* **3**(6) (2007) 910–918.
122. X. Wang, J.S. Nyman, X. Dong, H. Leng, M. Reyes, *Fundamental Biomechanics in Bone Tissue Engineering*, K.A. Athanasiou (Ed.), Morgan & Claypool, LaVergne, TN, 2010.
123. X. Cai, H. Tong, X. Shen, W. Chen, J. Yan, J. Hu. *Acta Biomater.* **5**(7) (2009) 2693–2703.
124. N. Vachiraroj, J. Ratanavaraporn, S. Damrongsakkul, R. Pichyangkura, T. Banaprasert, S. Kanokpanont. *Int. J. Biol. Macromol.* **45**(5) (2009) 470–477.
125. W.W. Thein-Han, R.D.K. Misra. *Acta Biomater* **5**(4) (2009) 1182–1197.
126. Z. Li, H.R. Ramay, K.D. Hauch, D. Xiao, M. Zhang. *Biomaterials* **26**(18) (2005) 3919–3928.
127. S. Panzavolta, M. Fini, A. Nicoletti, B. Bracci, K. Rubini, R. Giardino, A. Bigi. *Acta Biomater.* **5**(2) (2009) 636–643.
128. H.W. Kim, H.E. Kim, V. Salih. *Biomaterials* **26**(25) (2005) 52.
129. H.W. Kim, H.E. Kim, V. Salih. *Biomaterials* **26**(25) (2005) 5221–5230.
130. K.S. Vecchio, X. Zhang, J.B. Massie, M. Wang, C.W. Kim. *Acta Biomater.* **3** (2007) 910–918.
131. N.A.M. Barakat, K.A., Khalil, F.A. Sheikh, A.M. Omran, B. Gaihre, S.M. Khil, H.Y. Kim. *Mater. Sci. Eng. C* **28** (2008) 1381–1387.
132. A. Di Martino, M. Sittinger, M.V. Risbud. *Biomaterials* **26** (2005) 5983–5990.
133. Q. Hu, B. Li, M. Wang, J. Shen. *Biomaterials* **25** (2004) 779–785.
134. S. Teng, E. Lee, B. Yoon, D. Shin, H. Kim, J. Oh. *J. Biomed. Mater. Res. Part A* **88** (2009) 569–580.
135. F. Chen, Z. Wang, C. Lin. *Mater. Lett.* **57** (2002) 858–861.
136. Y. Zhang, J.R. Venugopal, A. El-Turki, S. Ramakrishna, B. Su, C.T. Lim. *Biomaterials* **29** (2008) 4314–4322.
137. C. Xianmiao, L. Yubao, Z. Yi, Z. Li, L. Jidong, W. Huanan. *Mater. Sci. Eng. C* **29** (2009) 29–35.
138. L. Kong, Y. Gao, W. Cao, Y. Gong, N. Zhao, X. Zhang. *J. Biomed. Mater. Res. Part A* **75A** (2005) 275–282.
139. I. Manjubala, I. Ponomarev, I. Wilke, K. Jandt. *J. Biomed. Mater. Res. Part A Appl. Biomater.* **8** (2008) 7–16.
140. W. Thein-Han, R. Misra. *Acta Biomater.* **5** (2009) 1182–1197.
141. J. Oliveira, M. Rodrigues, S. Silva, P. Malafaya, M. Gomes, C. Viegas, I. Dias, J. Azevedo, J. Mano, R. Reis. *Biomaterials* **27** (2006) 6123–6137.
142. S. Ding. *J. Non-Cryst. Solids* **353** (2007) 2367–2373.
143. I. Yamaguchi, K. Tokuchi, H. Fukuzaki, Y. Koyama, K. Takakuda, H. Monma, J. Tanaka. *J. Biomed. Mater. Res.* **55** (2001) 20–27.
144. S.-K. Kim, E. Mendis. *Food Res. Int.* **39** (2006) 383–393.
145. S. Nath, A. Dey, A.K. Mukhopadhyay, B. Basu. *Mater. Sci. Eng. A* (2009) 513–514, 197–201.
146. P. Ajayan, O. Zhou. *Carbon Nanotubes* **80** (2001) 391–425.
147. S. Samal, S. Bal. *J. Miner. Mater. Character. Eng.* **7** (2008) 355–370.
148. S.F. Wang, L. Shen, W.D. Zhang, Y.J. Tong. *Biomacromolecules* **6** (2005) 3067–3072.

149. Y. Usui, K. Aoki, N. Narita, N. Murakami, I. Nakamura, K. Nakamura, N. Ishigaki, H. Yamazaki, H. Horiuchi, H. Kato. *Small* **4** (2008) 240.
150. M. Terada, S. Abe, T. Akasaka, M. Uo, Y. Kitagawa, F. Watari. *Dental Mater. J.* **28** (2009) 82–88.

**FROM THE MACROSCALE TO
NANOSTRUCTURES: CAN TISSUE
ENGINEERING RECREATE BONE FEATURES?**

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12.1. BONE BIOLOGY

Skeleton plays several important roles in the human body: while it houses and protects from injuries critical internal organs and tissues (*e.g.* brain, heart, lungs, bone marrow and spinal cord), it provides, as well, body mechanical support, movements and locomotion capability. Bones supply insertion sites for ligaments and muscles and, acting as levers, permit, through the joints, the transmission of muscular forces between the different parts of the body. Besides the role of internal framework, human skeleton has also crucial metabolic functions, which include those of mineral storage and maintenance of calcium homeostasis, endocrine regulation of chemical energy (by the release of osteocalcin), acid-base balance, and blood cells production (hematopoiesis processes) that takes place in the bone marrow niche [1].

According to their localization, bones origin from three lineages: the somites, the lateral plate mesoderm, and the cranial neural crest. Bone tissue can be formed following two main pathways, which are represented by intramembranous ossification (for some skull, facial and pelvic bones), or by endochondral ossification (mainly in appendicular and axial skeleton, and in some cranial bones). Whereas both processes embrace mesenchymal precursors as origin of bones, they differ in the characteristics of osteogenesis: during intramembranous ossification, bone progenitors directly differentiate into osteoblasts; on the other hand, during endochondral ossification, mesenchymal cells aggregate, and further differentiate into chondrocytes, up the more differentiated states, called hypertrophied- and post-hypertrophied chondrocytes, that form a mineralized template for the ingrowth of newly differentiated osteoblasts, before dying by apoptosis [2]. Gradually, cartilage/bone become greatly supplied with blood. Bone is, in effect, a highly vascularized tissue, and this aspect is crucial to bear bone development, to maintain bone homeostasis and to support bone repair, since blood and endothelial cells serve as suppliers of nutrient, oxygen, hormones and growth factors, and for the elimination of wastes and of the main toxic end products. Osteoclasts are also recruited from blood precursors in the site of bone formation for remodeling the extracellular matrix (ECM) of the newly synthesized bone into a solid bone matrix. Osteoclasts attach to the bone, dissolve the mineral matrix and, subsequently, the organic matrix, to let free hole for osteoblasts to deposit organic matrix to form osteoid [3-5]. The mineralization of this bone ECM begins several days after osteoblasts have deposited the collagenous matrix. The mineralization process, as well as the osteoblast differentiation, are, at least partially, coordinated by the gap junction-mediated intercellular communication (GJIC), and, more particularly, by the connexin 43 (cx43) component [6]. The biomineralization process is a complex of events that, besides the fine regulation of inorganic phosphate/pyrophosphate balance and the calcium/phosphate supply, is

controlled by a variety of mechanisms. These mechanisms include: chemical effectors (*e.g.* ECM, with its proteins, serves as an activator, and as a source / reservoir of growth and differentiating factors, and enzymes cofactors), physical stimuli (*e.g.* mechanic and electrical forces created by the interactions between cells and cell-matrix), morphological features and structural aspects (cyto-architecture and tissue geometry, known to be involved in the regulation of gene expression), and by the peculiar characteristics of the microenvironment (*e.g.* proteolytic enzymes, collagen, and matrix vesicles) [7,8]. Other important paracrine regulators of bone homeostasis are present in the bone microenvironment, and include bone morphogenetic proteins (BMPs, osteogenic factors), fibroblasts growth factors (FGFs, mitogen and angiogenic factors), insulin like growth factor (IGF, osteogenic factor), Wnt (mitogen and osteogenic factor), Indian hedgehog proteins (Ihh), or hormonal regulators of osteogenesis, such as 1,25 Dihydroxy-vitamin D (anabolic effect and promotion of osteogenic differentiation), parathyroid hormone-related peptide (PTHrP), estrogens (inhibitors of bone resorption), androgens (inhibitors of bone turnover and resorption, and promoters of a subsequent increased in trabecular and cortical bone mass), calcitonin (inhibitor of osteoclasts secretions, and activator of osteoblasts differentiation), growth hormone (GH), and glucocorticoids (activators of bone resorption) [9-12]. Moreover, 1,25-Dihydroxy-vitamin D3 was also shown to stimulate osteoblast for synthesizing vascular endothelial growth factor (VEGF) and, consequently, endothelial cells. Some membrane-associated proteoglycans are supposed to control osteogenesis by interacting as co-receptors with growth factors [13]. In the end, it is important to keep in mind that, in addition to cell-ECM interface, cell-cell interactions are essential for bone formation and maturation. Besides the GJIC, that directly joins two juxtaposed cells and allows the direct exchange of molecular signaling from cytoplasm to cytoplasm, cadherins play a key role in regulating cell condensation, osteogenesis and bone mass [14-17]. Deletion of cx43 in knockout (KO) mice induces a delay in ossification and craniofacial abnormalities [18]. Concerning cadherins, the “block” of *N*-cadherin (by a specific inhibitor or by an antibody) results in the decrease of adherens junctions between osteoblasts, and in a down-regulation of osteoblastic gene expression [19].

Bone is constantly remodeled, in order to better face the daily physical activity, which is often accompanied by “microcracks”. To perform such turnover, a basic multicellular unit – comprising bone cells, chemical factors (*i.e.* growth factors, chemokines, cytokines, systemic hormones), cell adhesion molecules and bone ECM – is essential to allow bone resorption (by osteoclasts), and consecutive bone formation (by osteoblasts). Moreover, the activity of this unit is regulated by numerous local and systemic parameters, such as physical conditions, electric charges and mechanical loads. Also microcapillaries were shown to be involved in the bone remodeling process: they serve either as

intermediators between the bone marrow and the bone surface to be remodeled, or for recruiting bone cell progenitors in the remodeling site [20]. A delicate equilibrium exists between bone building and bone resorption, but, due to the difference in their kinetics, even a minor imbalance *versus* resorption can result in dramatic consequences.

12.1.1. Bone: a composite biomaterial

Bones are particularly dense connective tissues, where the specific ECM represents about the 92–95 % of the whole mass. Bone ECM consists of two phases: organic (32 % of volume, and about 40 % of bone dry weight) and inorganic (43 % of volume, and about 60 % of bone dry weight) [21,13]. ECM is responsible for guiding cells behavior by furnishing chemical, physical, and topological instructions. Moreover, aqueous phase is also very important for bone homeostasis, since the loading–unloading cycles induce interstitial flows and pressure gradients [22]. This hydrodynamic flow – and others resulting forces (*i.e.* matrix/cell strains, shear stress, hydrostatic forces and electrical fields) – regulate the behavior of mechanosensing cells, such as osteocytes, and, furthermore, because of the high mass transfer generated around the cells, they also bring nutrient, hormones, minerals and oxygen to all bone components. Concerning osteocytes, Klein-Nulend and co-workers also hypothesized that this cell type could also sense loading stimuli thanks to its capability to adjust its own morphological configuration, and the distribution of cytoskeletal proteins, according to the mechanical loading pattern it is subjected to [23,24].

The organic components of bone ECM include structural fibrillar proteins, such as collagen (type I collagen represents about the 90 % of the organic part of bone, while type V and type III collagens are present in a few amount), and elastin, that are embedded in an amorphous matrix, made of non-collagenous proteins. The most abundant non-collagenous protein is osteonectin (ONN), a SPARC matricellular protein (SPARC, Secreted Protein Acidic and Rich in Cysteine); important are also fibronectin (FN), thrombospondin, osteocalcin (OCN) and matrix GLA protein, two gamma-carboxyglutamic acid-containing proteins, fibrillin-1 and -2, alkaline phosphatase (ALP), glycosaminoglycans (GAGs), and proteoglycans (hyaluronan, versican, decorin and biglycan) [25,7]. Furthermore, bone ECM also comprises cell surface receptors involved in cell attachment, and integrins, which interact with the Arg-Gly-Asn (RGD) sequence of ECM proteins (type I collagen, thrombospondin, FN, *etc.*). These interactions between integrins and ECM, play a crucial role in the osteoblastic differentiation process [26]. Osteopontin (OPN) and bone sialoproteins (BSPs) are also involved in the binding of osteoclasts to bone EMC. Collagen orientation and its organization as lamellar material are responsible for bone properties, and for its flexibility and tensile strength, whereas little is known regarding the role of non-collagenous proteins to this concern. ECM proteoglycans are known for their osmotic capacity, that, in response to the

application of mechanical forces, allow the diffusion of nutrients and gases within bone tissue; the mineralization process extremely decreases this movement of molecules (and cells) [13]. GAGs containing heparin sulfate are, for example, involved in the role of ECM reservoir, since they bind growth factors by non-covalent links.

The inorganic component of bone ECM represents the mineral phase of this tissue, and, mainly, comprises phosphate and carbonate of calcium, crystals of hydroxyapatite (HA), pyrophosphate, di-phosphate and phosphate esters. It also includes other ions, such as magnesium, sodium, fluoride, zinc, manganese, and iron. The inorganic component of ECM contributes to the compressive strength, as well as to the stiffness of bone.

Lamellar bones are made of two different components. The main material is represented by orientated type I collagen fibrils, assembled in well-ordered arrays, and including proteoglycans and non-collagenous proteins. In these arrays, HA is within collagen fibrils. The second material is disordered, and comprises individual collagen fibrils, without ordered-arrangement, proteoglycans, non-collagenous proteins, and an ample ground mass. This more randomly distributed material includes HA crystals inside and outside of the fibrils, and incorporates canaliculi and osteocytes' processes [27].

12.1.2. Bone properties

The anisotropic and mechanical properties of bones are related not only to their 3D structure, but also to their composition, and to the arrangement and the linkage between their different molecular components. For example, the mechanical properties of bone change, according to the deposition of apatite / HA crystals [28]; they also depend on bone porosity. The porosity of bones greatly varies according to the site, and to the type of bone: it is comprised between 5–30 % in the compact bone, whereas it is about 30–90 % in the trabecular one [29]. Moreover, bone characteristics can vary from bone to bone (anatomical location), but also within the same bone (bone region), according to age, gender, nutritional state, state of health, calcium availability, sedentary vs. intense physical activities, loading vs. unloading [30]. Finally, bone mass is regulated by leptin, that interacts with its receptor on hypothalamic neurons, and inhibits bone formation (probably by a serotonin-dependent mechanism/s), and should be further mediated by the β_2 adrenergic receptor [3,31,32]. Ghrelin, known to counteract leptin action, was shown to regulate bone mass by activating osteoblasts and, thus, bone building [33,34].

Mechanical loading is transduced from mechanosensing cells (osteoblasts and osteocytes) into a physiological input, that allows the regulation of bone mass, homeostasis, healing and repair. Focal adhesion kinase (FAK) is believed to be important in mediating mechanotransduction in bone cells, at least when the stimulus arises from shear stress [35]; however, Castillo and co-workers seem

to indicate that FAK activation could be important only in *in vitro* systems, rather than in the *in vivo* situation [36]. Other membrane structures sense mechanical stimuli. They include cell glycocalyx, adherens-junctions, integrins, connexins (mainly cx43), and stretch-activated cation channels (L-type voltage-sensitive Ca^{2+} channels) [37,38]. Mechanical loading stimulates bone formation either directly, by activating osteoblasts and inducing their differentiation, or indirectly, after osteocytes' activation [39]. Mechanical loading also induces the synthesis of prostaglandins, ALP and type I collagen. Mechanical strain was shown to play a crucial role in bone homeostasis and repair. *In vitro*, MC3T3-E1 osteoblastic cells, stimulated by growing tensile strains, increased the mRNA synthesis of osteoprotegerin (OPG), an inhibitor of the osteoclastogenesis, while decreased receptor activator of nuclear factor kappa B ligand (RANKL) expression [40], thus suggesting that mechanical strain can regulate osteoclastogenesis and, at a higher scale, bone modeling / remodeling. The main molecular mediators involved in mechanotransduction are related to nitric oxide (NO), Wnt and prostaglandins pathways. Mechanical stretching and cell tension were also shown as potentially more potent than chemical signals to guide cell behavior and even stem cell commitment [41,42]. The mechanotransduction from loading to physiological response of bone, *in vivo* or *in vitro*, is regulated at different levels, from cytoskeleton, cell shape and elasticity, and it is also related to the Wnt/ β -catenin signaling pathway [43,44]. *In vitro*, mechanical stimuli (oscillatory fluid flow) seem to increase osteogenic differentiation of mouse bone marrow (BM)-derived stem cells, at least by epigenic processes (DNA methylation) [45].

Bone stiffness and load-bearing strength are mainly determined by the inorganic components of bone and, more particularly, by the concentration of minerals in the ECM. On the contrary, the organic phase of bone is responsible for the elasticity and flexibility properties of the tissue. The Tensile Strength (TS) is provided by collagen, according to its organization, its orientation, and to the distance between the fibrils; the resistance is also related to matrix organization and collagen cross-links [46]. From the organic matrix, proteoglycans are responsible for the resistance to compressive forces.

12.1.3. Bone cell types

Whereas in the bone marrow cells such as fibroblasts, adipocytes, progenitor cells and stem cells can also be found, the typical bone cells are represented by osteoblasts, osteocytes, osteoclasts, and by the resting surface cells. Bone is a dynamic tissue in a permanent renewal and remodeling state. Such a renew / adaptation state is allowed, in normal adult bones, by a delicate coupling between osteoclast-mediated resorption activity and osteoblast-mediated bone formation. Bone remodeling depends on mechanical and biological / chemical cues.

Osteoblasts arise from pluripotent mesenchymal precursors, and are highly specialized cells, involved in bone building. Osteoblastogenesis is under the control of hormones and cytokines (parathyroid hormone, prostaglandins, insulin-like growth factor-1 (IGF-1), transforming growth factor beta (TGF- β), and interleukin 11 (IL11)), as well as under that of Wnt/ β -catenin pathway, Twist family factors and BMPs [47-49]. Osteoblastic differentiation from precursors is inhibited by the macrophage colony stimulating factor (M-CSF) [50], while runt-related transcription factor 2 (RUNX2) (controlled by the osterix proteins activator of bone formation) represents its main transcriptional up-regulator [51]. Osteoblasts are responsible for the synthesis of bone ECM (type I collagen, fibronectin (FN), ALP, osteopontin (OSP), osteocalcin (OSC), and bone sialoproteins (BSPs)). The regulation of collagen deposition by osteoblasts (orientation, localization, and speed of build-up of fibrils) involves their cytoskeleton, and it is important for the further mineralization of the ECM and, thus, for the mechanical properties of the bone. When ECM becomes mineralized, osteoblasts continue to differentiate and to synthesize ALP and, consecutively, OSC and OSP. By mediating receptor activator of nuclear factor (NF)- κ B ligand (RANKL) and OPN synthesis, osteoblasts play also a fundamental role in regulating osteoclastogenesis [52].

During the elaboration of the new bone, some osteoblasts remain entrapped into the ECM, and terminally differentiate into osteocytes. *In vitro*, such a differentiation process was shown to depend on the ECM stiffness, and on the distance between the osteoblastic cells (MC3T3-E1 line) [53,54]. Osteocytes are non-proliferating cells, and represent the most abundant cell type of the bone (more than the 90 % of the total number of bone cells). They are embedded in spaces called *lacunae*, and are interconnected by means of their canalicular systems, forming, therefore, a syncytial network. This organization also allows osteocytes to communicate between themselves, and with the other bone cell types, in part also thanks to the efficiency of the gap junction-mediated communication (GJIC) and to the presence of functional junctional hemichannels [55]. Osteocytes can interplay with other cells by direct cell-cell contact, or, indirectly, by paracrine factors [56,57]. They are responsible for the osteoclastic activity during bone repair. Osteocytes are mechanosensing cells: thanks to their long cell processes, they form a network that conducts in the canalicular system the transductive information resulting from loading or other mechanical stimuli (strain detection). To sense biomechanical stimuli, osteocytes processes are anchored to the bone ECM, at least *via* β 3 integrins; the interaction between ECM molecules and integrins could then amplify their mechanosensing capacity [58]. It seems that, when osteocytes undergo apoptotic process in response to different physiological events (unloading, estrogen deficiency, fatigue cracks, *etc.*), neighborhood osteocytes transfer specific signals in order to recruit osteoclasts. Moreover, osteocytes express sclerostin (a Wnt antagonist protein), and, by this way, they can control, in part, bone formation. The shape of both osteocytes and their *lacunae* influence

the mechanosensing process, and, then, the correlated processes of bone remodeling and repair [23,59-61].

Osteoclasts are multinucleated cells, derived from hematopoietic stem cells, and resulting (under the control of the macrophage colony-stimulating factor, M-CSF) from the differentiation of the macrophage / monocytes precursor cells at the bone surface. They are responsible for the resorption of bone, performed by acidification and further dissolution of the bone matrix in the “sealing zone”, characterized by a particular specialization of the osteoclast membrane [62]. Osteoclasts also secrete proteases (*e.g.* cathepsin K and matrix metalloproteinases, MMPs) and tartrate-resistant acid phosphatase (TRAP) that actively disrupt the collagen framework. Osteoclastogenesis depends on different crucial factors. First of all, osteoclasts differentiation cannot occur without their interaction with other bone cells (mainly osteoblasts, and bone marrow or stromal cells) [7,63,64]. In addition, the chemotactic recruitment of osteoclasts, and their further development, are supported by several cytokines and hormones, such as interleukines (IL1, IL6, IL11), the receptor RANK (receptor activator of NF- κ B factor), the colony stimulating factors-1 (CSF-1), proteins from the tumor necrosis factor superfamily (TNF α , RANKL), parathormone (PTH), 1,25-(OH) $_2$ -Vitamin D, and calcitonin [7,65,66]. RANKL is crucial for the osteoclastogenesis and for the survival and function of osteoclasts [67]. The alternative NF- κ B pathway, and, mainly, the p100 component, was also shown to be crucial for osteogenesis [68]. On the other hand, OPG inhibits the activation of the NF- κ B pathway. Osteoclasts activity is controlled by PTH, 1,25(OH) $_2$ vitamin D, calcitonin, cytokines and chemokines [69]. The complex and numerous functions of osteoclasts have been attractively reviewed by Cappariello and co-workers [70], who present in detail the involvement of osteoclasts in bone renewal and in osteoblastogenesis processes, in the regulation of calcemia and phosphatemia, in cooperating with the immune system and regulating angiogenesis, and in maintaining the homeostasis of the hematopoietic niche.

12.1.4. Bone hierarchy, structure and topology

Bone acts as a multiscale biocomposite, with a well-organized structure distinguishable at seven different hierarchic levels. These include: I) the macrostructural level (> 1 cm), consisting in the division between cortical and trabecular arrangement of bone structure; II) the mesoscale level (2–10 μ m), which is represented by the intrinsic *lamellae* network; III) the micrometer scale level (10–500 μ m), composed of Haversian systems (or osteons), and single *trabeculae*; IV) the sub-microscale level (1–10 μ m), formed by single Haversian canals or single *lamellae*; V) the nanometer scale level ($< 0,1$ μ m), comprising collagen fibers and mineral phase; VI) the sub-nanostructural level ($< \text{few nm}$), formed by collagen fibrils. The VII) and smallest length scale of the bone hierarchy, consists of the ultrastructural level, and comprises molecular constituents, such as minerals, water, collagen molecules, and non-collagenous

organic proteins [71-74]. All these structures and components intimately interact altogether in order to give bone its biomechanical properties.

At the macrostructural level, most of bones appear formed by two different structures: the outer one, consisting of compact bone, while the inner one made of cancellous bone, which represent about the 80 % and the 20 % of the skeleton mass, respectively [75]. Bones interface with other bones, muscles, joints, ligaments and tendons. On its outer side, cortical bone is covered by the *periosteum* (except for the epiphysis region, where it is coated by articular cartilage), whereas in correspondence to the medullar cavity, it is delimited by the *endosteum*. The mature bone is characterized by its lamellar organization. Whereas in the trabecular bone, *lamellae* do not form an organized structure, in the compact bone *lamellae* are parallelly organized. Trabecular bone is organized as a network of plate-like and rod structures. The microstructure of the trabecular part of the bone is composed of thin *trabeculae*, made of dense and sinuous *lamellae*, which form a 3 dimensional complex that holds the bone marrow. Trabecular bone is present, for example, at the epiphyses of long bones. On the other hand, the compact bone is the dense part of bone tissue, and, at the microscale level, it is composed of regular, cylindrically shaped *lamellae*, arranged around the Haversian canals. Compact bone is mainly localized in the diaphysis of long bones, and blood is mainly supplied throughout the Volkmann's canals. Haversian and Volkmann's canals form a continuous network, where metabolic and gas exchange, as well as waste removal, can take place. The osteon (chief structural unit of compact bone) also includes osteocytes and the network of their relative cell processes. Cortical and trabecular bones are found in almost all bones, but in a ratio that differs according to the site, the localization, and the type of bones. The topics treated in this chapter will be mainly focused on long bones characteristics.

The microscopic level of bone structural hierarchy concerns the biocomposites of type I collagen fibrils / fibers, more or less mineralized. Bone ECM can be classified according to the organization of collagen fibers it is composed of (immature bone, with its unorganized collagen fibers that formed the woven bone; mature bone, composed of collagens organized as concentric *lamellae*, that formed the osteons of the lamellar bone). Only the mature bone present high resistance properties, due to ECM geometry, organization and composition.

The scale ranging from molecular- to nanometer-sized component, comprises, as already indicated, the molecules of type I collagen, and their associated HA nano-crystals. The conformation, arrangement and deposition of collagen, together with the intra-collagen fiber orderliness of HA minerals, are responsible for the mechanical properties of bones. Concerning apatite crystals, they influence the mechanical properties of bone, according to their shape, their organization, their orientation and their nanometer-scale thickness [76-78].

12.2. BONE TISSUE ENGINEERING

When bone requires repairing, a complex scenario takes place, including a number of physiological events such as inflammation, healing by endochondral and intramembraneous ossification, and remodeling (Figure 1).

Figure 1. Bone injury, the facts. *Healthy bone* is characterized by a complex macro-, micro- and nano- environment, where cells, as well as humoral factors, play a crucial role in preserving bone homeostasis. The process of *bony healing* is complex, and requires a spatio-temporal combination of events to reach an effective bone repair.

When bone is unable to counteract tissue damage, *bone tissue engineering* could represent an effective therapeutic strategy, if performed by respecting some basal principles.

Bone healing involves a complex microenvironment (Figure 2), composed of a network of cells (osteoblasts, osteoclasts and osteoclasts precursors, endothelial progenitors, lymphocytes, fibroblasts and mesenchymal progenitors, macrophages), growth/differentiating factors, chemokines, cytokines, signaling molecules, mineral elements and ECM macro-, micro-, and nano-components [79,80]. Furthermore, this multifactorial process is characterized by an orchestrated succession of events that includes mediators of inflammation, cell chemiotaxis, migration, proliferation, differentiation,

mechanosensing and tissue remodeling. The existence or the development of adequate blood flow is also essential to sustain full bone development, homeostasis and repair [81,82]. Moreover, the unsuccessful penetration of vasculature into the bone graft also leads to unsatisfactory repair and even bone necrosis. Thus, factors involved in *in vivo* angiogenesis [VEGF, basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), and angiopoietin-1 (Ang-1)] could be fundamental for supporting bone repair, also in the field of orthopaedic tissue engineering. Endothelial cells can regulate osteoblasts functions by secreting other growth factors, such as endothelin-1 and IGFs [83]. Furthermore, also mechanical stimuli and loading are of crucial importance to improve bone healing.

the Actors:

Figure 2. Bone healing, the actors. In order to heal, bone needs strict crosstalks between all the actors of the skeletal micro-environmental network. Cells (stem or mature cells), ECM (with its soluble and insoluble components), and mediators (autocrine, paracrine or humoral chemical signals) are the main actors of bone healing, and their specificity (and intervention timing) must be recognized, in order to optimize the efficacy of tissue engineering-based treatments.

Even if the more traditional treatment of bone defects is, first, represented by the autologous graft and, secondly, by bone allograft or distraction osteogenesis, several drawbacks can be encountered. These include, for

example, the insufficient size of bone to be grafted (as in the case of large bone defects due to tumor resection, trauma or infection), or when the host site of injury is altered by avascular necrosis, or by a pronounced osteoporosis. Other obstacles arise when blood and cell supply in the wound-healing site is too scarce, if the osteointegration is incomplete, in the case of donor site morbidity, or in the presence of an adverse immune response. To overcome such limits, the tissue-engineering field offers the possibility to amplify cells number *in vitro*, and, successively, to use them in order to generate a tissue substitute, ultimately disposable for graft. Alternatively, acellular scaffolds can be directly implanted in the site of injury, awaiting its colonization by host cells. As for scaffolds carrying living cells before they are implanted into bone defects, also these acellular scaffolds can be manufactured by demineralized matrix [84], natural or synthetic polymers, biocomposites, ceramics, and, often, they need to be functionalized in order to optimize local cell ingrowth, proliferation and differentiation (Figure 3).

the Scenarios:

Figure 3. Tissue engineering-based orthopedics strategies. If a scaffold is used, orthopedics can also choose to introduce (and model) a scaffold, free of cells, in the site of bone injury. Bone repair should be observed after the colonization of this scaffold by local cells and precursors. Alternatively, bone cell precursors/stem cells can be amplified *in vitro*, guided to colonize the scaffold (3D culture gives better results) and, finally, the cellularized scaffold can be implanted in the site of injury. These processes can be supported by a supplement of specific chemical mediators, previously enclosed in the scaffold (“functionalized” scaffold).

12.2.1. What are the specific requirements of skeletal tissue engineering *in vivo* and *in vitro*?

Tissue engineering is intended for developing tissue substitutes, able to restore, maintain and/or increase tissue functions *in vivo* [85]. To reach this objective, two main key factors should be taken into account: i) the scaffold, that will serve as physical support, and as supplier of molecular factors able to induce cell proliferation and/or to guide cells into specific lineages of differentiation, and ii) the cells, that must invade the scaffold and produce the neo-tissue. For the *in vitro* development of engineered tissue, a bioreactor, able to support neo-tissue formation and growth, is also necessary. The scaffolds produced by tissue engineering for regenerative medicine aims, must be, obviously, biocompatible and sterilizable, in order to avoid any induction of inflammatory and/or immune response. Concerning, bone tissue engineering, the main crucial points are summarized in Figure 4.

Figure 4. Bone tissue engineering: an example of translation medicine from bedside to bench prior the return to the boosted bedside part. As shown in Figures 1 & 2, bone healing involves number of actors, and all of them need to be taken into account during the whole tissue engineering procedure to better mimic the *in vivo* conditions. Thus, regarding bone engineering, the main parameters, that must be finely defined to reproduce as much as possible the *in vivo* situation, are the cells (origins, their history, their commitment *etc.*), the bioreactors, the optimal microenvironment (where osteoconduction, osteoinduction, osteointegration properties of the scaffold are promoted by the induction of the optimal biosignalling pathways, and the reproduction of the native bone physical, chemical, topographical and biological cues).

They include the interaction of cells, usually precursors of bone tissue or endothelium, with an adequate microenvironment. This last comprises a scaffold, harboring various chemical, physical, and mechanical properties, as well as bioactive molecules, involved in the recruitment and differentiation of cells (in the scaffold and/or in the site of implantation). Lastly, particular growth and angiogenic factors can be used, in order to recruit endothelial cells, and to further favor bone formation and survival, since nutrient and gas supply can be performed by diffusion processes only up to a tract of 200 μm [86-88].

12.2.1.1. Scaffolds

The ideal scaffold for bone tissue engineering should mimic the native macroscopic bone organization, as well as the bone microenvironment and features, since these conditions allow the natural cell recruitment, adhesion, migration, proliferation and differentiation. As already mentioned above, bone cells' behavior is finely regulated by ECM properties (biochemical, physical, geometrical, and topographical aspects). The surface of biomaterials must, then, possess well-defined characteristics, since substrate chemistry, tension and topography play a fundamental role in regulating cell behavior and bone tissue features [89,90]. The perfect scaffold (Figure 5) usable for bone tissue engineering, has, in addition, to possess mechanical properties close to those of the *in vivo* tissue, shall recapitulate its 3D architecture, must favor cell adhesion / attachment, osteoconduction (related to the capacity of biomaterial surface to allow cell growth), osteoinduction (related to the capacity of biomaterials to guide stem and bone precursor cells toward the osteogenic pathway of differentiation), and cell functions [91]. Osteoconduction, osteoinduction and the further peri-bioengineered-implant healing, as well as osseointegration, are fundamental for regenerative orthopaedic procedures. Along with the 3D organization, it is necessary to take into account also the need of specific bone growth factors, which, in addition, must be released gradually, according to a well-defined spatio-temporal schema. Moreover, because of the need of nutrients and gas supply, a scaffold favoring an early angiogenesis in the neo-tissue is desirable. In effect, co-cultures of osteoblasts (or osteoblastic precursors) with endothelial cells (or endothelial precursors) have been shown to favor cell communication mediated by gap junctions (GJIC) between these cell types, and to support osteoblastic differentiation [92 93]. Kim and co-workers [94] confirmed this support for bone formation after the seeding of adipose-derived MSCs and human umbelical vein endothelial cells (HUVEC) in solid free form-based poly(ϵ -caprolactone)(PCL) / poly(lactic-co-glycolic acid) (PLGA) / tricalcium phosphate (TCP) scaffolds. After 8 and 12 weeks of implantation in a rat calvaria defect, the implants with co-seeded cells reinforce osteogenesis, bone-like formation, and mineral deposition, as compared to monotypic (osteoblasts-only) seeded scaffolds.

the Context:

NATURAL BONE	BIOMATERIALS	MAIN PROPERTIES	OPTIMISATION FOR BONE TISSUE ENGINEERING	TOPOGRAPHY	CHEMISTRY
<p>Natural 3D biocomposite scaffold</p> <p>organic and inorganic ECM components</p>	<ul style="list-style-type: none"> • porosity (cortical bone: low; trabecular bone: high) • interconnectivity • modeling/remodeling • mechanics 	PRP or growth/differentiating factors spatio-temporally supplied with nano-vehicles	<ul style="list-style-type: none"> • naturally rough, due to the molecular association of ECM components • presence of whole-scale length (macro-, meso-, micro-, nano-) and correct molecular arrangement 	ECM naturally contains adhesive, chemoattractive, growth-stimulating, and differentiating factors
ARTIFICIAL SCAFFOLD	BIOMATERIALS	MAIN PROPERTIES	OPTIMISATION FOR BONE TISSUE ENGINEERING	TOPOGRAPHY	CHEMISTRY
<ul style="list-style-type: none"> • metals • ceramics • bioglasses • natural polymers • synthetic polymers • biomimetic materials 	<ul style="list-style-type: none"> • porosity (macro-/micro-scale) • interconnectivity and open pores • biodegradability and harmless of by products • mechanical properties 	<ul style="list-style-type: none"> • biocomposites • surface coating • spatio-temporally release of growth/differentiating factors 	<ul style="list-style-type: none"> • doughness (micro- and nano- scale) • discontinuous 	adsorption of molecules (or proteins/DNA/miRNA sequences) to increase cell adhesion - osteoconduction (FN, laminin, collagens, hyaluran, heparin, poly-lysine, etc.) and osteinduction (rhBMPs, etc.)

Figure 5. How is reproduced the *in vivo* skeleton context in tissue engineering and scaffolding? Number of parameters are now well-known for their fundamental role in regulating bone repair. Concerning the development of scaffold usable in orthopedics, a lot of biomaterials, based on native bone properties, have been created and modified to reach a better level of osteointegration, osteoconduction and osteoinduction.

The properties of engineered tissue requirements (mainly strength) vary because not all bones have to support the same loading forces, and, then, different scaffolds may be used. So, it demands considering a wide range of biomaterials as suitable ones.

12.2.1.2. Cells

The need of reliable source of cells is important. The main, fundamental property of these cells should be the absence of immune response induction. Thus, the best way to avoid any adverse immunizing capacity, is to use cells from autologous donors. Since osteoblasts are very difficult to isolate and, further, to amplify *in vitro*, the best choice is the use of adult stem cells, or osteoprogenitors able to differentiate *versus* the osteoblastic phenotype.

The multipotency of adult stem cells and their high capacity to proliferate render their use very promising for engineering bone. Another advantage of adult stem cells is that, bone marrow-derived stem cells, as placenta's one, for example, present an immunomodulator effect, that renders them attractive

also for allo-transplants [95-97]. Adipose-, umbilical cord- or placenta-derived stem cells represent an easy-to-use source of cells, as compared to bone marrow-derived cells [98]. But, even if almost all mesenchymal stem cells (MSCs) are able to differentiate into different lineages, not all the sources of MSCs present the same plasticity to follow the osteoblastic lineage up to the terminally differentiated phenotype [99]. Endothelial progenitor cells (EPCs) were also used for bone healing, and a synergic effect was observed when both MSCs and EPCs were co-seeded with β -TCP granules [100]. Endothelial progenitor CD34⁺ population was also directly injected in a rat bone fracture site. Both neo-angiogenesis and osteogenesis were activated and resulted in bone healing [101]. The co-culture of MSCs and monocytes was shown to favor MSCs differentiation toward osteoblastic lineage, against adipocyte lineage. Moreover, osteoblasts downregulated the number of osteoclast-like cells, so mimicking the fine equilibrium between these two cell types, and suggesting that also osteoclast precursors could play an important role in developing bone neo-tissue [102]. Another strategy should be the recruitment of host endothelial progenitors (or mature cells), as well as of native MSCs, into a scaffold, serving as bioactive molecules carrier, and as temporary template and structural support for bone formation.

For exploiting engineered bone tissue as *ex vivo* model to perform studies on bone physiopathology or pharmacotoxicology, cell lines can be used instead of stem cells. The available cell lines are numerous, and each of them possesses individual properties. The most known cell lines are: the UMR106, the MC3T3-E1 and the MG-63 cells for the pre-osteoblastic and the osteoblastic lineages; the MLO-Y4 cells and the FLG 29.1 cells for the osteocytic and for the osteoclastic lineage, respectively. Moreover, there is a growing interest concerning human-induced pluripotent stem cells; these reprogrammed cells can differentiate into many cell lineages, comprising bony lineage, but their use for human implants still need further investigation [103].

12.2.1.3. Bioreactors applied to bone tissue engineering

Bioreactors are devices able to reproduce/maintain a tissue-like microenvironment, and to provide controlled culture conditions (*e.g.* temperature, pH, O₂/CO₂ and nutrients supply, flow rate, chemical/mechanical stimuli, *etc.*). Bioreactors need to favor mass transfer (nutrient and gas supply + waste removal), without exposing cells to high shear stress forces, that may be risky for their function and survival. This high-mass transfer/low-shear stress operational regimen can be attained by devices able to generate fluid-dynamic culture conditions (an interesting review, published by Rhiel and Lim, illustrates the importance of macro- and micro-fluidic flows on bone tissue formation, describing also the importance of the flow dimensionality on regulating cell/tissue behavior) [104]. Moreover, bioreactors usually furnish the possibility to maintain or recreate the three-dimension (3D) microenvironment generally present within a tissue. This

dimensionality is also important, because it allows the establishment of interactions between cells and ECM, which are very different from those created in the classical, static 2D culture condition. In such 3D conditions, cells sense each other, receive/exchange biochemical and biophysical cues from ECM, adapt their behavior, and, in turn, can change ECM properties. This is of great importance when mechanosensing cells or bone tissues must be studied. At last, the highly controlled fluid-dynamic conditions reached within specific bioreactors are necessary to improve cell seeding, proliferation, migration, and their further differentiation onto / into the scaffolds.

Rotating wall vessels. One example of fluid-dynamic bioreactors are the rotary cell culture system (RCCS™) devices, developed by the N.A.S.A.'s Johnson Space Center technological research (Synthecon Inc., U.S.A.). These bioreactors are composed of a horizontally rotating culture vessel, which angular speed can be regulated in order to reach optimal hydrodynamic conditions for cell/tissue culturing or, alternatively, to reach a peculiar equilibrium between the main forces acting on the cells (gravitational, centrifugal and fluid drag forces), that simulates some features of microgravity [105-107]. The vessel is fully filled with culture medium, avoiding the presence of air bubbles and, thus, deleterious turbulent forces. Therefore, this bioreactor can effectively provide cultured cells with a low-shear stress and a high-mass transfer microenvironment. In such a 3D hydrodynamic culture conditions, isolated cells or tissues explants remain alive, and express their tissue-specific phenotype from several days up to weeks of culture [106,108-112]. The RCCS™ bioreactor was also successfully applied, also by our group, to the study of the skeletal system on isolated cells, in the presence or in the absence of scaffolds, or on bone explants [107,113-117]. This bioreactor was also shown to speed up cell aggregation, osteoblastogenesis, osteoblast differentiation, and subsequent mineralization of mesenchymal or embryonic stem cells [109,115]. Goldstein and co-workers [118], contrariwise, demonstrated that, when seeded on PLGA scaffolds, rat osteoblastic cells better differentiated if kept in spinner flasks or in perfusion bioreactor, rather than in the RCCS™ device. According to our experience, it is possible that the results obtained by Goldstein's group were due to the specificity of the scaffold they used (*i.e.* density, surface conformation, size, interaction with fluid flow and relative deleterious forces). Even if not always easy to use for growing large engineered tissue masses, Hidaka and co-workers succeeded in transplanting RCCS™-engineered bone tissue in a rat calvarial bone defect, since this neo-tissue was further replaced by a mature bone after 2 months [119]. Song and co-workers [120] performed a comparative study between static conditions (T-Flasks), and dynamic conditions (spinner flasks and RCCS™ bioreactors). They showed that the osteoblasts seeded within an acellular bone-derived scaffold could reach a better state of differentiation when maintained in the RCCS™ device. Another example of rotating vessel is the BIOSTATS® Bplus RBS bioreactor, a cylindrical rotating bed system, which

allows the seeding of the MC3T3-E1 pre-osteoblastic cell line onto a ceramic carrier (Sponceram®). These conditions were more effective than the static ones, and, after several days of culture, cell proliferated and further differentiated, expressing OCN and type I collagen, and depositating mineralized matrix [121,122].

Perfusion bioreactors. They are basically organized as one or more culture chambers coupled up to tubing circuits and culture medium reservoirs. The whole system forms a closed circuit media loop, regulated by a peristaltic pump. This last allows perfusing cells dynamically, even when grown into a scaffold. As compared to the RCCS™ bioreactor, the regulation of fluid flow is finer in classical perfusion bioreactors; however, it also exists a Perfused RCCS™ device, in which the laminar flow created by the rotation of the vessel and culture medium is almost unaffected by the perfusion process.

The perfusion bioreactors, in their several versions, recapitulate, at least in part, the potential interstitial fluid flow. As *in vivo*, flow characteristics depend on scaffold properties (structure, porosity and interconnectivity). There are different types of perfused bioreactors. In a perfused chamber, the MC3T3-E1 osteoblastic cell line was exposed to a fluid shear of 1 dyn cm⁻², 12 dyn cm⁻² or 25 dyn cm⁻². In these conditions Ryder and Duncan [123] observed a magnitude-dependent increase in the intracellular concentration of calcium; this effect was significantly higher when the cells were stimulated with PTH, suggesting that cell response induced by shear and PTH is mediated by the modulation of mechanosensitive cation-selective channels, and L-type voltage-sensitive channels. The culture of rat BM-MSCs in perfused bioreactors without the addition of dexamethasone (a supplement usually employed to induce stem cell differentiation in osteoblasts), was shown to allow the differentiation of cells in the osteoblastic lineage; in the presence of both perfusion and dexamethasone, the differentiative process was enhanced [124]. Oscillatory perfusion was shown to be more efficient in engineering bone from MC 3T3-E1 osteoblastic-like cells seeded on porous ceramic, than the conditions generated by unidirectional or static bioreactors [125,126].

Spinner flasks. These bioreactors produce a dynamic flow, due to the mixing of medium by a stirring element localized at the bottom of the tank, whereas scaffolds are usually anchored to a needle, connected to the cap of the flask. These bioreactors are more difficult for monitoring hydrodynamic flow. Thus, turbulent forces and shear stress could be created, inducing cell sufferance. In such a bioreactor the Saos-2 osteoblastic cell line was shown to attach to HA-incorporating microcarriers and proliferate in a more efficient way than when the microcarriers were kept in static conditions [127]. Seeded into a decellularized bone-derived scaffold, adipose tissue-derived stem cells are able to adhere, migrate, grow and differentiate into osteoblastic cells expressing ALP. In these 3D hydrodynamic culture conditions, the level of differentiation was higher than in static conditions [128,129]. However, Rat BM-derived MSCs

better differentiated when maintained in a perfused system, rather than in spinner flasks [130].

Applying an adequate biomechanical stimulus, in order to better recapitulate the dynamic bone activities, is also of importance in *in vitro*, as well as in *in vivo* conditions. Usually, *in vitro*, the main mechanical strains that are reproduced are compression, stretching, contraction, and bending. Cyclic compression and cell perfusion enhanced BM-derived MSCs commitment and cell proliferation into a decellularised ECM scaffold, respectively [131]. BM-derived MSCs were shown to positively respond to these two stimuli by increasing collagen synthesis, also with polyurethane scaffolds. However, whereas the initial tensile modulus was elevated, it drastically decreased after two weeks of culture [132]. Contrariwise, Sittichockechaiwut and co-workers [133] showed that, as compared to unloaded cells, the MLO-5 osteoblastic-like cells were able to increase bone matrix synthesis (collagens, mineralization, OPN and OCN) when kept into a polyurethane scaffold and exposed to cyclic compressive stimuli. Moreover, with time, the samples acquired a higher stiffness. Elongation strains were also applied to MSCs, with or without dexamethasone [41]. Cyclical stretching together with dexamethasone favored osteogenic commitment of BM-derived MSCs, whereas mechanical stimulation alone significantly stimulated cell differentiation (increase in ALP levels and in collagen and OCN synthesis). The results confirmed that the mechanical stimulus could be as effective as the chemical one to induce bone cell differentiation [134]. Akhouayri and co-workers [135] evaluated the effect of contractive forces onto ROS 17/2.8 osteosarcoma-derived cells. Different contractile conditions were reached in type I collagen gels (freely retracted gels, stretching of the tense gel, periodic stress). The results showed that cell response (proliferation, morphology, ALP activity, OCN secretion, and ECM mineralization) depends on the attributes of the contractile stimulus. Static and dynamic mechanical forces differentially regulate cell behavior and phenotype. As an example, the osteoblastic differentiation induced by contractile collagen gels is mediated by the MMP-2/MMP-13/MT1-MMP cascade of metalloproteases [136], while a bending stimulus, tested on BM-derived MSCs seeded in biomimetic material, is able to upregulate osteogenic differentiation in the presence of dexamethasone [137].

12.2.1.4. Scaffolds – ECM/microenvironment

Biomaterials can be prepared as fillers for injection in injured sites, or as scaffolds for cell repopulation procedures. Whereas scaffolds were initially developed to sustain cell viability, technical progresses have allowed the development of more dynamic scaffolds, whose properties can be regulated to guide cell behavior (*in vitro* and *in vivo*), and to sustain the further osseointegration process. As already known, *in vivo*, cells are embedded in a 3D-organized ECM, the roles of which are to support cell adhesion, to promote cell organization, polarization and growth, to guide cell migration, to favor

nutrients' diffusion up to all bone cells, to provide growth factors and serve as mineral reservoir, and to be responsible for the structural and mechanical properties of the tissue. *In vivo*, the ECM is fundamental for the chemical, physical and topographical cues that provide to bone cells. Scaffolds designed for bone tissue engineering should then recapitulate such signals and properties and, consequently, in their conception, the following characteristics should be considered.

Porosity and connectivity. Scaffolds need to present open cavities (pores) to favour cell adhesion and proliferation. Bone cells are sensitive to the gross morphology of scaffolds. For osteoblasts, the suitable size of pores was shown to range between 100–400 μm in diameter. According to the cell type, the interconnectivity of these pores or / and additional micropores (important for an efficient mass transport), can promote homogeneous cell repartition, nutrients supply, and metabolic waste removal. Moreover, a pore diameter greater than 100 μm was shown to favor blood vessel ingrowth [138]. For a bone tissue analogous, the highly porous structure also better mimics the natural 3D architecture of the tissue.

Biodegradability or permanency. According to the aim to be attained, the scaffold can be either bioresorbable or permanent. The scaffold should, in effect, temporary behave as a template, allowing the ingrowth of bone cells, the expression of specific cell functions, the growth of neo-formed bone tissue and, eventually, the ingrowth of vascular components. When implanted in bone defects, it is of importance to finely regulate the degradation rate of the scaffold, in order to obtain a complete substitution of the biomaterial by the newly formed bone, and to avoid its collapse. The development of new biodegradable materials requires also to think about the byproducts of their degradation, since these last can induce a change in the graft microenvironment, and, as consequence, hinder transplant osteointegration and / or bone formation [139].

Mechanical properties. One of the major problems encountered for developing scaffolds suitable to be used in bone tissue engineering is to find the just balance between a scaffold harboring a high porosity, and the necessity that it can also maintain, at the same time, high stability, stiffness, strength, and toughness properties. Whereas for the *in vitro* studies engineered bone can present less strength capacities, for the *in vivo* use the engineered tissue must be able to face the natural mechanical forces experienced during the daily activity of the body (load, torsion, compression, *etc.*). The more the bone defect is wide, the more the biomaterials must be mechanically stable, and it is more so true if a biodegradable scaffold is used. Another strategy to increase the mechanical features of the bone micro-environment, is to use a calcium channel agonist, that favours bone formation.

Surface roughness was, as well, shown to influence cell behavior [140,141]. Even if both amplitude and organization characteristic of roughness variation

in surface texture or microtopography can affect the cellular response to an implant, it seems that the organization plays a more important role [142]. When examining the ability of osteoblast-like cell to attach to titanium surfaces, it was found that a higher percentage of cells attached to the rougher surface [143,144]. However, it remains difficult to strictly monitor cell behavior and, since experimental conditions vary from lab-to-lab, the significance of roughness effect on bone cell homeostasis is not always evident.

Surface tailoring. As for a number of other cell types, either the (bio)chemical or the physical (topographical) properties of the scaffold surface can modulate and guide bone cell behavior [145]. Functionalization of the scaffold surface allows regulating adhesion and migration of cells onto the scaffold (osteoconduction), as well as stem or progenitor cells differentiation (osteinduction).

Combination of scaffolds with soluble and bioactive molecules was shown to control bone cell behavior. In order to increase osteogenic properties of the scaffold, it may be integrated with osteoinductive growth factors and morphogens, which provide physiological regulation of bone features (*e.g.* TGF- β , BMPs, VEGF), or with drugs / polymeric carriers, used for gene delivery. To drive cell performance in order to optimize bone repair, different growth factors were tested in humans. The main bioactive molecules, which demonstrated to be effectively able to improve bone healing, are some members of the TGF- β superfamily of proteins (BMPs, TGF- β 1, TGF- β 3), members of the parathyroid hormone family (PTH, PTHrP), and platelet-derived growth factor (PDGF). However, many bioactive molecules were tested *in vivo* (on animals) or *in vitro*. In addition to the precedent ones, they include IGF, bFGF and the Wnt signaling proteins. BMPs (the most osteoinductive proteins are BMP 2, 4, 6, 7), TGF- β , FGF, IGF-I/-II, and PDGF are the most commonly used bioactive molecules employed in bone tissue engineering [146]. BMPs represent fundamental proteins, and are directly involved in the whole osteogenesis process. The key role of one of them, BMP-7, was demonstrated by the transdifferentiation of BMP-7 transduced fibroblasts into osteoblasts [147]. Carriers usually associated for bioactive molecules delivery are often polymer-based biomaterials. Natural carriers that can be, for example, used to this aim, are collagen and hyaluran; among synthetic polymers, poly(lactic acid) (PLA), poly(glycolic acid) (PGA) and poly(lactic-co-glycolic acid) (PGLA) are the most widely used. Recombinant human bone morphogenetic protein-7 (rhBMP-7) was also demonstrated to be able to induce cell proliferation and osteoblastic differentiation [148]. Dadsetan and co-workers [149] recently proved that the coating of poly(propylene fumarate) scaffolds with carbonated HA and rhBMP-2 results, after 6 weeks of implantation in calvaria defect, in a significant increase in bone formation, and in an effective osseointegration. These findings suggest that rhBMP-2 and HA, added together, could represent a good strategy to repair large bone defect. Zhang and co-workers [150] also demonstrated that rhBPM-2 loaded calcium

silicate/calcium phosphate cement stimulate cell adhesion to the scaffold, osteogenesis and bone repair, with a higher efficiency, as compared to either the cements alone, or the growth factor alone. These results confirm that bioactive molecules are necessary to furnish biological cues to cells, but the macro- and micro-porous arrangement of the scaffold is fundamental, as well. Moreover, as for healthy bone, where the mechanical properties result from the synergic effect of the whole component of bone tissue, in this particular case, the effect resulting from the combination of cement-based biomaterial and growth factor is higher than that of the single components. Another strategy that is currently of a great interest in tissue repair and tissue engineering strategies is the use of platelet-rich plasma (PRP). This preparation is rich in the growth factors that are commonly involved in bone formation and homeostasis *in vivo*, as, for example, TGF- β 1, bFGF, PDGF, IGF-1, EGF, VEGF, Factor V, Factor XI, Factor XIII, fibrinogen and von Willebrand factor (vWF), OSC, FN and Angiotensin-2 (Ang-2) [151,152]. Nevertheless, the results obtained with PRP are still controversial, probably due to the difference in PRP preparation between the different laboratories. Clinical trials are necessary to ascertain the most efficient PRP preparation protocol, and its real effect on bone healing process. For mimicking cell adhesion mediated by integrins, RGD sequences can also be incorporated into biomaterials. HA bio-functionalised with peptides harboring the RGD sequences allows a better adhesion of osteoprogenitors [153]. The adsorption of bioadhesive molecules, such as FN or laminin, could also enhance the interaction cell-scaffold [154]. Another interesting strategy, developed by Yang and collaborators [155] and by Wood and collaborators [156], is based on the use of a voltage-operated calcium channels agonist, delivered to mouse MG63 or rat ROS 17/2.8 cells exposed to a mechanical stimulus by a poly(L-lactic acid) (PLLA) scaffold. The results obtained showed that this scaffold modulates the mechanosensitivity of mouse osteoblasts, and increases cell differentiation (type I collagen and ALP production), as compared to the osteoblasts that received only the mechanical stimulus. Moreover, the PLLA porous biomaterial allowed a long-term release of the agonist, since it was stable over the 28 days culture period. Lastly, in addition to DNA delivery, miRNA delivery seems a very promising resource for applications in bone formation strategies, at least for the possibility it may give for regulating osteogenesis and bone cell differentiation *in vitro* [157].

Therefore, in order to be suitable for bone tissue engineering, a scaffold should integrate, as much as possible, the properties of native bone tissue. The scaffold should then closely reproduce tissue architecture, and combine the nanoscale topography of ECM with its relative higher-scale micro- / meso- / and macro-structures [158-160], since surface topography influences the rate of bone differentiation, formation, repair, and angiogenesis, as well [161-164]. Surface topography can also control BM-derived MSCs proliferation and differentiation, and it has been shown to play a major role than surface chemistry in regulating cell commitment. These processes are, at least in part,

regulated by cell shape, and could involve cytoskeleton organization, membrane tension, cell adhesion molecules and cell-cell interactions [42,145,165].

Newborn-rat calvaria-derived osteogenic cells, as well as MG63 cells, were seeded on titanium or titanium alloy surfaces harboring different topographies [166,167]. Cell behavior [adhesion, differentiation, matrix mineralization and bioactive molecules synthesis (VEGF, TGF- β 1, FGF-2, and angiopoietin-1), were differently controlled by the characteristics of the surface, rough surface favoring always the expression of the differentiated phenotype. Osteoblasts are sensitive to surface roughness, and the discontinuous-type topographies, by altering cell adhesion, shape, migration and differentiation (and acting, in part, by signaling pathways involving α 2 β 1 integrins, FAK and phosphorylative processes), could represent an efficient strategy for coating low osteoconductive biomaterials and for increasing osseointegration [168-171].

Both biochemical and biophysical aspects of ECM should be taken into account to reach a better cell homeostasis and to favour ulterior bone integration.

12.2.1.5. Scaffold composition

The main components of materials used as scaffolds and developed for bone engineering, are metals, polymeric materials (natural or synthetics), and ceramics.

12.2.1.5.1. Metals

Even if metals present the advantage to furnish an immediate mechanical support, their use is limited by their poor integration with the host tissue, by the release of ions, and by the possible bone collapse because of secondary fatigue loading [172]. Whereas stainless steel was highly used in the past, titanium and, mainly, titanium alloys are, at present, the most widely studied metal components for bone engineering techniques. Metal surfaces have been modified by changing their local topography and / or chemistry, in order to increase the possibility of cell interactions with the metallic supports, the rate of mesenchymal cell differentiation, and to facilitate implant integration in the site of injured tissues [86,173-176]. *In vitro* studies demonstrated that the osteoblastic cell line MG63 behaves differently, according to the roughness of titanium, and of titanium alloys [177-179]. Microroughness was shown to favor osteoblastic proliferation and differentiation, while cell number was decreased. Moreover, these processes are, at least in part, mediated by α 2 β 1 integrins [86,180,181]. However, as noticeably reviewed by Anselme and coworkers [182], results are often contradictory, because of a scarce homogeneity in the scaffold preparation and roughness characterization.

12.2.1.5.2. Polymers

Natural polymers. Natural biopolymers can be made of various molecules (*e.g.* collagen, fibrinogen, chitosan, alginate, starch, hyaluronic acid, silk, fibrin and poly(hydroxybutyrate)) [172,183-185]. They present the great advantages to be osteoinductive materials and naturally biodegradable substances. They generally support well cell attachment, they can guide cell migration and differentiation, but are often too weak to face the biomechanical forces imposed by daily activities. To overcome such a limit, and in order to reinforce material stability, natural biomaterial can be cross-linked, or associated with synthetic polymers or ceramics / bioactive glasses, in order to create biocomposite materials [186,187]. Kane and co-workers [187] reinforced collagen scaffolds with HA, and obtained a highly porous and interconnected biomaterial, able to support angiogenic and osteogenic differentiation after implantation in ectopic site (*in vivo*), or to favor the differentiation of adipose-derived MSCs (*in vitro*). It is of importance to keep in mind that the structural state of the polymers can profoundly modify the characteristics of the resulting scaffold. For example, Tsai and co-workers [186] demonstrated that, whereas soluble and fibrillar forms of type I collagen were able to stimulate the growth of MG-63 cells, the denatured form was more adequate in guiding cell differentiation. Biopolymers can also be used to cover hard implant surface, in order to increase osteoconduction, osteoinduction, and osseointegration. The 3D macroporous arrangement of silk-based or biocomposite scaffolds made up of collagen and HA is very interesting, since it mimics some aspects of bone porosity, and favors bone repair [189,190].

Synthetic polymers. Synthetic polymers represent the most widely used biomaterials in tissue engineering. Many techniques can be used as a means to prepare synthetic polymer-based scaffolds [191]. Synthetic biomaterials include poly(α -hydroxy acids), PCL, poly(propylene-fumarates), polycarbonates, polyphosphazenes, and polyanhydrides [172]. The poly(α -hydroxy acids) group comprises PLA, PGA, and their co-polymer PLGA. These synthetic polymers usually present the characteristic to be hydrolyzed into non-toxic byproducts (minimal systemic toxicity), and a poor immunoreactivity. PGLA copolymer was already tested in human patients, and, even if more slowly than autologous bone graft, it supported bone healing [192]. *In vitro*, a composite biomaterial made of chitosan and PLGA was shown to harbor some mechanical properties of trabecular bone, in addition to be osteoconductive and to support bone cells phenotype expression (production of ALP, OSP, BSPs) [193]. The functionalization of the chitosan / PLGA scaffold with a high loading of heparin, further stimulates MC3T3-E1 cells proliferation and differentiation [194]. Smith and co-workers [195] also demonstrated that the etching of PLGA with NaOH modifies the nano-scale surface topography of the manufact, and differently regulate cell behavior, according the cell type. Results from other authors suggest that, in addition to allow the spatio-temporal release of bioactive molecules, it is possible to devise a cell-type specific scaffold. Smith and co-workers [195] think that it could be

feasible to create a biphasic scaffold made up, first, of fibroblasts, in order to generate fibrocartilage, and, on the other pole, the scaffold could be treated with NaOH, in order to allow osteoblasts adhesion and bone formation.

However, PLGA polymers, for example, could release acidic byproducts, rendering the local microenvironment inadequate for bone integration. To overcome such a limit, polymer blends were developed; the mixture of PGLA and polyphosphazenes was shown to neutralize the environment of biomaterial degradation [196,197]. PLLA is widely used in bioengineering, since, in addition to the fact of having been approved by the Food and Drug Administration (FDA), it is biocompatible and biodegradable. However, its poor mechanical properties hinder its routine use in orthopedics [198]; this is the reason why this polymer was further reinforced with fibers or with inorganic components, such as HA or bioactive glass. PLGA combined with bioactive glasses was shown to possess higher compressive properties, and to support osteoblasts attachment and differentiation [199].

12.2.1.5.3. Ceramics

HA, calcium phosphate, calcium sulphate, alumina, zirconia, and coralline are also widely used as scaffolds for bone engineering [126,200]. They can be elaborated from natural or synthetic [*e.g.* sintered HA (sHA) and beta-tricalcium phosphate (β -TCP)] materials. Ceramics can be subdivided in inert, bioactive (sHA), and resorbable (β -TCP) products, depending on their main properties. As already illustrated, different types of substances are available, the most common being phosphate calcium ceramics, but they do not possess identical surface chemistry, surface charges, wet ability, and, consequently, the same capability to interact with cells [201]. Among all ceramics, sHA and β -TCP are the most studied formulae; they can be used alone, or mixed together, with a ratio variable between the 2 components [202]. When they are grafted, they give rise to a bone-like apatite layer that favors the ulterior osseointegration. sHA presents the advantage to allow the creation of a 3D stable and rigid scaffold, with a defined porosity. β -TCP is resorbed quicker than sHA, and it is less tough. sHA is widely used for bone regeneration, because of its good biocompatibility and high osteoconduction attributes, while is too fragile and hard, and not sufficiently elastic to be used to repair large defects in load-bearing bones [203,204]. Biphasic forms of calcium phosphate component were developed, in order to optimize the osteogenic and mechanic properties of the biomaterial, as well as its biodegradability. Arinzeh and co-workers [205] compared 6 different biomaterials made up of sHA, β -TCP, or of composites of these two substances (in different ratios). They showed that, after ectopic implantation (in severe combined immunodeficiency (SCID) mice), the composite with 20 % sHA and 80 % β -TCP better supports BM-MSCs, a faster osteoinduction, and the correlated differentiation into osteoblasts. The incorporation of rat or human MSCs into a composite biomaterial harboring a ratio of 60 % sHA / 40 % β -TCP

was shown to support the bridging of critical-sized defects [206]. Even if the resorbability of these scaffolds can be controlled by setting differently the mass ratio of sHA / β -TCP, it is difficult to fully direct the degradation of these ceramics, since they show a too limited resistance for being used in human patients with critical-sized bone defects. However, they can be poorly osteoinductive, and need to be functionalized. To counteract the poor efficiency in driving bone neo-formation, Choy and co-workers [207], incorporate RANKL onto β -TCP ceramic scaffold. Murine osteoclasts precursors seeded onto this support were able to mature, and to give rise to active osteoclasts expressing cathepsin K, calcitonin receptor, the sodium / hydrogen exchanger NHA2 and an active TRAP.

Concerning the problem of friability, sometimes met with porous bioceramics, the coating of ceramics with biological compounds (*i.e.* gelatin or alginate) can notably increase their mechanical properties (either compressive strength, or elastic modulus) [186,208]. Biocomposites made of sHA or of calcium cements and natural (*e.g.* gelatin, collagen, chitosan) or synthetic [*e.g.* PLA, PLLA, PLGA, PCL, poly(propylene carbonate)] elastic polymers were also developed, in order to better mimic the physiological biocomposite bone structure, to reduce ceramic weakness, and to facilitate the shaping of the scaffold by surgeons. This association was largely shown to increase the efficiency of osteogenesis and mineralization (MSCs-derived cells), as well as the differentiation of osteoclasts [209-217]. The coating of PLLA fibers with sHA allowed human cord blood-derived stem cells to form bone-like structures when scaffolds and cells were implanted subcutaneously, avoiding also the induction of excessive inflammatory reaction [214]. By substituting phosphate ions with silicate ions, different bioceramic materials have been developed. The investigation *in vivo* (sheep) showed that also this peculiar ceramics are able to increase bone formation and development [218]. Another strategy was to develop bioactive glasses, or glass-ceramics materials, by incorporating, for example, silicon into calcium phosphate materials [219,220]. These silica-based bioglasses are able to quickly bond to bone through a carbonated apatite layer that forms on the surface of the biomaterial after its interaction with the body fluids. These bioglasses are degraded faster than mixture of glass and ceramic, and than, of course, sHA. According to Oonishi and co-workers [221], the rate of silica dissolution is correlated with the rate of bone differentiation and growth, rendering this kind of biomaterials interesting to be used for bone tissue engineering. The results obtained suggested, moreover, that the higher rate of bone formation could be due to the silica incorporated into the biomaterials. The bioactive glasses can also be prepared with a macroporous configuration that was usually shown to increase the formation of bone. In addition, bioactive glasses can be added to PLGA films, further increasing their mechanical properties and osteoconductivity, and the proliferation / differentiation rate of associated human osteoblasts [139]. One

limit of these bioactive-glasses is their brittleness, and the difficulty to be properly shaped by the surgeon [222].

12.2.1.5.4. Biomimetics

De-cellularized / de-mineralized trabecular or cortical bones can be used as biomaterials, as well. They present the advantage to recapitulate the natural ECM features, and allow, therefore, attachment, proliferation and further differentiation of adipose-derived stem cells [129]. Usually, acellular ECM can be obtained after physical, chemical or enzymatic treatments [223,224]. According to the processing of demineralized bone, osteoconductive and osteoinductive properties can vary [84,225]. Moreover, following the treatments for preparing demineralized bone, a decrease of bone interconnectivity due to pore obstruction may occur. Bone-based scaffold can be also demineralized and functionalized with bioactive molecules [226]. Kim and co-workers [227] used another source of acellular ECM. The human lung fibroblast ECM can be used as natural scaffold, coupled with synthetic polymers (PLA, PLGA), and functionalized with the BMP-2 bioactive molecule. This composite material was shown to favor osteogenic differentiation, either *in vitro* or *in vivo*. In effect, the ectopic implantation of such a scaffold in the SCID mice, resulted in an increase in bone formation, while its graft in a rat calvarial bone defect promoted bone healing. However, whereas demineralized bone matrix is able to provide biochemical cues, it is not able to serve as a tough structural support.

12.2.1.6. Nanoscale

Nanoparticles or nanocapsules can be proposed as molecule delivery systems, able to allow the biochemical control of spatial and temporal release of signaling molecules, such as members of the TGF- β , BMP-2, and BMP-7 family, or of other bioactive molecules, such as proinflammatory cytokines and growth factors [*e.g.* TNF- α , interleukins, interferon gamma (IFN γ), prostaglandins]. These “nanosystems” have been shown to regulate recruitment, migration and differentiation of bone cells, and, as in the *in vivo* conditions, to favor cell survival and to guide bone healing [228-231]. At the nanometer scale, it becomes possible to regulate cell interactions by means of specific proteins, so mimicking the nanocomposite structure of bone tissue. Nanostructured groove patterned biomaterial was shown to be able to induce osteoblasts to mimic, at least in part, their physiological behavior in response to a mechanical stimulus (longitudinal stretching), whereas microstructured biomaterials did not induce such a response [232], then suggesting that, for a proper bone cells activity, the nanotopography of scaffolds needs to be taken into consideration. Nanobiomaterials can be produced in different structures / shapes, such as nanofibers, nanotubes, nanowires, nanoparticles, nanocrystals or nanocomposites. Because of their nanometer scale, scaffold properties can be optimized by engineering their surface topography and chemistry, taken into

account that wetness and surface energy become, at the nanometer length scale, important parameters involved in the control of cell behavior. Nanostructured scaffolds were developed to optimize cell-scaffold interactions, and to physically guide the differentiation of mesenchymal stem cells and bone progenitors. The nanometer level (< 100 nm for the minor dimension) is of importance, since it is the level at which cells-biomaterials can interact, due to the possibility to adsorb proteins on the surface and, then, to sustain a further development of the engineered tissue-biocomposite assembly [233,234]. Nanotopography and nanostructure of scaffold surface were shown to direct cell behavior (thus determining / conditioning the features), even in the absence of growth factors or of other chemiotactic agents [163,235], underlining the importance of such parameters in developing new scaffolds.

The progress of technologies allows now to create new and efficient nanomaterials able to mimic, as much as possible, the native bone hierarchy.

12.2.1.6.1. Nanofibers and nanotubes

This group of nanomaterials was developed because these nano-scaled biomaterials present a structure very similar to some ECM components, such as collagen fibrils. They also present a high surface area to volume ratio, that, after the functionalization of the biomaterial surface, it is important in order to increase osteoconduction and / or osteoinduction. Kumar and co-workers [145] tested a broad range of biomaterials for their capacity to interact with BM-derived MSCs. They showed that the only scaffolds able to guide MSCs through the osteoblastic lineage were the nanofibrous arranged biomaterials. The main polymers used to electrospun nanofibers are PLA, PGA, PLGA and PCL, but some of them still present limitations relating to cell adhesion (caused by their hydrophobic characteristics), and restrictions due to the harmless of their degradations byproducts. On the contrary, PCL nanofibers allow rat BM-derived MSCs to adhere, to proliferate up to form cell multilayer coating of the scaffold, and, after 4 weeks of culture, to express markers of differentiated osteoblasts (*i.e.* mineralization and synthesis of type I collagen) [236]. Carbon nanotubes present optimal electrical and mechanical properties that can be exploited for bone engineering, even if they need to be functionalized, in order to be able to better favor calcium mineralization, and to reduce any possible cytotoxic outcome [237]. The conjugation of carbone nanotubes with different molecular groups (*e.g.* COOH or NH₂) is possible, and favors HA mineralization process. The functionalization, in addition to increasing the biocompatibility of the biomaterial, can also regulate the strength of the fibers. Another important attribute of functionalization is that it can modify the surface free energies, shown to play an important role in cell adhesion and functions [238-241]. Another advantage of the electrospun nanofibers is that, in the presence of osteogenic culture medium, they regulate cell morphology, proliferation and sustain stem cells differentiation, in a manner dependent on fibers' alignment and diameter [242,243]. This fact may allow for guiding progenitor / stem cell

differentiation by modulating the manufacture of the nanofibers. However, this kind of nanofibrous scaffold presents a poor resistance to mechanical stress. On the contrary, nano-composites scaffolds made of β -TCP and sHA nanofibers, were shown to be tougher than non-composite biomaterials (β -TCP ceramics alone), and to reach compressive properties close to the ones of the trabecular bone *in vivo* [244]. Venugopal and Vadgama [245] maintained in culture human fetal osteoblasts on collagen / PLC / sHA composite nanofibers and showed that such organic-inorganic meld favors cell adhesion, migration, proliferation and mineralization. A biocomposite made of electrospun PCL nanofibers and nanoparticles of allogenic bone ECM has been demonstrated to highly favor the *in vivo* osteogenic differentiation of adipose-derived stem cells, when compared to the influence of PCL fibers without ECM nanoparticles on the same cell type [246]. The versatility of electrospun scaffolds is also based on their capacity to be processed with bioactive molecules and / or HA. As an example, a silk poly(ethylene oxide) (PEO) scaffold, harboring both BMP-2 and HA nanoparticles, was proved to induce human BM-derived MSCs to differentiate into osteoblastic cells, which, with respect to controls, were characterized by the expression of BSP, type I collagen, and by a significant increase of BMP-2 expression and matrix mineralization [247].

12.2.1.6.2. Nanoparticles

sHA was developed as nanocrystalline sHA to better mimic the *in vivo* situation, and was used to improve materials' biocompatibility and conduction. When MG-63 cells were seeded onto films made of various-sized sHA, the results indicated that the nanoparticles of sHA support cell proliferation and inhibit apoptosis better than the microparticles [248]. Nanoparticles of sHA has been shown to be osteoconductive as amorphous calcium phosphate, functionalized with the RGD sequences [249]. The nanoparticles of sHA are particularly appreciated for the great surface area they expose, for the roughness of their surface, and, also, for their surface energy and the arranged distribution of their electrons [250]. Moreover, in addition to coat biomaterials, sHA nanoparticles can also be used as fillers. Many techniques can be used to prepare sHA nanoparticles, and each of them gives its own sHA conformation, size and the possibility to create biocomposites [251].

12.2.1.6.3. Nanostructured biomaterials

ECM Hydrogels could better recapitulate some bone matrix properties, such as structure and high porosity, and provide a large surface area that sustains the adsorption of adhesive proteins. Hydrogels usually consent an easy exchange of nutrients and gas up the central part of the scaffolds. They are, furthermore, easy to manipulate: for example, the linking of protein receptors to the scaffold can control cell behavior (shape, growth, differentiation, functions) [252]. Co-polymerization of degradable and non-degradable macromers permits to monitor hydrogel degradation and concomitant expression of osteoblastic

phenotype and mineral deposit [253]. Concerning fabrication, among the various materials that can be used to create nanostructured hydrogels, some are synthetic polymers [such as poly(vinyl alcohol) (PVA) or PEO], while others are of natural origin (collagen, alginate, chitosan, fibrin, silk, fibrinogen, gellan gum, *etc.*). However, hydrogels present often too poor mechanical properties to be used in orthopedic field. This is the reason why they are often combined with other biomaterials (*e.g.* inorganic substances such as sHA, β -TCP or bioactive glasses) in order to improve their strength [254]. As an example, gellan-gum hydrogels reinforced by the addition of bioactive glass nanoparticles, became able to sustain cell adhesion and viability [254].

Another application of nanostructured surfaces in bone repair was developed by Rani and co-workers [255], who showed that the fabrication of non-periodic nanostructures renders biocompatible the scaffold that they cover. Moreover, according to the arrangement of the nanostructures (nanoscaffolds, nanoleaves, or nanoneedles), the expression of some osteoblastic markers is distinctly regulated, as their osteointegration differs in efficiency.

Nanocomposite materials. First of all, being nanocomposites formed by, at least, two materials, the resultant of the sum of the properties of the biomaterials they are composed of, should surpass (in number or quality) the complex of properties of each individual element. For example, sintering HA bioceramics, thanks to the high surface energy encountered in nanomaterials, can increase the mechanical properties of the biomaterial. Combining sHA particles and chitosan, has permitted to prepare nanocomposites rods with stronger mechanical properties [256]. Biocomposites scaffolds made of nanostructured phosphates and natural biopolymers (nanoHA-Si-Mg-Zn / type I collagen / chitosan) and organized according the layer-by-layer method, were able to allow BM-derived MSCs adhesion, remodeling of the scaffold surface by cells into a more bone-like structure [257]. Bhattacharya and Chaudhry [258] succeeded in preparing nanocomposites made of silica nanoparticles and PVA and presenting bone similar properties. The osteoinductivity of microscale-bioactive glasses can be optimized, by switching from the micro- to the nano-scale level [259]. Bearing in mind that bone is a composite material made of inorganic and organic phases, it is straightforward to consider that sHA or bioactive glasses could be combined with nanofibers of polymers to form nanocomposites. In addition to sHA, polymers that can be used are, for example, synthetic polymers (PLGA, PLLA, PCL, PVA) and natural-based polymers (starch, chitosan, silk, collagen). Nanoceramic composites of PLGA and nano-HA were used to increase biocompatibility of the PLGA component. The 3D culture of mesenchymal stem cells with these biocomposite scaffolds in the fluid-dynamic RCCS™ bioreactor, sustains good osteoconduction and osteoinduction, and an efficient osteogenesis [260]. Recently, scaffolds made of poly(D,L-lactide) (PDLLA), carbon nanotubes and nano-HA have been developed, and shown to be able to promote cell viability,

differentiation, and matrix mineralization. *In vivo*, such scaffolds allowed the formation of an immature bone, indicating that the combination of several components can improve tissue engineering efficiency [261].

12.3. CONCLUSIONS

Regulation of bone homeostasis and healing is complex, and encompasses a multiplicity of actors that intimately interact and cooperate to reach the best bone quality in health and illness. When nature is no more able to counteract bone injury, tissue engineering must take the measures, bearing in mind the multi-signaling networks involved in bone formation, maturation and functions, which include biochemical, physical, mechanical, topographical, and electrical inputs. As described previously, bone is a hierarchically organized, composite biomaterial, which includes tissue-specific ECM and multiple cell types: osteoblasts, osteocytes, osteoclasts, lining cells, and precursors. The 3D tissue organization, the defined cell polarization, the spatial and temporal localization of biochemical and mechanical signals, the specific cell-cell and cell-ECM molecular interactions, are fundamental characteristics (and requirements) for optimal tissue features and responses to various stimuli. Bone cells interact with a number of other cell types (*e.g.* endothelial cells, bone marrow cells, *etc.*), and bioactive molecules (*e.g.* cytokines, growth factors, hormones), synthesized by bone tissue-specific cells, as well as by neighboring or distant cells. Many engineering strategies have been developed during the last decades, in order to facilitate / permit the repair of injured bone unable to self-repair, either since affected by severe pathologies (*e.g.* osteoporosis, ischemic necrosis, neoplasia), or due to large loss of substance (large-sized defect).

Even if great efforts have been addressed in order to optimize bone cell culturing / transplantation strategies and the design / fabrication techniques for the manufacture of suitable scaffolds / bioreactors, translational medicine (and bone tissue engineering) still needs new approaches, in order to allow a more efficient treatment of orthopaedic patients.

A significant number of nanomaterials has been already conceived and developed in recent years, aimed at optimizing bone tissue engineering strategies; nevertheless, none of these products has, at present, been successfully used in humans for bone repairing procedures. Table 1 shows some examples of already developed nanomaterials, with their relative “field(s) of application” (basic research, *in vitro/in vivo* pre-clinical study, and translational use at clinical level). Among all the various nanomaterials, nanoparticles are, at present, the only ones employed in the medical context, as drug or growth factor vehicles; bone cements and injectable materials (not necessarily nanoscaled), able to polymerize and harden *in situ*, are becoming very interesting, even if not yet ready to be used in orthopaedic contexts.

Table 1. Examples of nanomaterials use in bone research, from bench to clinic

	Nanomaterials for coating Metals and Alloys implants (USE: material coating)	Nano-particles (USE as: filler, drug delivery systems, gene delivery (e.g. plasmid carrying DNA, siRNA))	Nano-ceramics (USE: increase protein adsorption & wettability)	Nano-composites (USE: reproduce bone composition)	Nano-tubes (USE: mimic the dimensions of bone ECM)	Nano-fibers (USE: increase the ratio surface area/volume; increase protein adsorption)
BASIC RESEARCH	In vitro	EFFECT ON CELLS: <ul style="list-style-type: none">✓ Ti-surface (from micro- to nano-scale); increase TGF-β1 and osteopontin synthesis✓ Ti- covered by carbon nanotubes permits to increase osteoblasts proliferation, calcium deposit & implant mechanical strength✓ Fluorinated hydroxyapatite (FHA) coating favors cell attachment & proliferation. The roughness of FHA determine the stability of alkaline phosphatase expression	EFFECT ON CELLS: <ul style="list-style-type: none">✓ Increase material biocompatibility✓ Increase cell differentiation & proliferation✓ Increase Osteoinductivity	EFFECT ON CELLS: <ul style="list-style-type: none">✓ Increase strength✓ Increase biocompatibility✓ Increase cell adhesion✓ Increase cell proliferation	EFFECT ON CELLS: <ul style="list-style-type: none">✓ TiO₂ nanotubes present specific nanotopography that aids MSCs adhesion, spreading, proliferation and differentiation	EFFECT ON CELLS: <ul style="list-style-type: none">✓ Increase :<ul style="list-style-type: none">- cell adhesion- cell proliferation- cell differentiation- cell mobility✓ Induce cell alignment✓ Good Biocompatibility✓ Biodegradability (±) Effects increase when nanofibers are composed of natural/synthetic nanomaterials and HA
	In vivo (pre-clinical investigations on large animal models to overcome the limits of the small animal models)	EFFECT ON CELLS/ TISSUE: <ul style="list-style-type: none">✓ Crystallized TiO₂ favor osteointegration✓ Nanopolymorphic HA-coated Ti implants increase osteoimplantation (rat, rabbit); HA shows efficient degradation✓ PDLLA/HA composite-coated Ti implants increase the quality of osteointegration (by favoring bone matrix formation and by decreasing fibroblastic connective formation; sheep).✓ Apatite coating increases bone integration (goat)	EFFECT ON CELLS/ TISSUE: <ul style="list-style-type: none">✓ Nanoparticle-coated implants,✓ Electrospayed CaP nanoparticles-coated implantsincrease cell adhesion and cell differentiation (increase of alkaline phosphatase activity & Ca²⁺ deposit)	EFFECT ON CELLS/ TISSUE: <p>Favor:</p> <ul style="list-style-type: none">✓ Bone integration✓ Bone ingrowth✓ Bone formation (rabbit femur)	EFFECT ON CELLS/ TISSUE: <ul style="list-style-type: none">✓ Repair of osteochondral injury (sheep)✓ Repair of calvarial defects (rat & rabbit)	EFFECT ON CELLS/ TISSUE: <ul style="list-style-type: none">✓ Nanostructured Ti nanotubes coatings and silver particles favor osteointegration and antibiotic activity (rat calvar bone)✓ Nanostructured carbon increases:<ul style="list-style-type: none">- biocompatibility- mechanical strength- bone formation (rabbit femoral condyle or domestic pig frontal skull)
CLINICAL TRANSLATION						
CLINICAL TRIALS	Patients	EFFECT ON CELLS/ TISSUE: <ul style="list-style-type: none">✓ Optimal mechanical support✓ Nano- materials increase osteointegration	EFFECT ON CELLS/ TISSUE: <p>Optimization of delivery (treatment) of Drugs (biphosphonates), Antibiotics, & Growth factors</p>	TO BE DONE...		
		We are far away from routine application in clinic. Even if nanomaterials offer good perspectives, almost all of them need: <ul style="list-style-type: none">✓ to be tested for their physical, chemical, topographical, biochemical, biological effects on human bone✓ to support osteointegration and bone formation. Nanometer scale will need to be integrated into macro- and micro-designed scaffolds to reach a better quality for orthopaedic use of biomaterials				

Contrary to nanomaterials, nano-coating technologies applied to metal or alloy implants are easier applicable in clinic, where the nanoscaled size of the coating has been already demonstrated to favor implant integration in the site of bone injury, thus suggesting that this strategy is consistently promising.

In conclusion, as bone properties result from the interactions of its cellular and organic / inorganic components at the macro-, meso-, micro- and nano-scale level, it is intuitive to expect that the perfect bone bio-substitute (graft extender, void filler, *etc.*) should imply that the bioengineering approach aimed at designing this complex tissue requires multiple assembly / fabrication controls, up to the nanoscale level. The extraordinary technological progress attained in the last 10 years, together with the advance in our knowledge of bone physiopathology (coupled with the new holistic visions from the fields of systems and emergence biology), will open wide perspectives to innovative applications of nanoscale structures to bone tissue engineering, thus providing new powerful tools for improving its strategies for medical applications.

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REFERENCES

1. K.J. Oldknow, V.E. MacRae, C. Farquharson. *J. Endocrinol.* **225** (2015) R1–R19.
2. Y. Bala, E. Seeman. *Calcif. Tissue Int.* (2015) [Epub ahead of print].
3. P. Ducy, M. Amling, S. Takeda, M. Priemel, A.F. Schilling, F.T. Beil, J. Shen, C. Vinson, J.M. Rueger, G. Karsenty. *Cell* **100** (2000) 197–207.
4. H.K. Väänänen, H. Zhao, M. Mulari, J.M. Halleen. *J Cell Sci.* **113** (2000) 377–381.
5. H.K. Väänänen, T. Laitala-Leinonen. *Arch. Biochem. Biophys.* **473** (2008) 132–138.
6. Y. Hashida, K. Nakahama, K. Shimizu, M. Akiyama, K. Harada, I. Morita. *Bone* **61** (2014) 19–26.
7. V.J. Vigorita, in *Orthopaedic Pathology, 2nd Ed.*, W. Kluwer (Ed.), Lippincott Williams & Wilkins, 2008, New York, USA, pp. 1–49.
8. A. Tampieri, S. Sprio, M. Sandri, F. Valentini. *Trends Biotechnol.* **29** (2011) 526–535.
9. D.A. Wang, M. Miura, H. Demura, K. Sato. *Endocrinology* **138** (1997) 2953–2962.
10. T.J. Martin, N. Udagawa. *TEM* **9**(1998) 6–12.
11. H.M. Kronenberg. *Nature* **423**(2003) 332–336.
12. M. Sinnesael, S. Boonen, F. Claessens, E. Gielen, D. Vanderschueren. *J. Osteoporosis* (2011) ID240328.
13. V. Toppets, V. Pastoret, V. De Behr, N. Antoine, C. Dessy, A. Gabriel. *Ann. Méd. Vét.* **148** (2004) 1–13.
14. R.S. Tuan. *J. Bone Joint Surg. Am.* **85-A** (2003) 137–141.
15. B. Guillotin, R. Bareille, C. Bourget, L. Bordenave, J. Amédée. *Bone* **42** (2008) 1080–1091.
16. M.M. Thi, M. Urban-Maldonado, D.C. Spay, S.O. Suadicani. *Am. J. Physiol. Cell Physiol.* **299** (2010) C994–C1006.
17. E. Hay, F.X. Dieudonné, Z. Saidak, C. Marty, J. Brun, S. Da Nascimento, P. Sonnet, P.J. Marie. *J. Cell Physiol.* **229** (2014):1765–1775.
18. F. Lecanda, P.M. Warlow, S. Sheikh, F. Furlan, T.H. Steinberg, R. Civitelli. *J. Cell Biol.* **151** (2000) 931–943.
19. A.R. Guntur, C.J. Rosen, M.C. Naski. *Bone* **50** (2012) 54–62.
20. T.L. Andersen, T.E. Sondergaard, K.E. Skorzynska, F. Dagnaes-Hansen, T.L. Plesner, E.M. Hauge, T. Plesner, J.M. Delaisse. *Am. J. Pathol.* **174**(2009) 239–247.
21. J.S. Nyman, M. Reyes, X. Wang. *Micron* **36** (2005) 566–582.
22. Y. Han, S.C. Cowin, M.B. Schaffler, S. Weinbaum. *Proc. Natl. Acad. Sci. USA* **101** (2004) 16689–16694.
23. A. Vatsa, R.G. Breuls, C.M. Semeins, P.L. Salmon, T.H. Smit, J. Klein-Nulend. *Bone* **43** (2008) 452–458.
24. A. Vatsa, C.M. Semeins, T.H. Smit, J. Klein-Nulend. *Biochem. Biophys. Res. Commun.* **377** (2008) 1019–1024.
25. J. Behari, *Biophysical Bone behavior: principles and applications*. John Wiley & Sons Pte Ltd, Chichester, UK, 2009, pp. 129–172.
26. R.K. Globus, A. Moursi, D. Zimmerman, J. Lull, C. Damsky. *ASGSB Bulletin* **8** (1995) 19–28.
27. N. Reznikov, R. Shahar, S. Weiner. *Acta. Biomaterialia* **10** (2014) 3815–3826.

28. L. Gibson, M. Ashby, In *Cellular Solids, Structure and Properties* (2nd Ed.), Cambridge University Press, Cambridge, UK, 1999.
29. D.R. Carter, D.M. Spengler. *Clin. Orthop. Relat. Res.* **135** (1978) 192–217.
30. M.W. Hamrick, J.G. Skedros, C. Pennington, P.L. McNeil. *J. Musculoskelet. Neuronal Interact.* **6**(2006) 258–263.
31. S. Takeda, F. Elefteriou, R. Levasseur, X. Liu, L. Zhao, K.L. Parker, D. Armstrong, P. Ducy, G. Karsenty. *Cell* **111**(2002) 305–317.
32. V.K. Yadav, F. Oury, N. Suda, Z.W. Liu, X.B. Gao, C. Confavreux, K.C. Klemenhausen, K.F. Tanaka, J.A. Gingrich, X.E. Guo, L.H. Tecott, J.J. Mann, R. Hen, T.L. Horvath, G. Karsenty. *Cell* **138** (2009) 976–989.
33. N. Fukushima, R. Hanada, H. Teranishi, Y. Fukue, T. Tachibana, H. Ishikawa, S. Takeda, Y. Takeuchi, S. Fukumoto, K. Kangawa, K. Nagata, M. Kojima. *J. Bone Miner. Res.* **20** (2005) 790–798.
34. C. Ma, T. Fukuda, H. Ochi, S. Sunamura, C. Xu, R. Xu, A. Okawa, S. Takeda. *Mol. Metab.* **4** (2015) 175–185.
35. S.R.L. Young, R. Gerard-O’Riley, J.B. Kim, F.M. Pavalko. *J. Bone Miner. Res.* **24** (2009) 411–424.
36. A.B. Castillo, J.T. Blundo, J.C. Chen, K.L. Lee, N.R. Yereddi, E. Jang, S. Kumar, W.J. Tang, S. Zarrin, J.B. Kim, C.R. Jacobs. *PLoS One* **7** (2012) e43291.
37. H.J. Donahue. *Bone* **26** (2000) 417–422.
38. A. Liedert, D. Kaspar, R. Blakytyn, L. Claes, A. Ignatius. *Biochem. Biophys. Res. Commun.* **349** (2006) 1–5.
39. Y. Mikuni-Takagaki. *J. Bone Miner. Metab.* **17** (1999) 57–60.
40. L. Tang, Z. Lin, Y.M. Li. *Biochem. Biophys. Res. Commun.* **344** (2006) 122–128.
41. M. Jagodzinski, M. Drescher, J. Zeichen, S. Hankemeier, C. Krettek, U. Bosch, M. van Griensven. *Eur. Cell Mater.* **7** (2004) 35–41.
42. R. McBeath, D.M. Pirone, C.M. Nelson, K. Bhadriraju, C.S. Chen. *Dev. Cell* **6** (2004) 483–495.
43. I. Titushkin, M. Cho. *Biophys. J.* **93** (2007) 3693–3702.
44. J.A. Robinson, M. Chatterjee-Kishore, P.J. Yaworsky, D.M. Cullen, W. Zhao, C. Li, Y. Kharode, L. Sauter, P. Babij, E.L. Brown, A.A. Hill, M.P. Akhter, M.L. Johnson, R.R. Recker, B.S. Komm, F.J. Bex. *J. Biol. Chem.* **281**(2006) 31720–31728.
45. E.J. Arnsdorf, P. Tummala, A.B. Castillo, F. Zhang, C.R. Jacobs. *J. Biomech.* **43** (2010) 2881–2886.
46. S. Viguet-Carrin, P. Garnero, P.D. Delmas. *Osteoporos. Int.* **17** (2006) 319–336.
47. M. Zaidi. *Nature Med.* **13**(2007) 791–801.
48. S. Isenmann, A. Arthur, A.C. Zannettino, J.L. Turner, S. Shi, C.A. Glackin, S. Gronthos. *Stem Cells* **27**(2009) 2457–2468.
49. S. Kido, R. Kuriwaka-Kido, T. Imamura, Y. Ito, D. Inoue, T. Matsumoto. *Bone* **45** (2009) 1125–1132.
50. M. Gyda, S. Corisdeo, M. Zaidi, B.R. Troen. *Biochem. Biophys. Res. Commun.* **285** (2001) 328–334.
51. K. Nakashima, X. Zhou, G. Kunkel, Z. Zhang, J.M. Deng, R.R. Behringer, B. de Crombrughe. *Cell* **108** (2002) 17–29.
52. T.J. Martin, N.A. Sims. *Rev. Endocr. Metab. Disord.* 2015 Jan 4 [Epub ahead of print].
53. C.B. Khatriwala, S.R. Peyton, A.J. Putnam. *Am. J. Physiol. Cell Physiol.* **290** (2006) C1640–C1650.

54. C.A. Mullen, M.G. Haugh, M.B. Schaffler, R.J. Majeska, L.M. McNamara. *J. Mech. Behav. Biomed. Mater.* **28** (2013) 183–194.
55. R. Civitelli. *Arch. Biochem. Biophys.* **473** (2008) 188–192.
56. T.J. Heino, T.A. Hentunen, H.K. Väänänen. *Exp. Cell Res.* **294** (2004) 458–468.
57. E. Birmingham, G.L. Niebur, P.E. McHugh, G. Shaw, F.P. Barry, L.M. McNamara. *Eur. Cell Mater.* **23** (2012) 13–27.
58. L.M. McNamara, R.J. Majeska, S. Weinbaum, V. Friedrich, M.B. Schaffler. *Anat. Rec. (Hoboken)* **292** (2009) 355–363.
59. L.E. Lanyon. *Calcif. Tissue Int.* **53** (1993) S102–S107.
60. R.G. Bacabac, D. Mizuno, C.F. Schmidt, F.C. MacKintosh, J.J. Van Loon, J. Klein-Nulend, T.H. Smit. *J. Biomech.* **41** (2008) 1590–1598.
61. R.F.M. Van Oers, H. Wang, R.G. Bacabac. *Curr. Osteoporos. Rep.* **13** (2015) 61–66.
62. W.J. Boyle, W.S. Simonet, D.L. Lacey. *Nature* **423** (2003) 337–342.
63. H. Yasuda, N. Shima, N. Nakagawa, K. Yamaguchi, M. Kinoshita, S. Mochizuki, A. Tomoyasu, K. Yano, M. Goto, A. Murakami, E. Tsuda, T. Morinaga, K. Higashio, N. Udagawa, N. Takahashi, T. Suda. *Proc. Natl. Acad. Sci. USA* **95** (1998) 3597–3602.
64. N. Udagawa, N. Takahashi, E. Jimi, K. Matsuzaki, T. Tsurukai, K. Itoh, N. Nakagawa, H. Yasuda, M. Goto, E. Tsuda, K. Higashio, M.T. Gillespie, T.J. Martin, T. Suda. *Bone* **25**(1999) 517–523.
65. S.C. Manolagas. *Bone* **17** (1995) 63S–67S.
66. P. Ducy. *Med. Sci.* **12** (2001) 1242–1251.
67. J.T. Warren, W. Zou, C.E. Decker, N. Rohatgi, C.A. Nelson, D.H. Fremont, S.L. Teitelbaum. *J. Cell Biochem.* (2015) [Epub ahead of print].
68. N.S. Soysa, N. Alles, D. Weih, A. Lovas, A.H. Mian, H. Shimokawa, H. Yasuda, F. Weih, E. Jimi, K. Ohya, K. Aoki. *J. Bone Miner. Res.* **25** (2010) 809–818.
69. G.D. Roodman. *Endocr. Rev.* **17** (1996) 308–332.
70. A. Cappariello, A. Maurizi, V. Veeriah, A. Teti. *Arch. Biochem. Biophys.* **561** (2014) 13–21.
71. J.Y. Rho, L. Kuhn-Spearing, P. Zioupos. *Med. Eng. Phys.* **20** (1998) 92–102.
72. S. Weiner, W. Traub. *FASEB J.* **6** (1992) 879–885.
73. S. Weiner, H.D. Wagner. *Ann. Rev. Mat. Sci.* **28** (1998) 271–298.
74. S.V. Dorozhkin. *Materials* **2** (2009) 399–498.
75. E. Seeman. *J. Gerontol. A Biol. Sci. Med. Sci.* **68**(2013) 1218–1225.
76. P. Fratzl, H.S. Gupta, E.P. Paschalis, P. Roschger. *J. Mater. Chem.* **14** (2004) 2115–2123.
77. D.R. Katti, S.M. Pradhan, K.S. Katti. *J. Biomech.* **43** (2010) 1723–1730.
78. Z. Qin, A. Gautieri, A.K. Nair, H. Inbar, M.J. Buehler. *Langmuir* **28** (2012) 1982–1992.
79. G.L. Barnes, P.J. Kostenuik, L.C. Gerstenfeld, T. A. Einhorn. *J. Bone Miner. Res.* **14** (1999) 1805–1815.
80. P. Guihard, M.A. Boutet, B. Brounais-Le Royer, A.L. Gamblin, J. Amiaud, A. Renaud, M. Berreur, F. Rédini, D. Heymann, P. Layrolle, F. Blanchard. *Am. J. Pathol.* **185** (2015) 765–775.
81. H.P. Gerber, T.H. Vu, A.M. Ryan, J. Kowalski, Z. Werb, N. Ferrara. *Nat. Med.* **5** (1999) 623–628.
82. E. Zelzer, W. McLean, Y.S. Ng, N. Fukai, A.M. Reginato, S. Lovejoy, P.A. D'Amore, B.R. Olsen. *Development* **129**(2002) 1893–1904.

83. X. Li, Z. Wang, L. Yang, Z. Li, Y. Ogata. *J. Oral Sci.* **52** (2010) 221–229.
84. J.L. Russell, J.E. Block. *Orthopedics* **22** (1999) 524–531.
85. R. Langer, J.P. Vacanti. *Science* **260** (1993) 920–926.
86. P. Carmeliet, R.K. Jain. *Nature* **407** (2000) 249–257.
87. J. Street, M. Bao, L. deGuzman, S. Bunting, F.V. Jr Peale, N. Ferrara, H. Steinmetz, J. Hoeffel, J.L. Cleland, A. Daugherty, N. van Bruggen, H.P. Redmond, R.A. Carano, E.H. Filvaroff. *Proc. Natl. Acad. Sci. USA* **99** (2002) 9656–9661.
88. A.L. Raines, R. Olivares-Navarrete, M. Wieland, D.L. Cochran, Z. Schwartz, B.D. Boyan. *Biomaterials* **31**(2010) 4909–4917.
89. D.A. Puleo, R. Bizios. *J. Biomed. Mater. Res.* **26** (1992) 291–301.
90. K. Anselme. *Biomaterials* **21** (2000) 667–681.
91. T. Albrektsson, C. Johansson. *Eur. Spin. J.* **10** (2001) S96–101.
92. Y. Zhang, A. Schedle, M. Matejka, X. Rausch-Fan, O. Andrukhov. *Cell. Mol. Biol. Lett.* **15** (2010) 517–529.
93. A. Hofmann, U. Ritz, S. Verrier, D. Eglin, M. Alini, S. Fuchs, C.J. Kirkpatrick, P.M. Rommens. *Biomaterials* **29** (2008) 4217–4226.
94. J.Y. Kim, G.Z. Jin, I.S. Park, J.N. Kim, S.Y. Chun, E.K. Park, S.Y. Kim, J. Yoo, S.H. Kim, J.W. Rhie, D.W. Cho. *Tissue Eng.* **16** (2010) 2229–2236.
95. M. Magatti, S. De Munari, E. Vertua, C. Nassuato, A. Albertini, G.S. Wengler, O. Parolini. *Cell Transplant* **18** (2009) 899–914.
96. R.A. Banas, C. Trumpower, C. Bentlejewski, V. Marshall, G. Sing, A. Zeevi. *Hum. Immunol.* **69** (2008) 321–328.
97. B.J. Jones, S.J. McTaggart. *Exp. Hematol.* **36** (2008) 733–741.
98. Y. Diao, Q. Ma, F. Cui, Y. Zhong. *J. Biomed. Mater. Res. A* **91** (2009) 123–131.
99. G.I. Im, Y.M. Shin, K.B. Lee. *Osteoarthritis Cartilage* **13** (2005) 845–853.
100. C. Seebach, D. Henrich, C. Kähling, K. Wilhelm, A.E. Tami, M. Alini, I. Marzi. *Tissue Eng. Part A* **16** (2010) 1961–1970.
101. T. Matsumoto, A. Kawamoto, R. Kuroda, M. Ishikawa, Y. Mifune, M. Iwasaki, M. Miwa, M. Horii, S. Hayashi, A. Oyamada, H. Nishimura, S. Murasawa, M. Doita, M. Kurosaka, T. Asahara. *Am. J. Pathol.* **169** (2006) 1440–1457.
102. A. Bernhardt, S. Thieme, H. Domaschke, H. Springer, A. Rosen-Wolff, M. Gelinsky. *J. Biomed. Mater. Res. Part A* **95A** (2010) 848–856.
103. G.M. de Peppo, I. Marcos-Campos, D.J. Kahler, D. Alsalman, L. Shang, G. Vunjak-Novakovic, D. Marolt. *Proc. Natl. Acad. Sci. USA* **110** (2013) 8680–8685.
104. B.D. Riehl, J.Y. Lim. *Cells* **1** (2012) 1225–1245.
105. P.S. Ayyaswamy, K. Mukundakrishnan. *Acta Astronaut* **60** (2007) 397–405.
106. F. Cosmi, N. Steimberg, D. Dreossi, G. Mazzoleni. *J. Mech. Behav. Biomed. Mater.* **2** (2009) 164–172.
107. F. Cosmi, N. Steimberg, G. Mazzoleni. *J. Mech. Behav. Biomed. Mater.* **44C** (2015) 61–70.
108. T.G. Hammond, J.M. Hammond. *Am. J. Physiol. Renal. Physiol.* **281** (2001) F12–F25.
109. X. Yu, E.A. Botchwey, E.M. Levine, S.R. Pollack, C.T. Laurencin. *Proc. Natl. Acad. Sci. USA* **101** (2004) 11203–11208.
110. C. Morabito, N. Steimberg, G. Mazzoleni, S. Guarnieri, G. Fanò-Illic, M.A. Mariggiò. *Biomed. Res. Int.* **2015** (2015) 754283.

111. C. Montani, N. Steimberg, J. Boniotti, G. Biasiotto, I. Zanella, G. Diafera, I. Biunno, L. Caimi, G. Mazzoleni, D. Di Lorenzo. *Toxicol. Appl. Pharmacol.* **280** (2014) 421–433.
112. M. Ferrarini, N. Steimberg, M. Ponzoni, D. Belloni, A. Berenzi, S. Girlanda, F. Caligaris-Cappio, G. Mazzoleni, E. Ferrero. *PLoS One* **8** (2013) e71613.
113. S.R. Facer, R.S. Zaharias, M.E. Andracki, J. Lafoon, S.K. Hunter, G.B. Schneider. *J. Dent. Res.* **84** (2005) 542–547.
114. Y.J. Ko, R.S. Zaharias, D.A. Seabold, J. Lafoon, G.B. Schneider. *J. Prosthodont.* **16** (2007) 431–438.
115. Y.S. Hwang, J. Cho, F. Tay, J.Y.Y. Heng, R. Ho, S.G. Kazarian, D.R. Williams, A.R. Boccaccini, J.M. Polak, A. Mantalaris. *Biomaterials* **30** (2009) 499–507.
116. G. Mazzoleni, F. Boukhechba, N. Steimberg, J. Boniotti, J.M. Boulter, N. Rochet. *Proc. Eng.* **10** (2011) 3662–3667.
117. N. Steimberg, J. Boniotti, G. Mazzoleni, In: *Methods in Bioengineering: Alternative Technologies to Animal Testing*, E. Novik, T. Maguire (Eds): Artech House publisher, USA, 2010, pp. 205–225.
118. A.S. Goldstein, T.M. Juarez, C.D. Helmke, M.C. Gustin, A.G. Mikos. *Biomaterials* **22** (2001) 1279–1288.
119. M. Hidaka, G.N. Su, J.K. Chen, K. Mukaisho, T. Hattori, G. Yamamoto. *In Vitro Cell Dev. Biol. Anim.* **43** (2007) 49–58.
120. K. Song, T. Liu, Z. Cui, X. Li, X. Ma. *J. Biomed. Mater. Res. A* **86** (2008) 323–332.
121. C. Kasper, K. Suck, F. Anton, T. Scheper, S. Kall, M. van Griensven, in *Topics in Tissue Engineering, Vol. 3*, N. Ashammakhi, R. Reis, E. Chiellini (Eds.), © 2007, pp. 1–15.
122. K. Suck, S. Roeker, S. Diederichs, F. Anton, J.A. Sanz-Herrera, I. Ochoa, M. Doblare, T. Scheper, M. van Griensven, C. Kasper. *Biotechnol. Prog.* **26** (2010) 671–678.
123. K.D. Ryder, R.L. Duncan. *J. Bone Miner. Res.* **16** (2001) 240–248.
124. H.L. Holtorf, J.A. Jansen, A.G. Mikos. *J. Biomed. Mater. Res. A* **72** (2005) 326–334.
125. D. Du, T. Asaoka, T. Ushida, K.S. Furukawa. *Biofabrication* **6** (2014) 045002.
126. D. Du, K.S. Furukawa, T. Ushida. *Biotechnol. Bioeng.* **102** (2009) 1670–1678.
127. R.A. Perez, K. Riccardi, G. Altankov, M.P. Ginebra. *J. Tissue Eng.* **5** (2014) 2041731414543965.
128. T. Mygind, M. Stiehler, A. Baatrup, H. Li, X. Zou, A. Flyvbjerg, M. Kassem, C. Büngrer. *Biomaterials* **28** (2007) 1036–1047.
129. S. Kedong, L. Wenfang, Z. Yanxia, W. Hong, Y. Ze, L. Mayasari, L. Tianqing. *Appl. Biochem. Biotechnol.* **174** (2014) 1331–1343.
130. H. Hosseinkhani, Y. Inatsugu, Y. Hiraoka, S. Inoue, Y. Tabata. *Tissue Eng.* **11** (2005) 1476–1488.
131. M. Jagodzinski, A. Breitbart, M. Wehmeier, E. Hesse, C. Haasper, C. Krettek, J. Zeichen, S. Hankemeier. *J. Biomech.* **41** (2008) 1885–1891.
132. C. Liu, R. Abedian, R. Meister, C. Haasper, C. Hurschler, C. Krettek, G. von Lewinski, M. Jagodzinski. *Biomaterials* **33** (2012) 1052–1064.
133. A. Sittichokechaiwut, A.M. Scutt, A.J. Ryan, L.F. Bonewald, G.C. Reilly. *Bone* **44** (2009) 822–829.
134. A. Sittichokechaiwut, J.H. Edwards, A.M. Scutt, G.C. Reilly. *Eur. Cell Mater.* **20** (2010) 45–57.
135. O. Akhouayri, M.H. Lafage-Proust, A. Rattner, N. Laroche, A. Caillot-Augusseau, C. Alexandre, L. Vico. *J. Cell Biochem.* **76** (1999) 217–230.

136. S. Barthelemi, J. Robinet, R. Garnotel, F. Antonicelli, E. Schittly, W. Hornebeck, S.J. Lorimier. *Cell. Biochem.* **113** (2012) 760–772.
137. J.R. Mauney, S. Sjostorm, J. Blumberg, R. Horan, J.P. O'Leary, G. Vunjak-Novakovic, V. Volloch, D.L. Kaplan. *Calcif. Tissue Int.* **74** (2004) 458–468.
138. N. Okii, S. Nishimura, K. Kurisu, Y. Takeshima, T. Uozumi. *Neurol. Med. Chir.* **41** (2001) 100–104.
139. C. Wu, Y. Ramaswamy, Y. Zhu, R. Zheng, R. Appleyard, A. Howard, H. Zreiqat. *Biomaterials* **30** (2009) 2199–2208.
140. B.D. Boyan, T.W. Hummert, D.D. Dean, Z. Schwartz. *Biomaterials* **17**(1996) 137–146.
141. A. Ponche, M. Bigerelle, K. Anselme. *Proc. Inst. Mech. Eng. H* **224** (2010) 1471–1486.
142. K. Anselme, M. Bigerelle. *Acta Biomater.* **1** (2005) 211–222.
143. C. Michaels, J. Keller, C. Stanford, M. Solursh. *J. Dent. Res.* **68** (1989) 276–281.
144. K.T. Bowers, J.C. Keller, B.A. Randolph, D.G. Wick, C.M. Michaels. *Int. J. Oral. Maxillofac. Implants* **7** (1992) 302–310.
145. G. Kumar, C.K. Tison, K. Chatterjee, P.S. Pine, J.H. McDaniel, M.L. Salit, M.F. Young, C.G. Simon. *Biomaterials* **32**(2011) 9188–9196.
146. I. Drosse, E. Volkmer, R. Capanna, P. De Biase, W. Mutschler, M. Schieker. *Injury* **39** (2008) S9–S20.
147. R.B. Rutherford, M. Moalli, R.T. Franceschi, D. Wang, K. Gu, P.H. Krebsbach. *Tissue Eng* **8** (2002) 441–452.
148. T.K. Sampath, J.C. Maliakal, P.V. Hauschka, W.K. Jones, H. Sasak, R.F. Tucker, K.H. White, J.E. Coughlin, M.M. Tucker, R.H. Pang, C. Corbett, E. Ozkaynak, H. Oppermann, D.C. Rueger. *J. Biol. Chem.* **267**(1992) 20352–20362.
149. M. Dadsetan, T. Guda, M.B. Runge, D. Mijares, R.Z. LeGeros, J.P. LeGeros, D.T. Silliman, L. Lu, J.C. Wenke, P.R. Brown Baer, M.J. Yaszemski. *Acta Biomater.* **18** (2015) 9–20.
150. J. Zhang, H. Zhou, K. Yang, Y. Yuan, C. Liu. *Biomaterials* **34** (2013) 9381–9392.
151. Y. Yamada, M. Ueda, T. Naiki, M. Takahashi, K. Hata, T. Nagasaka. *Tissue Eng.* **10** (2004) 955–964.
152. G. Intini. *Biomaterials* **30** (2009) 4956–4966.
153. M.C. Durrieu, S. Pallu, F. Guillemot, R. Bareille, J. Amédée, C.H. Baquey, C. Labrugère, M. Dard. *J. Mater. Sci. Mater. Med.* **15** (2004) 779–786.
154. Y. Yamada, K. Hozumi, F. Katagiri, Y. Kikkawa, M. Nomizu. *Biopolymers* **94** (2010) 711–720.
155. Y. Yang, J.L. Magnay, L. Cooling, A.J. El Haj. *Biomaterials* **23**(2002) 2119–2126.
156. M.A. Wood, S. Hughes, Y. Yang, A.J. El Haj. *J. Control Rel.* **112** (2006) 96–102.
157. M. Sriram, R. Sainitya, V. Kalyanaraman, S. Dhivya, N. Selvamurugan. *Int. J. Biol. Macromol.* **74** (2015) 404–412.
158. L. Wang, R.L. Carrier, *Advances in Biomimetics*, A. George (Ed.), InTech, 2011, Croatia. Available from: <http://www.intechopen.com/books/advances-in-biomimetics/biomimetic-topography-bioinspired-cell-culture-substrates-and-scaffolds>.
159. P.G. Coelho, S.J. Hollister, C.L. Flanagan, P.R. Fernandes. *Med. Eng. Phys.* **37** (2015) 287–296.
160. L. Cui, N. Zhang, W. Cui, P. Zhang, X. Chen. *J. Bionic. Eng.* **12** (2015) 117–128.

161. C. Larsson, P. Thomsen, J. Lausmaa, M. Rodahl, B. Kasemo, L.E. Ericson. *Biomaterials* **15** (1994) 1062–1074.
162. M.J. Dalby, D. McCloy, M. Robertson, H. Agheli, D. Sutherland, S. Affrossman, R.O. Oreffo. *Biomaterials* **27** (2006) 2980–2987.
163. M.J. Dalby, N. Gadegaard, R. Tare, A. Andar, M.O. Riehle, P. Herzyk, C.D. Wilkinson, R.O. Oreffo. *Nat. Mater.* **6** (2007) 997–1003.
164. S. Song, E.J. Kim, C.S. Bahney, T. Miclau, R. Marcucio, S. Roy. *Acta. Biomaterialia* **18** (2015) 100–111.
165. J. Settleman. *Mol. Cell.* **14** (2004) 148–150.
166. M. Wieland, M. Textor, B. Chehroudi, D.M. Brunette. *Biomaterials* **26** (2005) 1119–1130.
167. O Zinger, G. Zhao, Z. Schwartz, J. Simpson, M. Wieland, D. Landolt, B. Boyan. *Biomaterials* **26** (2005) 1837–1847.
168. R. Olivares-Navarrete, P. Raz, G. Zhao, J. Chen, M. Wieland, D.L. Cochran, R.A. Chaudhri, A. Ornoy, B.D. Boyan, Z. Schwartz. *Proc. Natl. Acad. Sci. USA* **105** (2008) 15767–15772.
169. R. Olivares-Navarrete, S.L. Hyzy, R.A. 1st Gittens, J.M. Schneider, D.A. Haithcock, P.F. Ullrich, P.J. Slosar, Z. Schwartz, B.D. Boyan. *Spine J.* **13** (2013) 1563–1570.
170. D.W. Hamilton, K.S. Wong, D.M. Brunette. *Calcif. Tissue Int.* **78** (2006) 314–325.
171. D.W. Hamilton, D.M. Brunette. *Biomaterials* **28** (2007) 1806–1819.
172. A.J. Salgado, O.P. Coutinho, R.L. Reis. *Macromol. Biosci.* **4** (2004) 743–765.
173. K. Anselme, P. Linez, M. Bigerelle, D. LeMaguer, A. Le Maguer, P. Hardouin, H.F. Hildebrand, A. Iost, J.M. Leroy. *Biomaterials* **21** (2000B) 1567–1577.
174. D.D. Deligianni, N. Katsala, D. Sotiropoulou, J. Amedee, Y.F. Missirlis. *Biomaterials* **22** (2001) 1241–1251.
175. M.S. Sader, A. Balduino, A. Soares Gde, R. Borojevic. *Clin. Oral Implants Res.* **16** (2005) 667–675.
176. R. Olivares-Navarrete, S.L. Hyzy, D.L. Hutton, C.P. Erdman, M. Wieland, B.D. Boyan, Z. Schwartz. *Biomaterials* **31** (2010) 2728–2735.
177. J.Y. Martin, Z. Schwartz, T.W. Hummert, D.M. Schraub, J. Simpson, J. Lankford Jr, D.D. Dean, D.L. Cochran, B.D. Boyan. *J. Biomed. Mater. Res.* **29** (1995) 389–401.
178. J. Lincks, B.D. Boyan, C.R. Blanchard, C.H. Lohmann, Y. Liu, D.L. Cochran, D.D. Dean, Z. Schwartz. *Biomaterials* **19** (1998) 2219–2232.
179. R.A. Gittens, T. McLachlan, R. Olivares-Navarrete, Y. Cai, S. Berner, R. Tannenbaum, Z. Schwartz, K.H. Sandhage, B.D. Boyan. *Biomaterials* **32** (2011) 3395–3403.
180. S.S. Hakki, S.B. Bozkurt, E.E. Hakki, P. Korkusuz, N. Purali, N. Koç, M. Timucin, A. Ozturk, F. Korkusuz. *Biomed. Mater.* **7** (2012) 045006.
181. M. Lai, C.D. Hermann, A. Cheng, R. Olivares-Navarrete, R.A. Gittens, M.M. Bird, M. Walker, Y. Cai, K. Cai, K.H. Sandhage, Z. Schwartz, B.D. Boyan. *J. Biomed. Mater. Res. A* **103** (2015) 564–573.
182. K. Anselme, A. Ponche, M. Bigerelle. *Proc. Inst. Mech. Eng. H* **224** (2010) 1487–1507.
183. A.J. Salgado, M.E. Gomes, A. Chou, O.P. Coutinho, R.L. Reis, D.W. Hutmacher. *Mater. Sci. Eng. C* **20** (2002) 27–33.
184. A.C. MacIntosh, V.R. Kearns, A. Crawford, P.V. Hatton. *J. Tissue Eng. Regen. Med.* **2** (2008) 71–80.

185. J.F.A. Valente, T.A.M. Valente, P. Alves, P. Ferreira, A. Silva, I.J. Correia. *Mater. Sci. Eng. C Mater. Biol. Appl.* **32** (2012) 2596–2603.
186. G.S. Lee, J.H. Park, J.E. Won, U.S. Shin, H.W. Kim. *J. Mater. Sci. Mater. Med.* **22** (2011) 1257–1268.
187. R.J. Kane, H.E. Weiss-Bilka, M.J. Meagher, Y. Liu, J.A. Gargac, G.L. Niebur, D.R. Wagner, R.K. Roeder. *Acta Biomaterialia* **17** (2015) 16–25.
188. S.W. Tsai, Y.H. Cheng, Y. Chang, H.L. Liu, W.B. Tsai. *J. Taiwan Inst. Chem. Eng. (DNLM)* **41** (2010) 247–251.
189. H. Kim, U. Kim, G. Leisk, C. Bayan, I. Georgakoudi, D. Kaplan. *Macromol. Biosci.* **7** (2007) 643–655.
190. H.J. Kim, U.J. Kim, G. Vunjak-Novakovic, B.H. Min, D.L. Kaplan. *Biomaterials* **26** (2005) 4442–4452.
191. H.J. Chung, T.G. Park. *Adv. Drug Deliv. Rev.* **59** (2007) 249–262.
192. C. Bertoldi, D. Zaffe, U. Consolo. *Biomaterials* **29** (2008) 1817–1823.
193. T. Jiang, W.I. Abdel-Fattah, C.T. Laurencin. *Biomaterials* **27** (2006) 4894–4903.
194. T. Jiang, Y. Khan, L.S. Nair, W.I. Abdel-Fattah, C.T. Laurencin. *J. Biomed. Mater. Res. A* **93** (2010) 1193–1208.
195. L.L. Smith, P.J. Niziolek, K.M. Haberstroh, E.A. Nauman, T.J. Webster. *Int. J. Nanomedicine* **2** (2007) 383–388.
196. M. Deng, L.S. Nair, S.P. Nukavarapu, S.G. Kumbar, T. Jiang, N.R. Krogman, A. Singh, H.R. Allcock, C.T. Laurencin. *Biomaterials* **29** (2008) 337–349.
197. N.R. Krogman, A.L. Weikel, K.A. Kristhart, S.P. Nukavarapu, M. Deng, L.S. Nair, C.T. Laurencin, H.R. Allcock. *Biomaterials* **30** (2009) 3035–3041.
198. D.W. Hutmacher. *Biomaterials* **21** (2000) 2529–2543.
199. H.H. Lu, S.F. El-Amin, K.D. Scott, C.T. Laurencin. *J. Biomed. Mater. Res. A* **64** (2003) 465–474.
200. J.R. McConnell, B.J. Freeman, U.K. Debnath, M.P. Grevitt, H.G. Prince, J.K. Webb. *Spine* **28** (2003) 317–323.
201. F. Barrère, C.A. van Blitterswijk, K. de Groot. *Int. J. Nanomedicine* **1** (2006) 317–332.
202. G. Daculsi. *Biomaterials* **19** (1998) 1473–1478.
203. C. Ohtsuki, M. Kamitakahara, T. Miyazaki. *J. R. Soc. Interface* **6** (2009) S349–S360.
204. L. Cheng, F. Ye, R. Yang, X. Lu, Y. Shi, L. Li, H. Fan, H. Bu. *Acta Biomater.* **6** (2010) 1569–1574.
205. T.L. Arinze, T. Tran, J. Mcalary, G. Daculsi. *Biomaterials* **26** (2005) 3631–3638.
206. T.L. Arinze. *Foot Ankle Clin.* **10** (2005) 651–665.
207. J. Choy, C.E. Albers, K.A. Siebenrock, S. Dolder, W. Hofstetter, F.M. Klenke. *Bone* **69** (2014) 80–88.
208. B. Liu, P. Lin, Y. Shen, Y. Dong. *J. Mater. Sci. Mater. Med.* **19** (2008) 1203–1207.
209. J. Venugopal, S. Low, A.T. Choon, A.B. Kumar, S. Ramakrishna. *Artif. Organs* **32** (2008) 388–397.
210. M.V. Jose, V. Thomas, K.T. Johnson, D.R. Dean, E. Nyairo. *Acta Biomater.* **5** (2009) 305–315.
211. M. Ngiam, S. Liao, A.J. Patil, Z. Cheng, C.K. Chan, S. Ramakrishna. *Bone* **45** (2009) 4–16.
212. M.P. Prabhakaran, J. Venugopal, S. Ramakrishna. *Acta Biomaterialia* **5** (2009) 2884–2893.

213. N. Rochet, T. Balaguer, F. Boukhechba, J.P. Laugier, D. Quincey, S. Goncalves, G.F. Carle. *Biomaterials* **30** (2009) 4260–4267.
214. E. Seyedjafari, M. Soleimani, N. Ghaemi, I. Shabani. *Biomacromolecules* **11** (2010) 3118–3125.
215. J. Zhao, W. Han, H. Chen, M. Tu, S. Huan, G. Miao, R. Zeng, H. Wu, Z. Cha, C. Zhou. *J. Mater. Sci. Mater. Med.* **23** (2012) 517–525.
216. C.C. Lin, S.J. Fu, Y.C. Lin, I.K. Yang, Y. Gu. *Int. J. Biol. Macromol.* **68** (2014) 39–47.
217. A.J. Ryan, J.P. Gleeson, A. Matsiko, E.M. Thompson, F.J. O'Brien. *J. Anat.* (2014) 1–14.
218. D.L. Wheeler, L.G. Jenis, M.E. Kovach, J.M. Marini, A.S. Turner. *Spine J.* **7** (2007) 308–317.
219. T. Gao, H.T. Aro, H. Ylanen, E. Vuorio. *Biomaterials* **22** (2001) 1475–1483.
220. J.R. Jones, S. Lin, S. Yue, P.D. Lee, J.V. Hanna, M.E. Smith, R.J. Newport. *Proc. Inst. Mech. Eng. H* **224**(2010) 1373–1387.
221. H. Oonishi, L.L. Hench, J. Wilson, F. Sugihara, E. Tsuji, M. Matsuura, S. Kin, T. Yamamoto, S. Mizokawa. *J. Biomed. Mater. Res.* **51** (2000) 37–46.
222. M. Peltola, I. Kinnunen, K. Aitasalo. *J. Oral Maxillofac. Surg.* **66** (2008) 639–646.
223. P.M. Crapo, T.W. Gilbert, S.F. Badylak. *Biomaterials* **32** (2011) 3233–3243.
224. T.W. Gilbert. *J. Cell Biochem.* **113** (2012) 2217–2222.
225. G.I. Drosos, K.I. Kazakos, P. Kouzoumpasis, D.A. Verettas. *Injury* **38** (2007) S13–S21.
226. J.J. Song, H.C. Ott. *Trends Mol. Med.* **17** (2011) 424–432.
227. I.G. Kim, M.P. Hwang, P. Du, J. Ko, C. W. Ha, S. H. Do, K. Park. *Biomaterials* **50** (2015) 75–86.
228. T.J. Cho, L.C. Gerstenfeld, T.A. Einhorn. *J. Bone Miner. Res.* **17** (2001) 513–520.
229. T.N. Vo, F.K. Kasper, A.G. Mikos. *Adv. Drug Deliv. Rev.* **64** (2012) 1292–1309.
230. K. Tsukii, N. Shima, S. Mochizuki, K. Yamaguchi, M. Kinoshita, K. Yano, O. Shibata, N. Udagawa, H. Yasuda, T. Suda, K. Higashio. *Biochem. Biophys. Res. Commun.* **246**(1998) 337–341.
231. R.F. Lai, Z.J. Li, Z.Y. Zhou, Z.Q. Feng, Q.T. Zhao. *Pac. J. Trop. Med.* **6** (2013) 884–888.
232. L. Prodanov, J. te Riet, E. Lamers, M. Domanski, R. Luttge, J.J. van Loon, J.A. Jansen, X.F. Walboomers. *Biomaterials* **31** (2010) 7758–7765.
233. T.J. Webster, C. Ergun, R.H. Doremus, R.W. Siegel, R. Bizios. *J. Biomed. Mater. Res.* **51** (2000) 475–483.
234. T.J. Webster, C. Ergun, R.H. Doremus, R.W. Siegel, R. Bizios. *Biomaterials* **21** (2000) 1803–1810.
235. E. Lamers, X.F. Walboomers, M. Domanski, J. te Riet, F.C. van Delft, R. Luttge, L.A. Winnubst, H.J. Gardeniers, J.A. Jansen. *Biomaterials* **31** (2010) 3307–3316.
236. H. Yoshimoto, Y.M. Shin, H. Terai, J.P. Vacanti. *Biomaterials* **24** (2003) 2077–2082.
237. B. Zhao, H. Hu, S.K. Mandal, R.C. Haddon. *Chem. Mater.* **17** (2005) 3235–3241.
238. D. Khang, J. Lu, C. Yao, K.M. Haberstroh, T.J. Webster. *Biomaterials* **29** (2008) 970–983.
239. R.L. Price, M.C. Waid, K.M. Haberstroh, T.J. Webster. *Biomaterials* **24** (2003) 1877–1887.
240. K.L. Elias, R.L. Price, T.J. Webster. *Biomaterials* **23** (2002) 3279–3287.
241. X. Liu, J. Y. Lim, H.J. Donahue, R. Dhurjati, A.M. Mastro, E.A. Vogler. *Biomaterials* **28** (2007) 4535–4550.

242. A.S. Badami, M.R. Kreke, M.S. Thompson, J.S. Riffle, A.S. Goldstein. *Biomaterials* **27** (2006) 596–606.
243. J.K. Wise, A.L. Yarin, C.M. Megaridis, M. Cho. *Tissue Eng. Part A* **15** (2009) 913–921.
244. H.R. Ramay, M. Zhang. *Biomaterials* **21** (2004) 171–180.
245. J. Venugopal, P. Vadgama, T.S. Sampath Kumar, S. Ramakrishna. *Nanotechnology* **18** (2007) 1–8.
246. M. Gibson, V. Beachley, J. Coburn, P.A. Bandinelli, H.Q. Mao, J. Elisseeff. *Biomed. Res. Int.* **2014** (2014) 469120.
247. C. Li, C. Vepari, H.J. Jin, H.J. Kim, D.L. Kaplan. *Biomaterials* **27** (2006) 3115–3124.
248. Z. Shi, X. Huang, Y. Cai, R. Tang, D. Yang. *Acta Biomater.* **5** (2009) 338–345.
249. G. Balasundaram, M. Sato, T.J. Webster. *Biomaterials* **27** (2006) 2798–2805.
250. S.B. Gayathri, P. Kamaraj. *Nano Vision* **1**(2011) 1–13.
251. H. Zhou, J. Lee. *Acta Biomaterialia* **7** (2011) 2769–2781.
252. K.A. Beningo, M. Dembo, Y.L. Wang. *Proc. Natl. Acad. Sci. USA* **101** (2004) 18024–18029.
253. D.S.W. Benoit, A.R. Durney, K.S. Anseth. *Tissue Eng.* **12** (2006) 1663–1673.
254. A. Gantar, L.P. da Silva, J.M. Oliveira, A.P. Marques, V.M. Correlo, S. Novak, R.L. Reis. *Mater. Sci. Eng. C Mater. Biol. Appl.* **43** (2014) 27–36.
255. V.V.D. Rani, L. Vinoth-Kumar, V.C. Anitha, K. Manzoor, M. Deepthy, V.N. Shantikumar. *Acta Biomaterialia* **8** (2012) 1976–1989.
256. Z. Wang, and Q. Hu. *Biomed. Mater.* **5** (2010) 045007.
257. G. Tomoaia, O. Soritau, M. Tomoaia-Cotisel, L.-B. Pop, A. Pop, A. Mocanu, O. Horovitz, L.-D. Bobos. *Powder Technol.* **238** (2013) 99–107.
258. M. Bhattacharya, S. Chaudry. *Mater. Sci. Eng. C Mater. Biol. Appl. C* **33** (2013) 2601–2610.
259. B. Marelli, C.E. Ghezzi, D. Mohn, W.J. Stark, J.E. Barralet, A.R. Boccaccini, S.N. Nazhat. *Biomaterials* **32** (2011) 8915–8926.
260. Q. Lv, M. Deng, B.D. Ulery, L.S. Nair, C.T. Laurencin. *Clin. Orthop. Relat. Res.* **471** (2013) 2422–2433.
261. I.A. Siqueira, M.A. Corat, B.D. Cavalcanti, W.A. Neto, A.A. Martin, R.E. Bretas, F.R. Marciano, A.O. Lobo. *ACS Appl. Mater. Interfaces* (2015) [Epub ahead of print].

**CRANIOFACIAL TISSUE RECONSTRUCTION
WITH MESENCHYMAL STEM CELLS DERIVED
FROM DENTAL TISSUE AND BONE MARROW**

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13.1. INTRODUCTION

In craniofacial region, there are a variety of specified tissues including bones, teeth, muscles, cartilages, blood vessels and nerves. Bones and teeth, which are parts of the craniofacial hard tissues, work as a functional unit and provide structural support, protection, sensation and allow movement. Defect and dysfunction of bones and teeth may occur due to pathological factors such as congenital malformations, progressive diseases, trauma and infections; or due to treatment procedure such as surgery. Whole or partial loss of bone or tooth structures has an enormous impact on patient's physical and psychological life. It is important to restore function and preserve esthetics during a craniofacial reconstruction to ensure patient's regaining self-esteem and maintaining a good quality of life [1].

For craniofacial reconstruction, bone grafting has become a major treatment modality in past decades. Various bone grafting materials, including autograft, allograft, xenograft, and alloplastic graft, provide a wide range of options to clinicians. Autologous grafting has been used as a gold standard in craniofacial reconstruction for years with its superior osteogenic, osteoinductive and osteoconductive properties. However, its further application is limited as a result of the potential donor site morbidity and insufficient amount of bone graft [2]. Although bone allograft materials such as demineralized bone matrix have good osteoconductive and osteoinductive capabilities, these foreign materials may induce immune rejection from the host. Immune suppression may also be caused by treatment alternatives to prevent this rejection [3]. Alloplastic grafts, which are usually synthetic materials such as hydroxyapatite and calcium carbonate, have an inferior osteoinductive and osteoconductive capability, but they can be customized to fill defects of different shapes [4].

In the last two decades, tissue engineering and regenerative medicine have been developed and advanced. Dr. Langer first defined tissue engineering as "an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function" [5]. For regenerative medicine, the principles of tissue engineering are utilized to regenerate human tissues or organs in order to restore normal functions [6]. Modern concept of tissue engineering was developed in the late 1980s, when synthetic biodegradable materials were introduced as scaffolds and stem cells technology was advanced. Since then, the cell-based tissue engineering modality along with a combination of stem cells, biomaterials and growth factors has been widely studied and applied. In this method, cells and growth factors are delivered to the defect site *via* scaffolds. Scaffolds not only support cell attachment and proliferation, but also contain and release growth factors to promote cell differentiation towards a specific lineage for tissue regeneration.

Tissue engineering using stem cells and biomaterials has been applied to the reconstruction of craniofacial bone defects. This chapter highlights the recent progress of tissue engineering in reconstruction of craniofacial bone and tooth tissues by focusing on two types of mesenchymal stem cells (MSCs): bone marrow mesenchymal stem cells (BMMSCs) and dental stem cells (DSCs).

13.2. MESENCHYMAL STEM CELLS

MSCs are a heterogeneous cell population with self-renewal, clonogenic and multipotent characteristics existing in almost all tissues. They were first isolated from hematopoietic tissues in 1974 [7]. Since then MSCs have been successfully isolated from a variety of tissues such as bone marrow, skeletal muscle, dermis, peripheral blood, adipose tissue, umbilical cord blood, amniotic fluid, cornea and tooth [8,9].

13.2.1. Origin of MSCs

MSCs have complex ontogeny. The exact histological origin of MSCs is still not well-known, mainly because of the lack of a unique MSC marker. Three possible origins of MSCs have been proposed.

a. Epithelial to mesenchymal transition (EMT)

EMT is a cellular process in which epithelial cells lose their epithelial traits and obtain mesenchymal cell properties. During EMT, cell polarization occurs and junctional structure of the epithelial cells gets lost, followed by cytoskeleton reorganization and transition to spindle shape mesenchymal-like cells [10]. Accompanying the morphological changes during EMT, the expression of epithelial related genes is down-regulated while the expression of MSC related gene is up-regulated. After the completion of EMT, the cells express MSC markers and exhibit the capability of multilineage differentiation [11-13].

b. Adventitial cells

Human vascular adventitial fibroblasts in pulmonary arteries are found to contain mesenchymal stem progenitor cells. These cells express MSC marker genes and undergo osteogenesis and adipogenesis under appropriate culture conditions [14]. Recently, a cell population characterized by CD34+ CD31- CD146- CD45- was isolated from the *tunica adventitia*. These cells expressed MSC markers and had a similar multipotency to BMMSCs [15].

c. Pericyte origin

In multiple human organs, MSCs also have a perivascular origin [8,16]. It is believed that stem cells reside in a specialized "stem cell niche" in perivascular areas, which was first discovered in bone marrow and later was confirmed in other tissues [17,18]. Pericytes are the contractile cells surrounding the endothelial cells in capillaries. Some surface markers of MSCs are expressed in

pericytes, including CD146 and PDGF-R β [19,20], and MSCs express some pericyte markers such as pericyte-associated antigen 3G5 [21], suggesting that pericytes have a potential link with MSCs. Pericytes have the ability of differentiation into osteoblasts, osteocytes, adipocytes, chondrocytes and nerve cells [22-26], indicating the potential perivascular origin of MSCs. Thus, two perivascular MSC progenitors may co-exist: adventitial cells around larger vessels and pericytes in capillaries. It should be noted that MSC-like cells can be as well derived from vascular tissues such as intervertebral discs. These cells not only express MSC markers CD146 and CD166, but also have the capability of multilineage differentiation [27-29].

13.2.2. Functions of MSCs

Although the exact mechanisms of MSCs are not fully clear yet, the functions of MSCs in tissue engineering can be concluded as the followings:

a. Homing effect

MSCs have a specific capability to migrate to injured or damaged tissue sites when they are introduced into the host body, which is defined as "homing efficiency" [30,31]. The migration is induced by a group of molecules called "homing-related molecules (HRMs)". HRMs have three major categories: (a) chemokines, such as chemokine ligand 12 (CCL-12)-chemokine receptor 4 (CXCR4), and chemokine ligand-2 (CCL-2)-chemokine receptor 2 (CCR2) [32-34]; (b) adhesion molecules, including intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) [35]; and (c) matrix metalloproteinases (MMPs) [36]. HRMs can be regulated by inflammatory cytokines. For example, tumor necrosis factor- α (TNF- α) and interleukin-1 (IL-1) up-regulate the expression of ICAM-1 and VCAM-1, leading to the MSCs chemotaxis [37,38].

b. Trophic effects

After delivered to the injured site, MSCs can be stimulated by the local micro-environment to produce trophic factors, such as insulin-like growth factor (IGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), interleukin-6 (IL-6) and CCL-2. The trophic factors can promote cell proliferation, anti-apoptosis and enhance angiogenesis [39,40]. For example, the conditioned media from mesenchymal stem cells have the effect of enhancing bone regeneration, suggesting that paracrine factors secreted by stem cells can promote osteogenesis [41].

c. Plasticity and multilineage differentiation

MSCs have the capability to differentiate into mature and functional cells of the same or different germ layer of origin, which is described as "plasticity" [42]. BMMSCs have been extensively studied, and their multipotency are proved to differentiate into different types of cells in bone, cartilage, adipose tissue and skeletal muscles [43-47]. BMMSCs can also differentiate into cardiomyocytes

[48], neural cells [49], epithelial cells [50,51], endothelial cells [52] and hepatocytes [53]. For regenerative medicine, the plasticity of MSCs provides the fundamental cellular basis. Four mechanisms of plasticity have been proposed: (a) stem cells from embryonic stage are persistent during the development and maintained in post-natal tissues; (b) "true" post-natal stem cells, such as multiple adult progenitor cells, reside in tissues [54]; (c) MSCs undergo de-differentiation, trans-differentiation or re-differentiation [55-57]; and (d) fusion of donor and host cells trigger the exchange of genetic information, leading to the MSCs trans-differentiation [58,59]. The plasticity of MSCs can be enhanced by environmental changes, stimulated by exogenous growth factors, or regulated by genetic manipulation. For example, the hypoxic condition can enhance osteogenic differentiation of BMMSCs [60], and genetically engineered MSCs with osteogenic factors bone morphogenetic protein-2 (BMP-2) promote osteogenesis [61,62].

d. Immunomodulatory effect

Studies have reported the immunosuppressive effect of MSCs [63,64]. MSCs can be stimulated to produce immunosuppressive mediators E2 by the inflammatory stimuli including nitric oxide and prostaglandin, as well as cytokines IFN- γ , TNF- α and IL-1 [65-67]. These stem cells regulate both of innate and adaptive immune systems. They are able to inhibit the maturation of monocytes [68], and also inhibit the proliferation of B cell and T cell [63,69]. Based on their immunosuppressive effect, MSCs have been suggested to be used to treat patients with immune diseases, such as systemic lupus erythematosus [70,71], rheumatoid arthritis [72] and multiple sclerosis [73]. However, higher level evidences are required to support the clinical use of MSCs as an immunosuppressive reagent, such as randomized and well controlled studies to understand their immunomodulatory effect further.

13.2.3. Isolation of MSCs

In order to isolate a population of MSCs, the first step is to separate cells from the tissue origins by either enzyme digestion or the explant culture [74]. Then the MSCs population can be isolated and identified from the entire cell population by the following methods: (a) selection based on physiological criteria including morphology, proliferation and differentiation properties; (b) immunohistochemical staining; (c) immunomagnetic bead selection; and (d) fluorescence-activated cell sorting (FACS).

Among these methods, FACS has become very popular, since it is sensitive, accurate and cost efficient. Immunohistochemistry method can help to confirm the location of MSCs in tissues. For example, immunohistochemical staining showed that a majority of STRO-1+ cells are found in the niche located in the perivascular and perineural sheath regions of the human dental pulp [75-79]; in the periodontal ligament, most cells with STRO-1+/CD146+/CD44+ are located in the perivascular regions [80].

13.3. BONE MARROW MESENCHYMAL STEM CELLS (BMMSCs)

Bone marrow contains heterogeneous populations of cells, in which only a small fraction are multipotent cells [7,43,45,81]. Two major distinct stem cell populations have been found in bone marrow: hematopoietic stem cells and mesenchymal stem cells. At present, BMMSC is one of the most well characterized postnatal stem cell populations that have been utilized for cell-based clinical therapies. BMMSCs express not only STRO-1, CD106 (VCAM-1), CD146 (MUC18), but also mesenchymal associated surface molecules CD49a, CD73, CD90, CD166, β -2 integrin (CD18), and endothelial progenitor related marker CD105 [45,82-86]. Cell flow cytometry is used to isolate different subpopulation of cells with desired stem cell characteristics with the help of single or a combination of these markers. Recently, a rare population of murine BMMSCs was discovered expressing high CD13 and stage-specific antigen I (SSEA-I). These cells, named as multipotent adult progenitor cells (MAPC), showed a very strong capacity of proliferation and differentiation towards cells originated from any of the three germ layers, indicating that MAPC had a great potential for cell-based stem cell therapy [54,87].

13.4. DENTAL STEM CELLS (DSCs)

Since the first isolation of human dental stem cells from dental pulp tissue in 2000 [75], at least five different types of mesenchymal stem cells have been isolated from tooth related tissues, including dental pulp stem cell (DPSC) [75], stem cells of human exfoliated deciduous teeth (SHED) [88], stem cells of the apical papilla (SCAP) [89], dental follicle progenitor cells (DFPC) [90] and stem cells from periodontal ligament (PDLSC) [91,92]. It has been reported that various types of DSCs exist in dental tissues such as dental pulp, periodontal ligament, apical papilla, tooth germ, dental follicle of mature and immature teeth, inflamed periapical [93,94]. Even induced bleeding during pulp regeneration procedure revealed a large amount of stem cells [95].

Similar to all other MSCs, DSCs reside in the perivascular and perineural sheath regions [76,77], and can be isolated by FACS based on the specific cell surface markers. The markers include but not limit to: positive markers such as STRO-1, CD13, CD44, CD24, CD29, CD73, CD90, CD105, CD106, CD146, Oct4, Nanog, β 2 integrin and 3G5; and negative markers such as CD14, CD34, CD45 and HLA-DR [96]. In recent studies, a subpopulation of cells expressing c-kit+/CD34+/STRO-1+ was isolated from perivascular niche of their respective tissues. These cells are believed to more accurately represent multipotent stem cells population and are therefore recommended to be used as DSCs [83,86,96-99].

DSCs have shown stem cell properties including self-renewal and multilineage differentiation. For example, under specific culture conditions *in vitro*, DPSCs can undergo osteogenic / odontogenic, adipogenic, chondrogenic or neurogenic differentiation [75,88].

13.5. COMPARISON OF BMMSCs AND DSCs

Both BMMSC and DSC are considered as a subpopulation of mesenchymal stem cells. Similarities and differences have been found between them (Table 1).

13.5.1. Gene expression profile

A similar pattern of gene expression between BMMSCs and DPSCs can be showed by microarray analysis. Shi *et al.* compared the gene expression profiles between DPSCs and BMMSCs using microarray, and found that more than 4000 known genes had a similar expression level between these two types of cells [100]. On the other side, DPSCs expressed higher level of Col18a1, IGF-2, discoidin domain tyrosine kinase 2 (DDR2), NAD(P)H menadione oxidoreductase (NMOR1), cyclin-dependent kinase 6 (CDK6), homolog 2 of drosophial large disk (DLG2); while the expression of Col1a2 and IGF binding protein 7 (IGFBP-7), were higher in BMMSCs. Moreover, no gene was expressed solely in either of these two cell types [100]. In another study by Yamada *et al.*, over 12,000 genes were compared between osteogenic induced DPSCs and BMMSCs. The result showed that after osteoinduction, DPSCs expressed higher level of ALP, DMP1 and DSPP. Cluster analysis revealed the difference of gene expressions existing in gene groups that involved cell signaling, cell communication or cell metabolism [101]. Kim *et al.* performed a GeneChip analysis to compare the gene expression profiles between STRO-1+ BMMSCs and STRO-1+ DSCs. They identified that in BMMSCs, 379 genes were up-regulated and 133 genes were down-regulated; in DPSCs, 218 genes were up-regulated and 231 genes were down-regulated [102].

13.5.2. Proteomic profile

A study used a large scale of 2-dimensional electrophoresis and liquid chromatography to compare the differential proteomic expression profiles of DPSCs and BMMSCs derived from an individual donor. Eighteen proteins were found upregulated in DPSCs relative to BMMSCs, while no protein upregulation was observed in BMMSCs compared with DPSCs [103]. Wei *et al.* identified 23 proteins related to odontoblastic differentiation of dental pulp cells (DPCs) *in vitro*, including cytoskeleton proteins, nuclear proteins, cell membrane-bound molecules, proteins involved in matrix synthesis, and metabolic enzymes [104]. Aforementioned results suggest that differences exist in the characteristic growth and development capacity of DPSCs and BMMSCs, which

needs to be considered when the cells are used for particular tissues regeneration.

13.5.3. Colony-forming unit / cell proliferation

The percentage of colony-forming cells derived from dental pulp tissue was significantly higher than that derived from bone marrow [75,105]. DPSCs exhibit a higher proliferation rate and grow faster compared to BMMSCs *in vitro* [75,93,105]. Flow cytometry study showed that DSCs contained a higher portion of cells that in G2 / M phase and lower portion of cells that in G0/G1 phase, suggesting more DSCs are undergoing mitosis [93].

13.5.4. Multilineage differentiation

a. Neural differentiation

DPSCs and BMMSCs are both capable of undergoing neural differentiation under defined conditions, which is evidenced by cell morphology change from fibroblast-like to neuron-like appearance with multipolarity and elongated processes. The differentiated cells have an increased level of neural markers nestin, β III-tubulin and NF-200 [93]. Karaöz *et al.* found that human DPSCs expressed several specific transcripts and proteins of neural stem cells and demonstrated better neural and epithelial stem cell properties than BMMSCs [106].

b. Osteogenic / odontogenic differentiation

Both DPSCs and BMMSCs are able to differentiate and form the alizarin-red positive tissues [93]. Batouli *et al.* implanted DPSC and BMMSC transplants respectively into mice, and found that BMMSCs tended to form more bone / marrow structures, whereas DPSC transplants more tended to form dentin / pulp complex. The same study also found that FGF and MMP-9 were highly expressed in the connective tissue compartment of BMMSC transplants; while dentin sialoprotein (DSP) was highly expressed in DPSC transplants [107]. In a study by Yu *et al.*, DPSCs or BMMSCs were cultured in renal capsule of rat for 14 days, and DPSCs were found to present more striking odontogenic capability than BMMSCs [108]. Zhang *et al.* seeded rat and human BMMSCs and DPSCs respectively on HA / TCP and cultured *in vitro*, or implanted them into nude mice *in vivo*. They found that *in vitro* both types of cells showed abundant cell growth and mineralization of extracellular matrix were noted, but *in vivo* BMMSCs displayed more potent capability to form mature bone-like structures than DPSCs [109].

c. Adipogenic differentiation

BMMSCs and DSCs both have capability to differentiate into oil-red O positive cells [93], although Zhang *et al.* observed that DPSCs and SCAPs had weaker capability of adipogenesis in comparison with BMMSCs [89,110].

d. Chondrogenic differentiation

Although BMMSCs and DPSCs are both able to differentiate into chondrogenic lineage [111-114], the chondrogenic potential of DPSCs appears weaker than BMMSCs [80,110].

e. Muscular differentiation

Both of BMMSCs and DPSCs can differentiate into muscular lineage and have the potential in craniofacial and cardiac repair and regeneration [115-119].

Table 1. Comparison between BMMSCs and DSCs

	BMMSCs	DSCs	Reference
General properties			
CFU	Lower (2.4–3.1 colonies / 10 ⁴ cells plated)	Higher (22–70 colonies / 10 ⁴ cellsplate d)	[75]
Proliferation rate	Lower (46 % BrdUrd-positive cells ± 1.96 SEM)	Higher (72 % BrdUrd-positive cells ± 3.48 SEM)	[75,93]
Cells in G0 / G1 phase	Higher	Lower	[93]
Cells in G2 / M phase	Lower	Higher	[93]
Surface marker			
CD44	+	+	[93]
CD29	–	+	[120]
CD13	+	+	[103]
CD90	+	+	[93]
CD105	+	+	[93]
CD106	+	–	[114]
CD14	–	–	[93,103]
CD34	–	–	[103]
CD45	–	–	[103]
STRO-1	+	+	[120]
Gene expression profile			
Nanog	+	+	[93,122]
Oct3 / 4	+	+	

	BMMSCs	DSCs	Reference
Sox2	+	+	
Col1a2	++	+	[103]
Insulin-like growth factor binding protein 7	++	+	[100]
Col 18a1	+	++	[99,102]
IGF-2	+	++	[100]
Discoidin domain tyrosine kinase 2	+	++	[100]
NADP	+	++	[100]
Cyclin-dependent kinase 6	+	++	[100]
Homolog 2 of drosophial large disk	+	++	[100]
vimentin	+	+	[123]
nestin	+	+	[123]
Proteomic expression			
Upregulation	0 protein upregulated relative to DPSC	18 proteins upregulated relative to BMMSC	[103]
Osteo/Odontogenic			
DMP1	+	++	[103]
ALP	+	++	[103]
BSP	+	+	[114]
OCN	+	+	[123]
Col1	+	+	[114]
Col3	+	+	[114]
Runx2	+	+	[123]
DSPP	–	++	[103]
Chondrogenic	++	+	[80,109]
Adipogenic	++	+	[38,109]
Neurogenic			
Nestin	+	+	[93]

	BMMSCs	DSCs	Reference
NF-200	+	+	[93]
Cytokine secretion			
VEGF	+	++	[106]
TGF- β 1	+	++	[106]

+: Expression tested positive; ++: expression tested positive and strong; -: expression tested negative

As a summary, several different gene and protein signatures have been exhibited in MSCs from bone marrow and dental tissue, which makes the stem cells competent to give rise to a specific lineage [120]. When applying these mesenchymal stem cells for craniofacial reconstruction, it is very important to recognize: (a) the differentiation capacity of cells may change along with cell passages. *In vivo* transplantation study showed that rat DPSCs at passage 1 could develop into dentin, bone and cartilage structures respectively, while DPSCs at passage 9 could only generate bone tissues [121]; (b) craniofacial bones are derived from neural crest cells, while long bones are derived from mesoderm [122]. The MSCs derived from bone marrows of these two origins may show different osteogenic potency [123,124]; (c) DSCs generally appear to be more committed to the odontogenic rather than the osteogenic lineage [79,88,125,126]; (d) DSCs from different tissue origins have difference regarding their potential in dental tissue regeneration. for example, PDLSCs are more potent to regenerate cementum / PDL-like structure, while DPSCs are more potent to regenerate dentin-pulp complex [114]; (e) The osteogenic potential of BMMSCs varies among different donors [127]; (f) Currently there is no unique master mesenchymal stem cell that is appropriate to regenerate all target tissues to treat various diseases.

13.6. REGENERATION OF CRANIOFACIAL TISSUE USING MSCs

Tissue engineering technology makes it possible to locally deliver MSCs and growth factors. MSCs and specific growth factors are first loaded into the biomaterial scaffold. The whole system is then delivered into the required sites [128,129]. This technology has been extensively applied to craniofacial tissues regeneration at both of the bench and clinical levels.

13.6.1. Regeneration of craniofacial bone tissues with BMMSCs

BMMSC is one of the most well characterized postnatal stem cells and have been used for cell based clinical therapies to regenerate hard tissues in craniofacial region.

13.6.1.1. Calvarial bone defect repair

The critical sized calvarial defect model is a well-established model to study the calvarial bone regeneration. A range of biomaterials have been used for calvarial bone defect regeneration, including silk fibroin, poly(lactic-co-glycolic acid) (PLGA), poly(vinylidene chloride), hydrogel, and minimally-invasive delivery of tissue-engineered bone (TEB). An injectable biopolymer scaffold seeded with BMMSCs and BMP-2 was used to repair rat calvarial defect, and significant regeneration of bone tissues was found at 4 weeks after implantation [130]. Lima *et al.* immobilized rat BMMSCs in the alginate beads before delivering them to bone defect area, and the results showed in accelerated bone regeneration [131]. Jiang *et al.* also reported that platelet-rich plasma scaffold loaded with BMMSCs promoted bone regeneration within 8 weeks in a rabbit model [132]. In a porcine model, a collagen scaffold cultured with BMMSCs led to the mineralization of newly formed bone tissue within 90 days after implantation [133]. BMMSCs can effectively support neovascularization mediated by endothelial cells, and promote angiogenesis for bone regeneration [134]. Furthermore, BMMSCs can be genetically modified to enhance their differentiation towards a specific lineage. Zou *et al.* reported that the transduced BMMSCs with angiogenic factor hypoxia-inducible factor-1 α (HIF-1 α) significantly improved blood vessel formation in calvarial defects [135].

13.6.1.2. Maxillary bone reconstruction

Maxillary sinus floor elevation is a common procedure for maxillary reconstruction. In a recent randomized controlled study, a bilateral sinus floor augmentation procedure was performed on 12 patients. The MSCs were seeded on BioOss® particles and then delivered to the defect sites. These cells were derived from either posterior iliac ridge or mandibular ridge in the retromolar areas. Both groups gained a sufficient volume of new bone formation, which enabled the reliable implants placement [136].

13.6.1.3. Mandibular bone reconstruction

Mandible reconstruction by tissue engineering has been reported on various animal models. In a study, BMMSCs were loaded on hydroxyapatite / tricalcium phosphate (PCL / TCP) to repair the mandibular defect in dogs, and significant bone formation was noted on day 21 [137]. To achieve vertical bone augmentation, Khojasteh *et al.* also loaded BMMSCs on PCL / TCP before implanting the system into mandible, and significant bone formation was observed in 8 weeks [138]. In another study, BMMSCs were loaded into PLGA scaffold and cultured for 10 days before being implanted into a mandibular defect site in minipig. The defect site was filled with bone-like tissues in 6 weeks [139]. Recently a clinical case of mandible reconstruction has been reported by Warnke *et al.*, in which a titanium mesh transplant was fabricated

by CT scanning and computer-aided design. The mesh was loaded with bone marrow and supplemented with BMP-7, followed by implantation into *latissimus dorsi* muscle for 7 weeks. It was then transplanted as a free bone-muscle flap to repair mandibular bone defect [140,141]. Hernández-Alfaro *et al.* reported another clinical case, in which bone marrow aspirate from the iliac crest was used to treat patient with mandibular bone defect. Bone marrow aspirate was supplemented with BMP-7 and seeded on a bovine xenograft blocks. The results showed adequate bone formation and recovery of esthetics and function [142].

13.6.1.4. Alveolar bone reconstruction

In secondary alveoloplasty, mesenchymal stem cells from a posterior iliac bone can be mounted on a biphasic scaffold combined with platelet derived growth factor (PDGF). In a recent study, the triads were placed into the alveolar cleft defects in patients. About 51.3 % of bone re-growth was observed in 3 months after the surgery [143].

13.6.2. Regeneration of craniofacial bone tissues with DSCs

DPCs provide an alternative MSC source to reconstruct craniofacial bone tissue. Otaki *et al.* reported that by mixing dental pulp cells with HA/TCP and subcutaneously implanting them to the immunocompromised mice, bone tissue was regenerated [144]. Yamada *et al.* demonstrated that stem cells from deciduous teeth mixed with platelet-rich plasma scaffold have the ability to form new bone to repair a significant osseous defect [145]. Tour *et al.* used periodontal ligament stem cells seeded on an extracellular matrix modified HA scaffold to repair calvarial critical-sized defect, resulting in a significant improvement of calvarial bone reparation [146]. In a clinical study, DPSCs were seeded on a collagen sponge scaffold to repair alveolar ridge defect, and optimal bone regeneration was observed one year after the grafting, indicating the combination of DPSCs with collagen sponge biocomplex completely repair human mandible bone defects [147].

13.6.3. Regeneration of dental tissue with BMMSCs

13.6.3.1. Regeneration of periodontium

Kawaguchi *et al.* demonstrated that new cementum, alveolar bone and periodontal ligament in class III periodontal defects in dogs could be regenerated by transplantations of *ex vivo* expanded autologous BMMSCs. A 20 % increase in new cementum length and bone area was revealed by morphometric analysis [148]. In a subsequent study, autologous BMMSCs with a collagen scaffold were transplanted to treat patients with periodontal osseous defects. The results showed a significantly improved bone formation [149]. Zhou *et al.* reported engraftments of BMMSCs were differentiated into

periodontal specific cells when BMMSCs were delivered to the injured dental tissue site, suggesting that BMMSCs may communicate with surrounding dental tissues to become tissue-specific mesenchymal progenitor cells, and participate into the regeneration process [150].

13.6.3.2. Regeneration of whole tooth

Ohazama *et al.* reported using cultured stem cells could induce a significant progress toward the creation of tissue-engineered embryonic tooth *primordia*. Various mixtures of non-dental-derived mesenchymal cells, such as embryonic stem cells, neural stem cells, and adult bone marrow cells, with embryonic oral epithelium cells were detected and transplanted into the renal capsules of adult mice. All mixtures led to the development of a tooth structure and bone. Interestingly, the fact was noted that the host tissues made no contribution to the donor tissue. Moreover, transfer of embryonic tooth *primordia* into the adult jaw resulted in the development of tooth structures, indicating that an embryonic *primordium* can develop in its adult environment [151].

Although it is believed that the ultimate goal of tooth regeneration is to develop a whole bioengineered tooth that is fully functional, at the current stage, growing a complete bioengineered tooth from a single kind of cells still remains very challenging [152].

13.6.4. Regeneration of dental tissues with DSCs

In periodontal field, guided bone regeneration (GBR) and guided tissue regeneration (GTR) are surgical procedures that utilize a barrier membrane to prevent the periodontal epithelium ingrowth, and to maintain the space for bone and tissue regeneration [153]. Various resorbable or nonresorbable membranes along with bone graft materials have been used for these procedures [154-162]. Additional growth factors can be added to scaffold to promote their regeneration capability [163]. In endodontic field, the routine root canal treatment for a necrotic tooth is very challenging, especially in the cases of immature permanent teeth with open apex. As a new treatment modality, regenerative endodontics is able to create and deliver tissues to replace diseased and missing pulp. The clinical procedure of regenerative endodontics was first described by Dr. Nygaard-Ostby [164] and further developed by Dr. Martin Trope [165]. Since then techniques and materials have been advanced to promote continued root formation and apical closure [166]. The principles of regenerative endodontics are to initiate bleeding into the root canal through mechanical irritation of the apex, thus producing a blood clot to form a scaffold. Stem cells from apical papilla were meanwhile introduced into the root canal by bleeding [95]. Numerous clinical case reports have shown the successful regain of root length and width [167,168]. Advancements have been made in regenerative endodontics *via* the tissue engineering technology. Huang *et al.* observed that DPSCs implantation in a mechanically enlarged root

canal led to the formation of dentin-pulp complex [169]. Recently, several novel scaffolds have been designed for pulp regeneration, such as the self-assembling peptide amphiphile nanofibers [170], and the electrospun nanocomposite composed of polydioxanone and halloysite nanotubes [171]. These scaffold materials have greatly promoted the tissue regeneration.

13.7. FUTURE PERSPECTIVE

In craniofacial hard tissue reconstruction, the future advancement of tissue engineering will continuously be centralized on three essential components: mesenchymal stem cells, scaffolds and growth factors. Before animal studies and preclinical models further contribute to the clinical practice of regeneration, it is necessary to address several major challenges.

13.7.1. Source of stem cells

Clinical application of allogeneic MSCs is limited by the potential immunosuppressive effect from the host. Even the MSCs with low immunogenic profile *in vitro* can still induce immune response when they are transplanted *in vivo* [172]. Autologous stem cells from the same patient are ideal to avoid immune rejection. The concept of "stem cell banking" has been raised, and it will potentially provide an essential infrastructure for cell-based tissue regeneration [173]. However, the effectiveness and economical aspects of stem cell banking, especially dental stem cell banking, need further evaluation. Recently researches on the induced pluripotent stem cells (iPS cells) from somatic [174,175] and dental tissues have advanced, and therefore provide an alternative source of MSCs [176].

13.7.2. Microbial control

Microbial control in oral cavity will remain as a major challenge for the dental tissues regeneration since microorganism invasion and biofilm formation are the fundamental etiologies of oral diseases, especially endodontic and periodontal diseases. A higher level of disinfection may be required in regenerative endodontics. A variety of antibacterial irrigants have been used in clinic, including sodium hypochlorite, chlorhexidine, ethylene diamine tetraacetic acid and iodine potassium iodide. As an alternative, mixture of multiple antibiotics has also been introduced to the clinical procedure of dental pulp regeneration [163]. It should be noted that the success of tissue engineering requires the survival and proliferation of stem cells. Therefore, the goal of microbial control should be to maximize the antimicrobial effect but meanwhile to minimize the harmful effects on stem cells [177].

13.7.3. Biomaterial scaffold

In tissue engineering, biomaterial based scaffolds play a critical role. Scaffolds are used to seed cells and act as extracellular matrix. The ultimate fate of stem cells is determined by the properties of scaffolds. Recently, the dynamic reciprocity between the MSCs and scaffolds has received increased attention. The reciprocity is not only referred to the chemical or biological characteristics of scaffolds, but also linked to its mechanical characteristics [178-181]. Scaffolds are used to integrate and release growth factors to guide stem cells differentiation. During stem cells differentiation, specific growth factors may be required only during a specific period of time and at a specific location. Therefore, obtaining the temporal and spatial control of growth factors release is still a challenge for designing a new scaffold in tissue engineering.

13.7.4. Regulation of stem cell differentiation

Currently BMSCs are the most widely used stem cells in craniofacial reconstruction [30,182,183], yet DSCs will greatly contribute to this field due to their origination from the same region and more commitment to the odontogenic lineage [79,88,125,126]. However, the detailed molecular signaling mechanisms of DSCs differentiation are still not fully clear. During stem cell differentiation towards a specific cell lineage, it is of great importance to understand the sequential activation or inactivation of signal cascades in order to help and promote tissue regeneration [184].

13.7.5. Risk of using stem cells

Stem cell laden scaffolds are not totally risk free but hold potential for malignancy. Currently most researchers focus on how to regenerate tissue. The next challenges will be how to control the process of regeneration, how to command the over-regeneration, and how to prevent the malignant transformation of cells. The genetic stability of these cells must be maintained during their differentiation.

13.8. CONCLUSION

Tissue engineering technology has been applied to the reconstruction of craniofacial tissue. The reconstruction of craniofacial tissue with MSCs has provided an alternative treatment modality, and various preclinical studies and case reports have proved its high potential of success. Stem cell-based tissue engineering in craniofacial reconstruction is promising, and it may finally replace autologous bone grafting to improve the life quality of patients. However, several challenges are still remaining. Successful craniofacial tissue regeneration depends on the appropriate selection of the source of stem cells. Dental tissue regeneration especially tooth regeneration needs the interaction

between epithelial and mesenchymal stem cells. It is challenging to control and guide the differentiation of MSCs towards the target craniofacial tissue, meanwhile to prevent their transformation during the differentiation.

The optimal combination of stem cells, growth factor and biomaterial scaffold is the current focus in order to achieve more advanced tissue regeneration. In the field of craniofacial tissue regeneration, researchers who are utilizing tissue engineering technology still face two major challenges: reestablishment of effective vascularization and functionality regaining of the bioengineered constructs. Various strategies have been proposed, including growth factor delivery, three dimensional culturing systems and biomimetic scaffold design. The spatial and temporal control of growth factors release from biomaterial scaffold is also a hot area of research. Furthermore, the translation of the cell-based technology from *in vitro* to *in vivo* is still a challenge. After transferring MSCs from *in vitro* to *in vivo* microenvironment, the interaction of MSCs and the microenvironment they reside, and the fate of stem cells *in vivo* need to be further studied. Safety precautions and regulations should also be considered before clinical application.

REFERENCES

1. M.K. Jeffcoat. *J. Bone Miner. Res.* **8**(2) (1993) S467–S473.
2. T.W. Bauer, G.F. Muschler. *Clin. Orthop. Relat. Res.* (2000) 10–27.
3. B.L. Eppley, W.S. Pietrzak, M.W. Blanton. *J. Craniofac. Surg.* **16** (2005) 981–989.
4. S. Sukumar, I. Drizhal. *Acta Medica (Hradec Kralove)* **51** (2008) 203–207.
5. R. Langer, J.P. Vacanti. *Science* **260** (1993) 920–926.
6. A. Atala, *Principles of regenerative medicine, 1st Ed.*, Elsevier/Academic Press, Amsterdam, Boston, 2008.
7. A.J. Friedenstein, R.K. Chailakhyan, N.V. Latsinik, A.F. Panasyuk, I.V. Keiliss-Borok. *Transplantation* **17** (1974) 331–340.
8. M. Crisan, S. Yap, L. Casteilla, C.W. Chen, M. Corselli, T.S. Park, G. Andriolo, B. Sun, B. Zheng, L. Zhang, C. Norotte, P.N. Teng, J. Traas, R. Schugar, B.M. Deasy, S. Badylak, H.J. Buhring, J.P. Giacobino, L. Lazzari, J. Huard, B. Peault. *Cell Stem Cell* **3** (2008) 301–313.
9. N. Pinnamaneni, J.L. Funderburgh. *Stem Cells* **30** (2012) 1059–1063.
10. E.D. Hay. *Acta Anat. (Basel)* **154** (1995) 8–20.
11. V.L. Battula, K.W. Evans, B.G. Hollier, Y. Shi, F.C. Marini, A. Ayyanan, R.Y. Wang, C. Briskin, R. Guerra, M. Andreeff, S.A. Mani. *Stem Cells* **28** (2010) 1435–1445.
12. C. Limbert, R. Ebert, T. Schilling, G. Path, P. Benisch, L. Klein-Hitpass, J. Seufert, F. Jakob. *Stem Cells Dev.* **19** (2010) 679–691.
13. D. Rubio, S. Garcia, T. De la Cueva, M.F. Paz, A.C. Lloyd, A. Bernad, J. Garcia-Castro. *Exp. Cell Res.* **314** (2008) 691–698.
14. A. Hoshino, H. Chiba, K. Nagai, G. Ishii, A. Ochiai. *Biochem. Biophys. Res. Commun.* **368** (2008) 305–310.
15. M. Corselli, C.W. Chen, B. Sun, S. Yap, J.P. Rubin, B. Peault. *Stem Cells Dev.* **21** (2012) 1299–1308.

16. B. Sacchetti, A. Funari, S. Michienzi, S. Di Cesare, S. Piersanti, I. Saggio, E. Tagliafico, S. Ferrari, P.G. Robey, M. Riminucci, P. Bianco. *Cell* **131** (2007) 324–336.
17. T. Yin, L. Li. *J. Clin. Invest.* **116** (2006) 1195–1201.
18. S. Yoshida, M. Sukeno, Y. Nabeshima. *Science* **317** (2007) 1722–1726.
19. K.E. Schwab, C.E. Gargett. *Hum. Reprod.* **22** (2007) 2903–2911.
20. W.A. Franklin, W.H. Christison, M. Colley, A.G. Montag, J.K. Stephens, C.E. Hart. *Cancer Res.* **50** (1990) 6344–6348.
21. W.S. Khan, A.B. Adesida, S.R. Tew, E.T. Lowe, T.E. Hardingham. *J. Orthop. Res.* **28** (2010) 834–840.
22. C. Farrington-Rock, N.J. Crofts, M.J. Doherty, B.A. Ashton, C. Griffin-Jones, A.E. Canfield. *Circulation* **110** (2004) 2226–2232.
23. P. Dore-Duffy, A. Katychew, X. Wang, E. Van Buren. *J. Cereb. Blood Flow Metab.* **26** (2006) 613–624.
24. X. Cai, Y. Lin, P.V. Hauschka, B.E. Grottkau. *Biol. Cell* **103** (2011) 435–447.
25. X. Cai, Y. Lin, C.C. Friedrich, C. Neville, I. Pomerantseva, C.A. Sundback, Z. Zhang, J.P. Vacanti, P.V. Hauschka, B.E. Grottkau. *Stem Cell Rev.* **5** (2009) 437–445.
26. M. Corselli, C.W. Chen, M. Crisan, L. Lazzari, B. Peault. *Arterioscler. Thromb. Vasc. Biol.* **30** (2010) 1104–1109.
27. H. Henriksson, M. Thormemo, C. Karlsson, O. Hagg, K. Junevik, A. Lindahl, H. Brisby. *Spine (Phila Pa 1976)* **34** (2009) 2278–2287.
28. M.V. Risbud, A. Guttapalli, T.T. Tsai, J.Y. Lee, K.G. Danielson, A.R. Vaccaro, T.J. Albert, Z. Gazit, D. Gazit, I.M. Shapiro. *Spine (Phila Pa 1976)* **32** (2007) 2537–2544.
29. J. Feng, A. Mantesso, C. De Bari, A. Nishiyama, P.T. Sharpe. *Proc. Natl. Acad. Sci. USA* **108** (2011) 6503–6508.
30. E.M. Horwitz, P.L. Gordon, W.K. Koo, J.C. Marx, M.D. Neel, R.Y. McNall, L. Muul, T. Hofmann. *Proc. Natl. Acad. Sci. USA* **99** (2002) 8932–8937.
31. A. Mahmood, D. Lu, M. Lu, M. Chopp. *Neurosurgery* **53** (2003) 697–702; discussion 702–693.
32. R.F. Wynn, C.A. Hart, C. Corradi-Perini, L. O'Neill, C.A. Evans, J.E. Wraith, L.J. Fairbairn, I. Bellantuono. *Blood* **104** (2004) 2643–2645.
33. Z. Cheng, L. Ou, X. Zhou, F. Li, X. Jia, Y. Zhang, X. Liu, Y. Li, C.A. Ward, L.G. Melo, D. Kong. *Mol. Ther.* **16** (2008) 571–579.
34. F. Belema-Bedada, S. Uchida, A. Martire, S. Kostin, T. Braun. *Cell Stem Cell* **2** (2008) 566–575.
35. B. Ruster, S. Gottig, R.J. Ludwig, R. Bistran, S. Muller, E. Seifried, J. Gille, R. Henschler. *Blood* **108** (2006) 3938–3944.
36. C. Ries, V. Egea, M. Karow, H. Kolb, M. Jochum, P. Neth. *Blood*. **109** (2007) 4055–4063.
37. M. Shi, J. Li, L. Liao, B. Chen, B. Li, L. Chen, H. Jia, R.C. Zhao. *Haematologica* **92** (2007) 897–904.
38. G. Ren, X. Zhao, L. Zhang, J. Zhang, A. L'Huillier, W. Ling, A.I. Roberts, A.D. Le, S. Shi, C. Shao, Y. Shi. *J. Immunol.* **184** (2010) 2321–2328.
39. A.I. Caplan, J.E. Dennis. *J. Cell Biochem.* **98** (2006) 1076–1084.
40. G. Xu, Y. Zhang, L. Zhang, G. Ren, Y. Shi. *Biochem. Biophys. Res. Commun.* **361** (2007) 745–750.

41. M. Osugi, W. Katagiri, R. Yoshimi, T. Inukai, H. Hibi, M. Ueda. *Tissue Eng. Part A* **18** (2012) 1479–1489.
42. A.Y. Leung, E.M. Mendenhall, T.T. Kwan, R. Liang, C. Eckfeldt, E. Chen, M. Hammerschmidt, S. Grindley, S.C. Ekker, C.M. Verfaillie. *Dev. Biol.* **277** (2005) 235–254.
43. H. Castro-Malaspina, R.E. Gay, G. Resnick, N. Kapoor, P. Meyers, D. Chiarieri, S. McKenzie, H.E. Broxmeyer, M.A. Moore. *Blood* **56** (1980) 289–301.
44. D.J. Prockop. *Science* **276** (1997) 71–74.
45. M.F. Pittenger, A.M. Mackay, S.C. Beck, R.K. Jaiswal, R. Douglas, J.D. Mosca, M.A. Moorman, D.W. Simonetti, S. Craig, D.R. Marshak, *Science* **284** (1999) 143–147.
46. B.E. Grottkau, P.P. Purudappa, Y.F. Lin *Int. J. Oral Sci.* **2** (2010) 21–27.
47. G. Ferrari, G. Cusella-De Angelis, M. Coletta, E. Paolucci, A. Stornaiuolo, G. Cossu, F. Mavilio. *Science* **279** (1998) 1528–1530.
48. S. Makino, K. Fukuda, S. Miyoshi, F. Konishi, H. Kodama, J. Pan, M. Sano, T. Takahashi, S. Hori, H. Abe, J. Hata, A. Umezawa, S. Ogawa. *J. Clin. Invest.* **103** (1999) 697–705.
49. M.A. Eglitis, E. Mezey. *Proc. Natl. Acad. Sci. USA* **94** (1997) 4080–4085.
50. D.S. Krause, N.D. Theise, M.I. Collector, O. Henegariu, S. Hwang, R. Gardner, S. Neutzel, S.J. Sharkis. *Cell* **105** (2001) 369–377.
51. E.A. Jones, S.E. Kinsey, A. English, R.A. Jones, L. Straszynski, D.M. Meredith, A.F. Markham, A. Jack, P. Emery, D. McGonagle. *Arthritis Rheum.* **46** (2002) 3349–3360.
52. M. Tomita, H. Yamada, Y. Adachi, Y. Cui, E. Yamada, A. Higuchi, K. Minamino, Y. Suzuki, M. Matsumura, S. Ikehara. *Stem Cells* **22** (2004) 21–26.
53. B.E. Petersen, W.C. Bowen, K.D. Patrene, W.M. Mars, A.K. Sullivan, N. Murase, S.S. Boggs, J.S. Greenberger, J.P. Goff. *Science* **284** (1999) 1168–1170.
54. Y. Jiang, B.N. Jahagirdar, R.L. Reinhardt, R.E. Schwartz, C.D. Keene, X.R. Ortiz-Gonzalez, M. Reyes, T. Lenvik, T. Lund, M. Blackstad, J. Du, S. Aldrich, A. Lisberg, W.C. Low, D.A. Largaespada, C.M. Verfaillie. *Nature* **418** (2002) 41–49.
55. J.P. Brockes. *Science* **276** (1997) 81–87.
56. U. Lakshminpathy, C. Verfaillie. *Blood Rev.* **19** (2005) 29–38.
57. K. Hochedlinger, R. Jaenisch. *Nature* **441** (2006) 1061–1067.
58. N. Terada, T. Hamazaki, M. Oka, M. Hoki, D.M. Mastalerz, Y. Nakano, E.M. Meyer, L. Morel, B.E. Petersen, E.W. Scott. *Nature* **416** (2002) 542–545.
59. Q.L. Ying, J. Nichols, E.P. Evans, A.G. Smith. *Nature* **416** (2002) 545–548.
60. T.L. Yew, T.F. Huang, H.L. Ma, Y.T. Hsu, C.C. Tsai, C.C. Chiang, W.M. Chen, S.C. Hung. *J. Orthop. Res.* **30** (2012) 1213–1220.
61. G. Pelled, J.G. Snedeker, A. Ben-Arav, S. Rigozzi, Y. Zilberman, N. Kimelman-Bleich, Z. Gazit, R. Muller, D. Gazit. *J. Orthop. Res.* **30** (2012) 1932–1939.
62. S. Jo, S. Kim, T.H. Cho, E. Shin, S.J. Hwang, I. Noh. *J. Biomed. Mater. Res. A* **101** (2013) 892–901.
63. A. Uccelli, D.J. Prockop. *Curr. Opin. Immunol.* **22** (2010) 768–774.
64. K. Nemeth, A. Leelahavanichkul, P.S. Yuen, B. Mayer, A. Parmelee, K. Doi, P.G. Robey, K. Leelahavanichkul, B.H. Koller, J.M. Brown, X. Hu, I. Jelinek, R.A. Star, E. Mezey. *Nat. Med.* **15** (2009) 42–49.

65. G. Ren, L. Zhang, X. Zhao, G. Xu, Y. Zhang, A.I. Roberts, R.C. Zhao, Y. Shi. *Cell Stem Cell* **2** (2008) 141–150.
66. M. Krampera, L. Cosmi, R. Angeli, A. Pasini, F. Liotta, A. Andreini, V. Santarlaschi, B. Mazzinghi, G. Pizzolo, F. Vinante, P. Romagnani, E. Maggi, S. Romagnani, F. Annunziato. *Stem Cells* **24** (2006) 386–398.
67. P.R. Crisostomo, Y. Wang, T.A. Markel, M. Wang, T. Lahm, D.R. Meldrum. *Am. J. Physiol. Cell Physiol.* **294** (2008) C675–C682.
68. K. English, A. French, K.J. Wood. *Cell Stem Cell* **7** (2010) 431–442.
69. S. Glennie, I. Soeiro, P.J. Dyson, E.W. Lam, F. Dazzi. *Blood* **105** (2005) 2821–2827.
70. J. Liang, H. Zhang, B. Hua, H. Wang, L. Lu, S. Shi, Y. Hou, X. Zeng, G.S. Gilkeson, L. Sun. *Ann. Rheum. Dis.* **69** (2010) 1423–1429.
71. D. Shi, D. Wang, X. Li, H. Zhang, N. Che, Z. Lu, L. Sun. *Clin. Rheumatol.* **31** (2012) 841–846.
72. B. Zhou, J. Yuan, Y. Zhou, M. Ghawji, Jr., Y.P. Deng, A.J. Lee, U. Nair, A.H. Kang, D.D. Brand, T.J. Yoo. *Clin. Immunol.* **141** (2011) 328–337.
73. B. Yamout, R. Hourani, H. Salti, W. Barada, T. El-Hajj, A. Al-Kutoubi, A. Herlopian, E.K. Baz, R. Mahfouz, R. Khalil-Hamdan, N.M. Kreidieh, M. El-Sabban, A. Bazarbachi. *J. Neuroimmunol.* **227** (2010) 185–189.
74. P. Hilken, P. Gervois, Y. Fanton, J. Vanormelingen, W. Martens, T. Struys, C. Politis, I. Lambrichts, A. Bronckaers. *Cell Tissue Res.* (2013).
75. S. Gronthos, M. Mankani, J. Brahimi, P.G. Robey, S. Shi. *Proc. Natl. Acad. Sci. USA* **97** (2000) 13625–13630.
76. A.C. Zannettino, S. Paton, A. Arthur, F. Khor, S. Itescu, J.M. Gimble, S. Gronthos. *J. Cell Physiol.* **214** (2008) 413–421.
77. S.C. Chen, V. Marino, S. Gronthos, P.M. Bartold. *J. Periodontal Res.* **41** (2006) 547–553.
78. G. Laino, A. Graziano, R. d'Aquino, G. Pirozzi, V. Lanza, S. Valiante, A. De Rosa, F. Naro, E. Vivarelli, G. Papaccio. *J. Cell Physiol.* **206** (2006) 693–701.
79. G. Laino, R. d'Aquino, A. Graziano, V. Lanza, F. Carinci, F. Naro, G. Pirozzi, G. Papaccio. *J. Bone Miner. Res.* **20** (2005) 1394–1402.
80. W. Sonoyama, Y. Liu, D. Fang, T. Yamaza, B.M. Seo, C. Zhang, H. Liu, S. Gronthos, C.Y. Wang, S. Wang, S. Shi. *PLoS One* **1** (2006) e79.
81. N. Falla, V. Van, J. Bierkens, B. Borremans, G. Schoeters, U. Van Gorp. *Blood* **82** (1993) 3580–3591.
82. P.J. Simmons, B. Torok-Storb. *Blood* **78** (1991) 55–62.
83. S. Gronthos, A.C. Zannettino, S.J. Hay, S. Shi, S.E. Graves, A. Kortessidis, P.J. Simmons. *J. Cell Sci.* **116** (2003) 1827–1835.
84. R.J. Filshie, A.C. Zannettino, V. Makrynika, S. Gronthos, A.J. Henniker, L.J. Bendall, D.J. Gottlieb, P.J. Simmons, K.F. Bradstock. *Leukemia* **12** (1998) 414–421.
85. P.A. Conget, J.J. Minguell. *J. Cell Physiol.* **181** (1999) 67–73.
86. Y. Miura, M. Miura, S. Gronthos, M.R. Allen, C. Cao, T.E. Uveges, Y. Bi, D. Ehrlich, A. Kortessidis, S. Shi, L. Zhang. *Proc. Natl. Acad. Sci. USA* **102** (2005) 14022–14027.
87. Y. Jiang, B. Vaessen, T. Lenvik, M. Blackstad, M. Reyes, C.M. Verfaillie. *Exp. Hematol.* **30** (2002) 896–904.
88. M. Miura, S. Gronthos, M. Zhao, B. Lu, L.W. Fisher, P.G. Robey, S. Shi. *Proc. Natl. Acad. Sci. USA* **100** (2003) 5807–5812.

89. W. Sonoyama, Y. Liu, T. Yamaza, R.S. Tuan, S. Wang, S. Shi, G.T. Huang. *J. Endod.* **34** (2008) 166–171.
90. C. Morsczeck, W. Gotz, J. Schierholz, F. Zeilhofer, U. Kuhn, C. Mohl, C. Sippel, K.H. Hoffmann. *Matrix Biol.* **24** (2005) 155–165.
91. B.M. Seo, M. Miura, S. Gronthos, P.M. Bartold, S. Batouli, J. Brahimi, M. Young, P.G. Robey, C.Y. Wang, S. Shi. *Lancet* **364** (2004) 149–155.
92. B.M. Seo, M. Miura, W. Sonoyama, C. Coppe, R. Stanyon, S. Shi. *J. Dent. Res.* **84** (2005) 907–912.
93. Y. Tamaki, T. Nakahara, H. Ishikawa, S. Sato. *Odontology* (2012).
94. K.M. Hargreaves, A. Diogenes, F.B. Teixeira. *Pediatr. Dent.* **35** (2013) 129–40.
95. T.W. Lovelace, M.A. Henry, K.M. Hargreaves, A. Diogenes. *J. Endod.* **37** (2011) 133–138.
96. S. Shi, S. Gronthos. *J. Bone Miner. Res.* **18** (2003) 696–704.
97. S.J. Greco, K. Liu, P. Rameshwar. *Stem Cells* **25** (2007) 3143–3154.
98. M. Dominici, K. Le Blanc, I. Mueller, I. Slaper-Cortenbach, F. Marini, D. Krause, R. Deans, A. Keating, D. Prockop, E. Horwitz. *Cytotherapy* **8** (2006) 315–317.
99. V.L. Battula, P.M. Bareiss, S. Treml, S. Conrad, I. Albert, S. Hojak, H. Abele, B. Schewe, L. Just, T. Skutella, H.J. Buhning. *Differentiation* **75** (2007) 279–291.
100. S. Shi, P.G. Robey, S. Gronthos. *Bone* **29** (2001) 532–539.
101. Y. Yamada, A. Fujimoto, A. Ito, R. Yoshimi, M. Ueda. *Biomaterials* **27** (2006) 3766–3781.
102. S.H. Kim, Y.S. Kim, S.Y. Lee, K.H. Kim, Y.M. Lee, W.K. Kim, Y.K. Lee. *J. Periodontal Implant. Sci.* **41** (2011) 192–200.
103. K.M. Mrozik, P.S. Zilm, C.J. Bagley, S. Hack, P. Hoffmann, S. Gronthos, P.M. Bartold. *Stem Cells Dev.* **19** (2010) 1485–1499.
104. X. Wei, L. Wu, J. Ling, L. Liu, S. Liu, W. Liu, M. Li, Y. Xiao. *J. Endod.* **34** (2008) 1077–1084.
105. D.L. Alge, D. Zhou, L.L. Adams, B.K. Wyss, M.D. Shadday, E.J. Woods, T.M. Gabriel Chu, W.S. Goebel. *J. Tissue Eng. Regen. Med.* **4** (2010) 73–81.
106. E. Karaoz, P.C. Demircan, O. Saglam, A. Aksoy, F. Kaymaz, G. Duruksu. *Histochem. Cell Biol.* **136** (2011) 455–473.
107. S. Batouli, M. Miura, J. Brahimi, T.W. Tsutsui, L.W. Fisher, S. Gronthos, P.G. Robey, S. Shi. *J. Dent. Res.* **82** (2003) 976–981.
108. J. Yu, Y. Wang, Z. Deng, L. Tang, Y. Li, J. Shi, Y. Jin. *Biol. Cell* **99** (2007) 465–474.
109. W. Zhang, X.F. Walboomers, G.J. van Osch, J. van den Dolder, J.A. Jansen. *Tissue Eng. Part A* **14** (2008) 285–294.
110. W. Zhang, X.F. Walboomers, S. Shi, M. Fan, J.A. Jansen. *Tissue Eng.* **12** (2006) 2813–2823.
111. O. Gurevitch, B.G. Kurkalli, T. Prigozhina, J. Kasir, A. Gaft, S. Slavin. *Stem Cells* **21** (2003) 588–597.
112. P.G. Robey, P. Bianco. *J. Am. Dent. Assoc.* **137** (2006) 961–972.
113. S. Saha, J. Kirkham, D. Wood, S. Curran, X.B. Yang. *Cell Tissue Res.* **352** (2013) 495–507.
114. G.T. Huang, S. Gronthos, S. Shi. *J. Dent. Res.* **88** (2009) 792–806.
115. G. Pagni, D. Kaigler, G. Rasperini, G. Avila-Ortiz, R. Bartel, W.V. Giannobile. *Adv. Drug Deliv. Rev.* **64** (2012) 1310–1319.
116. F. Schneider, F. Saucy, R. de Blic, J. Dai, F. Mohand, H. Rouard, J.B. Ricco, J.P. Becquemin, M. Gervais, E. Allaire. *Eur. J. Vasc. Endovasc. Surg.* **45** (2013) 666–672.

117. D. Dey, L. Han, M. Bauer, F. Sanada, A. Oikonomopoulos, T. Hosoda, K. Unno, P. De Almeida, A. Leri, J.C. Wu. *Circ. Res.* **112** (2013) 1253–1262.
118. R. Nakatsuka, T. Nozaki, Y. Uemura, Y. Matsuoka, Y. Sasaki, M. Shinohara, K. Ohura, Y. Sonoda. *Arch. Oral Biol.* **55** (2010) 350–357.
119. L. Spath, V. Rotilio, M. Alessandrini, G. Gambarà, L. De Angelis, M. Mancini, T.A. Mitsiadis, E. Vivarelli, F. Naro, A. Filippini, G. Papaccio. *J. Cell Mol. Med.* **14** (2010) 1635–1644.
120. N.H. Abu Kasim, V. Govindasamy, N. Gnanasegaran, S. Musa, P.J. Pradeep, T.C. Sriyaya, Z.A. Aziz. *J. Tissue Eng. Regen. Med.* (2012).
121. J. Yu, H. He, C. Tang, G. Zhang, Y. Li, R. Wang, J. Shi, Y. Jin. *BMC Cell Biol.* **11** (2010) 32.
122. Y. Chai, X. Jiang, Y. Ito, P. Bringas, Jr., J. Han, D.H. Rowitch, P. Soriano, A.P. McMahon, H.M. Sucov. *Development* **127** (2000) 1671–1679.
123. T. Matsubara, K. Suardita, M. Ishii, M. Sugiyama, A. Igarashi, R. Oda, M. Nishimura, M. Saito, K. Nakagawa, K. Yamanaka, K. Miyazaki, M. Shimizu, U.K. Bhawal, K. Tsuji, K. Nakamura, Y. Kato. *J. Bone Miner. Res.* **20** (2005) 399–409.
124. S.O. Akintoye, T. Lam, S. Shi, J. Brahim, M.T. Collins, P.G. Robey. *Bone* **38** (2006) 758–768.
125. I. Kerkis, A. Kerkis, D. Dozortsev, G.C. Stukart-Parsons, S.M. Gomes Massironi, L.V. Pereira, A.I. Caplan, H.F. Cerruti. *Cells Tissues Organs* **184** (2006) 105–116.
126. A. Arthur, G. Rychkov, S. Shi, S.A. Koblar, S. Gronthos. *Stem Cells* **26** (2008) 1787–1795.
127. D.G. Phinney, G. Kopen, W. Richter, S. Webster, N. Tremain, D.J. Prockop. *J. Cell Biochem.* **75** (1999) 424–436.
128. J.E. Dennis, E.K. Konstantakos, D. Arm, A.I. Caplan. *Biomaterials* **19** (1998) 1323–1328.
129. H. Ohgushi, N. Kotobuki, H. Funaoka, H. Machida, M. Hirose, Y. Tanaka, Y. Takakura. *Biomaterials* **26** (2005) 4654–4661.
130. S.J. Stephan, S.S. Tholpady, B. Gross, C.E. Petrie-Aronin, E.A. Botchway, L.S. Nair, R.C. Ogle, S.S. Park. *Laryngoscope* **120** (2010) 895–901.
131. A.C. Lima, P. Batista, T.A. Valente, A.S. Silva, I.J. Correia, J.F. Mano. *Tissue Eng. Part A* **19** (2013) 1175–1187.
132. Z.Q. Jiang, H.Y. Liu, L.P. Zhang, Z.Q. Wu, D.Z. Shang. *Oral Surg. Oral Med. Oral Pathol. Oral Radiol.* **113** (2012) 327–333.
133. P. Stockmann, J. Park, C. von Wilmowsky, E. Nkenke, E. Felszeghy, J.F. Dehner, C. Schmitt, C. Tudor, K.A. Schlegel. *J. Craniomaxillofac. Surg.* **40** (2012) 310–320.
134. S. Koob, N. Torio-Padron, G.B. Stark, C. Hannig, Z. Stankovic, G. Finkenzeller. *Tissue Eng. Part A* **17** (2011) 311–321.
135. D. Zou, Z. Zhang, J. He, K. Zhang, D. Ye, W. Han, J. Zhou, Y. Wang, Q. Li, X. Liu, X. Zhang, S. Wang, J. Hu, C. Zhu, W. Zhang, Y. Zhou, H. Fu, Y. Huang, X. Jiang. *Biomaterials* **33** (2012) 2097–2108.
136. D. Rickert, S. Sauerbier, H. Nagursky, D. Menne, A. Vissink, G.M. Raghoobar. *Clin. Oral Implants Res.* **22** (2011) 251–258.
137. I.J. De Kok, S.J. Drapeau, R. Young, L.F. Cooper. *Int. J. Oral Maxillofac. Implants* **20** (2005) 511–518.
138. A. Khojasteh, H. Behnia, F.S. Hosseini, M.M. Dehghan, P. Abbasnia, F.M. Abbas. *J. Biomed. Mater. Res. B Appl. Biomater.* (2013).

139. H. Abukawa, M. Shin, W.B. Williams, J.P. Vacanti, L.B. Kaban, M.J. Troulis. *J. Oral Maxillofac. Surg.* **62** (2004) 601–606.
140. P.H. Warnke, I.N. Springer, J. Wiltfang, Y. Acil, H. Eufinger, M. Wehmoller, P.A. Russo, H. Bolte, E. Sherry, E. Behrens, H. Terheyden. *Lancet* **364** (2004) 766–770.
141. P.H. Warnke, J. Wiltfang, I. Springer, Y. Acil, H. Bolte, M. Kosmahl, P.A. Russo, E. Sherry, U. Lutzen, S. Wolfart, H. Terheyden. *Biomaterials* **27** (2006) 3163–3167.
142. F. Hernandez-Alfaro, V. Ruiz-Magaz, P. Chatakun, R. Guijarro-Martinez. *Int. J. Periodont. Restor. Dent.* **32** (2012) e82–e86.
143. H. Behnia, A. Khojasteh, M. Soleimani, A. Tehranchi, A. Atashi. *J. Craniomaxillofac. Surg.* **40** (2012) 2–7.
144. S. Otaki, S. Ueshima, K. Shiraishi, K. Sugiyama, S. Hamada, M. Yorimoto, O. Matsuo. *Cell Biol. Int.* **31** (2007) 1191–1197.
145. Y. Yamada, K. Ito, S. Nakamura, M. Ueda, T. Nagasaka. *Cell Transplant.* **20** (2011) 1003–1013.
146. G. Tour, M. Wendel, G. Moll, I. Tcacencu. *J. Dent. Res.* **91** (2012) 789–794.
147. R. d'Aquino, A. De Rosa, V. Lanza, V. Tirino, L. Laino, A. Graziano, V. Desiderio, G. Laino, G. Papaccio. *Eur. Cell Mater.* **18** (2009) 75–83.
148. H. Kawaguchi, A. Hirachi, N. Hasegawa, T. Iwata, H. Hamaguchi, H. Shiba, T. Takata, Y. Kato, H. Kurihara. *J. Periodontol.* **75** (2004) 1281–1287.
149. H. Kawaguchi, H. Hayashi, N. Mizuno, T. Fujita, N. Hasegawa, H. Shiba, S. Nakamura, T. Hino, H. Yoshino, H. Kurihara, H. Tanaka, A. Kimura, K. Tsuji, Y. Kato. *Clin. Calcium* **15** (2005) 99–104.
150. J. Zhou, S. Shi, Y. Shi, H. Xie, L. Chen, Y. He, W. Guo, L. Wen, Y. Jin. *J. Cell Physiol.* **226** (2011) 2081–2090.
151. A. Ohazama, S.A. Modino, I. Miletich, P.T. Sharpe. *J. Dent. Res.* **83** (2004) 518–522.
152. E. Ikeda, T. Tsuji. *Exp. Opin. Biol. Ther.* **8** (2008) 735–744.
153. S. Nyman, J. Gottlow, T. Karring, J. Lindhe. *J. Clin. Periodontol.* **9** (1982) 257–265.
154. J.S. Boo, Y. Yamada, Y. Okazaki, Y. Hibino, K. Okada, K.-I. Hata, T. Yoshikawa, Y. Sugiura, M. Ueda. *J. Craniofac. Surg.* **13** (2002) 231–239.
155. J.C. Fricain, S. Schlaubitz, C. Le Visage, I. Arnault, S.M. Derkaoui, R. Siadous, S. Catros, C. Lalande, R. Bareille, M. Renard, T. Fabre, S. Cornet, M. Durand, A. Léonard, N. Sahraoui, D. Letourneur, J. Amédée. *Biomaterials* **34** (2013) 2947–2959.
156. O.V. Horst, M.G. Chavez, A.H. Jheon, T. Desai, O.D. Klein. *Dent. Clin. North Am.* **56** (2012) 495–520.
157. K. Ito, Y. Yamada, T. Naiki, M. Ueda. *Clin. Oral Implants Res.* **17** (2006) 579–586.
158. A. Khojasteh, H. Behnia, F.S. Hosseini, M.M. Dehghan, P. Abbasnia, F.M. Abbas. *J. Biomed. Mater. Res. B Appl. Biomater.* **29** (2013) 32889.
159. J.B. Lee, H.N. Park, W.K. Ko, M.S. Bae, D.N. Heo, D.H. Yang, I.K. Kwon. *J. Biomed. Nanotechnol.* **9** (2013) 424–429.
160. S.C.C.C. Miranda, G.A.B. Silva, R.M. Mendes, F.A.M. Abreu, M.V. Caliar, J.B. Alves, A.M. Goes. *J. Biomed. Mater. Res. Part A* **100A** (2012) 2775–2786.
161. A. Moshaverinia, C. Chen, K. Akiyama, S. Ansari, X. Xu, W.W. Chee, S.R. Schrick, S. Shi. *J. Mater. Sci. Mater. Med.* **23** (2012) 3041–3051.

162. N. Tovar, S. Bourke, M. Jaffe, N.S. Murthy, J. Kohn, C. Gatt, M.G. Dunn. *J. Biomed. Mater. Res. A* **93** (2010) 738–747.
163. M.J. Whitaker, R.A. Quirk, S.M. Howdle, K.M. Shakesheff. *J. Pharm. Pharmacol.* **53** (2001) 1427–1437.
164. B.N. Ostby. *Acta. Odontol. Scand.* **19** (1961) 324–353.
165. F. Banchs, M. Trope. *J. Endod.* **30** (2004) 196–200.
166. P.E. Murray, F. Garcia-Godoy, K.M. Hargreaves. *J. Endod.* **33** (2007) 377–390.
167. M. Torabinejad, H. Faras. *J. Endod.* **38** (2012) 864–868.
168. E. Shimizu, G. Jong, N. Partridge, P.A. Rosenberg, L.M. Lin. *J. Endod.* **38** (2012) 1293–1297.
169. G.T. Huang, T. Yamaza, L.D. Shea, F. Djouad, N.Z. Kuhn, R.S. Tuan, S. Shi. *Tissue Eng. Part A* **16** (2010) 605–615.
170. K.M. Galler, A. Cavender, V. Yuwono, H. Dong, S. Shi, G. Schmalz, J.D. Hartgerink, R.N. D'Souza. *Tissue Eng. Part A* **14** (2008) 2051–2058.
171. M.C. Bottino, G.H. Yassen, J.A. Platt, N. Labban, L.J. Windsor, K.J. Spolnik, A.H. Bressiani. *J. Tissue Eng. Regen. Med.* (2013).
172. A.J. Poncelet, J. Vercruysse, A. Saliez, P. Gianello. *Transplantation* **83** (2007) 783–790.
173. V. Arora, P. Arora, A.K. Munshi. *J. Clin. Pediatr. Dent.* **33** (2009) 289–294.
174. K. Takahashi, K. Tanabe, M. Ohnuki, M. Narita, T. Ichisaka, K. Tomoda, S. Yamanaka. *Cell* **131** (2007) 861–872.
175. J. Yu, M.A. Vodyanik, K. Smuga-Otto, J. Antosiewicz-Bourget, J.L. Frane, S. Tian, J. Nie, G.A. Jonsdottir, V. Ruotti, R. Stewart, Slukvin, II, J.A. Thomson. *Science* **318** (2007) 1917–1920.
176. X. Yan, H. Qin, C. Qu, R.S. Tuan, S. Shi, G.T. Huang. *Stem Cells Dev.* **19** (2010) 469–480.
177. A.F. Fouad. *Adv. Dent. Res.* **23** (2011) 285–289.
178. L. Saldana, L. Crespo, F. Bensiamar, M. Arruebo, N. Vilaboa. *J. Biomed. Mater. Res. A* **24** (2013) 34674.
179. J. Seong, N. Wang, Y. Wang. *J. Cell Mol. Med.* **17** (2013) 597–604.
180. G. Chen, Y. Lv, P. Guo, C. Lin, X. Zhang, L. Yang, Z. Xu. *Curr. Stem Cell Res. Ther.* **8** (2013) 8.
181. D.P. Pioletti. *Comput. Met. Biomech. Biomed. Eng.* **26** (2013) 26.
182. K.W. Liechty, T.C. MacKenzie, A.F. Shaaban, A. Radu, A.M. Moseley, R. Deans, D.R. Marshak, A.W. Flake. *Nat. Med.* **6** (2000) 1282–1286.
183. H. Kawada, J. Fujita, K. Kinjo, Y. Matsuzaki, M. Tsuma, H. Miyatake, Y. Muguruma, K. Tsuboi, Y. Itabashi, Y. Ikeda, S. Ogawa, H. Okano, T. Hotta, K. Ando, K. Fukuda. *Blood* **104** (2004) 3581–3587.
184. C.M. Kolf, E. Cho, R.S. Tuan. *Arthritis Res. Ther.* **9** (2007) 204.

**ENCAPSULATION TECHNOLOGIES IN BETA
CELL REPLACEMENT THERAPIES
FOR TYPE 1 DIABETES**

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14.1. INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disorder characterized by β -cell dysfunction, immune-mediated β -cell destruction within the islets of Langerhans in the pancreas, resulting in a rapid decline in insulin secretion, ultimately resulting in insulin deficiency [1]. The current management paradigm for T1D is strict glycemic control through injectable exogenous insulin, often administered subcutaneously using insulin injections or pumps. An alternative to exogenous insulin administration is islet allotransplantation, a surgical procedure that attempts to replenish the depleted β -cell reserve by transplantation of islets isolated from cadaver human donors into the patient's portal vein. Over the last decade, this procedure has undergone several modifications and refinements, such that current recipients demonstrate insulin independence for a reasonable period of time.

Unfortunately, only about one in every 1000 type 1 diabetics is able to avail of this treatment modality due to scarcity of donors. Even those fortunate enough to be able to receive a transplant require islets from multiple donors. These disadvantages, coupled with inconsistent islet yields, and the need for a multi-drug immunosuppressive regimen with significant adverse effects has prevented the widespread application of this safe and efficacious therapeutic option [2]. While the problems with human islet donor scarcity seem insurmountable, the need for chronic immunosuppression can be circumvented, or at the very least, reduced by encapsulating these islets before transplantation.

Encapsulation of cells and tissue in biocompatible polymers has been extensively evaluated by the scientific community over the last three decades. Encapsulation serves to create a shield against immune cells and molecules either of which could be potentially cytotoxic, thus protecting the encapsulated tissue from immune-mediated graft rejection while still permitting the influx of glucose, amino acids, micronutrients, oxygen and the efflux of insulin, urea, metabolites and carbon dioxide. Although encapsulation was first described in 1933, research into using encapsulation technologies in developing therapies for T1D has only been prevalent over the last two decades. This chapter will provide an update on advances in this field while expounding on current trends and future directions.

14.2. A BRIEF HISTORY OF ENCAPSULATION IN ISLET AND STEM CELL THERAPY

14.2.1. Small and large animal trials

Bisceglie *et al.*, demonstrated in 1933 that mouse tumor cells encapsulated within a membranous biopolymer, could be transplanted safely into the abdominal cavity of a guinea pig. He observed that the cells were able to successfully evade the immune system and remained viable for several months. Today, this report is widely regarded as the first scientific document describing an attempt to transplant cells encapsulated within bioartificial membranes [3].

In 1980, Lim *et al.* reported that transplantation of 2,000–3,000 islet equivalents (IEQ) of islets encapsulated within alginate-poly(L-lysine)-alginate (APA) microcapsules into streptozotocin (STZ)-induced diabetic rats was able to reverse hyperglycemia for a three week period post-transplantation [4]. The hydrogel used in this study was alginate, which is an umbrella term that refers to a number of complex polysaccharides most of which are commercially extracted from seaweed, such as kelp (brown algae) and certain cases, from bacteria (*Pseudomonas* and *Azotobacter spp.*). Alginate is composed of linear, binary copolymers of β -D-mannuronic (M) and α -L-guluronic (G) acid and at certain ratios of M to G, alginate exhibits a favorable immunologic profile provided it has undergone extensive purification [3-5]. Several other researchers have reported that islets demonstrate prolonged function after transplantation into small [6-11] and large animals [12-17] after encapsulation. In 2000, Ramiya *et al.* reported the first instance where 'islet-like' clusters generated *in vitro* from pancreatic stem cells could reverse hyperglycemia in non-obese diabetic (NOD) mice [18]. In 2010, Tuch *et al.* demonstrated that encapsulated pancreatic progenitor cells can normalize blood glucose levels in NOD mice [19].

14.2.2. Human clinical trials

A few early-phase human clinical trials with alginate encapsulated islets demonstrated prolonged graft survival, reductions in hemoglobin A1C (HbA1c) levels and lower insulin requirements [20-23]. In 1994, the first clinical trial using human islets was reported where a 38 year old diabetic patient was transplanted with 10,000 IEQ kg⁻¹ body weight alginate-encapsulated human islet allografts while also concurrently receiving low-dose immunosuppression. After transplantation, the recipient reported that he was insulin-free for 9 months [24]. In 2006, Calafiore *et al.* reported two subjects that received encapsulated human islets. Both demonstrated lower insulin requirements and improved glycemic profiles, but no insulin independence [25]. In 2013, Sernova Corp. (Edmonton, Canada) reported that phase I / II clinical trials on the Sernova Cell Pouch™ on 20 T1D patients demonstrated

that the device was safe for use in a subcutaneous transplant site and demonstrated superior biocompatibility [26]. In August 2014, Viacyte Inc., a privately-held research company secured approval from the U.S. Food and Drug Administration (FDA) to conduct the first clinical trial using encapsulated stem cell-derived insulin producing cells on human patients in San Diego [27].

14.3. BIOMATERIAL SELECTION IN ENCAPSULATED-CELL TRANSPLANTATION

Devices used in cell encapsulation today can broadly be classified into macroscale, microscale, and nanoscale devices. These implantable devices could be implanted at intravascular or extravascular sites anywhere in the body. Several encapsulation devices currently being under evaluation are shown in Figure 1.

Figure 1. Encapsulation devices currently being evaluated for use with stem cells and islets. VC-01™ [185] (A). β -Air [186] (B). Sernova Cell Pouch System™ [182] (C). Double encapsulated islet [P. de Vos, personal communication] (D). Various Theracyte™ models [132] (E). Single encapsulated islet (F). Nanoencapsulated islet (G) (Ricordi C, personal communication).

14.3.1. Intravascular devices

Intravascular devices contain islets encapsulated within biocompatible tubes or fibers which are then directly connected to the recipient's vascular system [28,29]. Although they present advantages over extravascular transplant sites, namely, the ability to sense instantaneous changes in blood glucose and respond appropriately and superior oxygen and nutrient diffusion facilitated

by being directly connected to blood vessels. However, they have a predilection to trigger intravascular thrombosis at the implant site, and thus require concomitant anticoagulation therapy [30,31]. These drawbacks make them poor candidates for use in stem cell transplantation.

14.3.2. Extravascular devices

A majority of macroencapsulation devices [32-40] that have been evaluated for use in islet and stem cell transplantation are implanted at extravascular sites. This is because recipients do not suffer the hemorrhagic complications seen with implantable intravascular devices. Consequently, such devices have been evaluated more extensively. They may be broadly classifiable into tubular and planar devices based on their morphology.

14.3.3. Tubular devices

Over the last two decades, studies conducted using extravascular islet-containing tubular devices and sealed hollow fiber devices [41-46] have demonstrated poorer outcomes compared to similar devices transplanted intravascularly. These results were presumed to be a result of inadequate oxygen and nutrient diffusion within tubular devices transplanted at extravascular sites. XM-50 Amicon hollow fiber macrocapsule implants (Amicon Corp, Danvers, MA), containing human [47] or canine islets transplanted into STZ-induced diabetic pigs and rodents respectively, demonstrated minimal fibrosis 5 months post transplantation in the peritoneal cavity, despite no immunosuppression [48]. In diabetic canine recipients, these devices demonstrated a 50 % success rate in achieving insulin independence for 7–12 week period, demonstrating efficacy in large animal models [49]. Subcutaneously implanted hollow fiber implants demonstrated better glycemic control, minimal fibrotic response and better protection from the immune system when they were manufactured with smooth outer surfaces compared to implants with rough or fenestrated outer surfaces [50-53]. Prevost *et al.* [54] reported that AN69 hollow fiber implants containing syngeneic islets demonstrated euglycemia for a 10 week period after transplantation into STZ-induced diabetic rats. Except for a thin layer of fibroblasts, no host reaction to the implant was observed. These fibers have been reported to have neovascularization potential [55], similar to intraperitoneally transplanted, smooth surface-cellulose fibers [56]. A recent *in vitro* study suggested that islets encapsulated in hollow fiber-devices are adequately oxygenated, comparable to levels found within microcapsules [57]. Hollow fiber devices have several advantages; they are easy to implant, retrievable, and versatile enough to be used at subcutaneous transplant sites. However, they are susceptible to damage after transplantation *in vivo* and require large doses of islets to achieve insulin independence [58]; this limits their widespread applicability.

14.3.4. Planar devices

Planar devices consist of two circular or rectangular flat sheets attached along a peripheral rim to obtain a chamber, within which islets or stem cells are contained. It is believed that this design offers greater stability than hollow-fiber chambers. These devices are commonly implanted in the subcutaneous tissue. Alternatively, a deeper location like the pre-peritoneal space can be chosen if their configuration and size would favor a subcutaneous location. In the case of prevascularized devices, the subcutaneous site is usually preferred, as a second procedure is often required to seed islets into the device a few weeks after the initial surgery. However, it has been observed that planar implants develop changes in shape and size after implantation [59] and studies have reported formation of a dense fibrotic capsule around these devices that ultimately results in graft failure and death [60,61]. Suboptimal nutrient and oxygen diffusion across the layers leading to reduced islet viability, poor graft function, and ultimately graft failure, limit the ability of these devices to achieve prolonged insulin independence. Despite these disadvantages, the ease with which they can be retrieved after implantation, for evaluation of cell viability [62] and function [63,64] has led to several biomaterial device engineers to prefer this conformation over all others. This includes the Sernova Cell Pouch™ (Sernova Corp, Canada), the VC-01™ device (Viacyte LLC, San Diego, CA) and the Theracyte device (Theracyte, Laguna Hills, CA) some of which can even be modified to promote vascularization while simultaneously providing effective immunoisolation [65,66].

14.3.5. Prevascularized devices

A 'prevascularized' device is one that is designed to increase transplant site vascularity by the administration of angiogenic factors, or by the induction of neovascularization by device preimplantation, followed by islet seeding several weeks later. Prevascularization is being evaluated as a possible solution to overcome the diffusional limitations noted with planar devices and to recreate the pancreatic vascular architecture, where islets are in close contact with the surrounding microvasculature [67,68]. Several studies have reported successful reversal of diabetes after implantation of prevascularized islet-containing devices in the peritoneal cavity, specifically in the greater omentum or other sites; subcutaneous implantation remains the most attractive location, and represents the safer, less invasive alternative with minimal adverse effects that also allows for continuous monitoring and effortless device retrieval [69,70]. Pepper *et al.* demonstrated that even in the absence of a device, prevascularization of a subcutaneous transplant site reversed hyperglycemia in over 90 % of diabetic mice after syngeneic islet transplantation [71]. Thus, the efficacy of this strategy cannot be overlooked, especially if the stem-cell containing device is being implanted in the subcutaneous space.

14.3.6. Vascularized devices

Over the last decade, researchers began devising ‘vascularized’ devices to improve nutrient and oxygen delivery to the islets, thus improving insulin release and increasing success rates after encapsulated islet transplantation. In order to achieve vascularization, researchers first implant a ‘scaffold’, usually a several days or weeks before stem cell implantation. The scaffold promotes neovascularization – the genesis of new capillaries in and around, and in some instances, into the implant – and helps ameliorate hypoxic injury to the cells, especially during the first few days after transplantation when they are most vulnerable to hypoxic stress. Studies evaluating poly(tetrafluoroethylene) (PTFE) scaffolds either alone [72], or coated with acidic fibroblast growth factor (a-FGF) [73] (implanted into recipient animals four weeks before seeding with islets) concluded that, in both cases, vascularization was induced at both the subcutaneous and peritoneal locations. After the islets were seeded, the diabetic recipients were noted to demonstrate sustained normoglycemia for up to 180 days after transplantation.

14.3.7. Microencapsulation

In the case of microencapsulation, islets are encapsulated inside micron-sized spherical devices made of alginate, agarose or another biocompatible material and implanted into the recipient. Compared to planar and tubular macro-scale devices, alginate microcapsules are less likely to deform due to mechanical forces after transplantation, are simple to construct, easy to administer, can be scaled to meet commercial needs, and most importantly, can be easily manipulated to adjust size and permeability. Consequently, they are the most popular bioencapsulation devices [74-76] being evaluated for use in islet and stem cell encapsulation. Since these capsules can be produced in large numbers using commercially developed air-pressure driven electrostatic droplet generators [77,78], they are inexpensive, and can be standardized for use with islets or stem cells. Their safety and efficacy has been evaluated in numerous small [79] and large animal trials [80] where reversal of experimentally-induced hyperglycemia was noted without the need for immunosuppressive therapy. Their safety has already been tested in a handful of human clinical trials [81]; they are safe for widespread application in clinical transplantation. Chayosumrit *et al.* reported that microencapsulation promoted differentiation of human embryonic stem cells (hESCs) into definitive endoderm and prevented the formation of teratomas after transplantation in animal models [82].

14.3.8. Nanoencapsulation

Nanoencapsulation is achieved by enveloping the stem cell or islet in a nano-scale immunoprotective layer that is conformally coated on the cell surface, thus significantly reducing diffusion barriers and permitting implantation into

sites normally not considered feasible [83]. This technique has several notable advantages over conventional micro- and macroencapsulation technologies, especially, rapid glucose response time, improved nutrient access, and the ability to control capsule permeability by adjusting the thickness and composition of the encapsulation layer without significantly increasing the size of the encapsulation coating. 'PEG'ylation or coating islets with poly(ethylene glycol) (PEG) is the most popular method of nanoencapsulation currently being evaluated in islet transplantation trials [84]. Exposure to ultraviolet or visible light enables PEG cross-linking resulting in the formation of a conformal coat (or nanocapsule) around the stem cell or islet with minimal tissue damage [85]. However, PEG-encapsulation is ineffective in protecting islets from cytokine-mediated cell injury and it has also been demonstrated that PEG is less biocompatible than alginate and similar hydrogels [86]. Nanolayers composed of alginate and chitosan [158], membranes made of polylysine and poly(glutamic acid) [87], poly(L-lysine)-poly(ethylene glycol) (biotin) (PPB) and streptavidin (SA) [160], complement receptor-1 and heparin [88], and PEG lipid and poly(vinyl alcohol) (PVA) [89,90] are some of the many biomaterials currently being evaluated for use in stem cell and islet nanoencapsulation.

14.4. FACTORS THAT IMPACT BIOENCAPSULATION DEVICE TRANSPLANT OUTCOME

14.4.1. Composition, stiffness & surface characteristics

Results from several *in vivo* studies have demonstrated that implant survival after encapsulation within biomaterials is heavily influenced by implant composition and surface. King *et al.* demonstrated that when islets were encapsulated within poly(L-lysine)-free (PLL-free) high mannuronate alginate (high M) capsules, they demonstrated sustained normoglycemia (up to 8 weeks) compared to high guluronate (high G) alginate capsules [91]. However, it has also been reported that high M, but not high G, alginate microcapsules stimulated increased pro-inflammatory cytokine release by monocytes, specifically TNF- α , IL-1, and IL-6, that would negatively impact islet survival [92,93]. Lanza *et al.* concluded that improved capsule stability, and prolonged graft function and survival could be achieved by increasing the concentration of alginate (from 0.75 % to 1.5 %) used for encapsulation [94].

The length and sequence of mannuronate and guluronate chains and ratio of mannuronate to guluronate (M/G ratio) in alginate hydrogels determines the mechanical strength, elasticity, durability, permeability, and swelling characteristics of the alginate [95,96]. Wilson *et al.* reported that encapsulation within high G alginate inhibited murine embryonic stem cell (mESC) differentiation while high M alginate promoted differentiation toward a primitive endoderm phenotype [97]. Thus, encapsulation material composition

can be used to direct stem cell fate down the desired lineage. Several chemical modifications to alginate composition have either positive or undesirable effects on its biocompatibility. The use of divalent cations (Ca^{2+} , Ba^{2+}), polycations (poly(L-lysine) or poly(L-ornithine)), and poly-electrolytes in alginate synthesis has been extensively studied [98,99]. APA capsules provide superior permselectivity, *i.e.* selective blocking of antibody entry into microcapsules [100,101]. They are also more stable and possess superior mechanical strength. Unfortunately, their use is associated with increased pericapsular cellular overgrowth [102,103], and macrophage activation [104,105], both of which are attributed to greater antigenicity [106,107].

Crosslinking alginate using divalent cations like barium or calcium eliminates these disadvantages. This is because crosslinking results in the formation of elastic alginate capsules with better stability and superior mechanical strength [108,109]. Barium crosslinking has been reported to be less immunogenic [110] than APA (or other polycation-linked) capsules, while providing sufficient protection from antibody and cytokine-mediated islet-damage. These results were surprising as they were previously shown to be more permeable to IgG antibodies than APA capsules [111].

Candiello *et al.* demonstrated that hESC gene expression was influenced by changes in the stiffness of the alginate hydrogel used. Gene expression of markers specific for the endodermal lineage were highly sensitive to small changes in substrate stiffness and it was hypothesized that substrate stiffness could be manipulated along with chemical signals to direct stem cell-lineage fates toward endodermal lines [112].

One study demonstrated that hollow fiber devices [113] with surface irregularities can trigger fibroblast activation and attachment which consequently lead to fibrosis and implant failure. It is expected that most planar and microencapsulation devices would demonstrate minimal fibroblast activation and fibrosis owing to their smooth outer layers. It has been reported that the geometry, rigidity and surface characteristics of biomaterial devices greatly influence macrophage proliferation and surface irregularities in the order of a few microns could trigger their differentiation into inflammatory phenotypes [114].

14.4.2. Synthetic scaffolds

Although alginate remains the most popular hydrogel of choice, agarose [115,116], chitosan [117], methacrylic acid [118], methyl methacrylate [119], polyamide [120,121], PVA [122], PEG [123,124], 2-hydroxyethyl methacrylate (HEMA) [125,126], AN69 (a copolymer of acrylonitrile and sodium-methallyl sulfonate) [127], and collagens type I and IV [128] are all being evaluated for use in islet and stem cell encapsulation. Stimuli-responsive synthetic hydrogels are commonly employed in cell encapsulation and tissue engineering. These include poly(vinylmethyl ether), polyacrylamide gels, PVA, polyphosphazene,

and other derivatives [129]. The single biggest drawback with synthetic biomaterials is their lower biocompatibility, *i.e.* they are more likely to engender a brisk host inflammatory response, leading to fibrosis, graft failure and cell death. Since these materials will ultimately be transplanted into humans, all synthetic constituents would need to pass extensive toxicological evaluation and the material as well as the manufacturing process cannot, at any stage of the process, use chemicals that are known carcinogens or toxic to humans. Most synthetic devices are typically modified to interact favorably with the environment and in certain cases, designed to degrade gradually under physiologic conditions.

14.4.3. Natural scaffolds

Unlike their synthetic counterparts, hydrogels derived entirely from natural sources (gelatin, fibrin, agarose, hyaluronate, chitosan, and alginate) are much less likely to induce a fibrotic or inflammatory host response, and are preferred for use in microencapsulation [130,131]. Alginate is the most common hydrogel, synthesized from naturally-derived sources, used in the generation of microcapsules. However, naturally-occurring hydrogels also have certain disadvantages: lower tensile strength, greater costs, larger lot-to-lot variability, and the need for extensive purification to get rid of contaminants, all of which significantly affect costs and affect optimization of the manufacturing process [132].

14.4.4. Permeability and permselectivity

Choosing the appropriate pore size is vital for the success of any bio-artificial encapsulation device. An exceedingly small pore size may impede inward nutrient and oxygen diffusion, and outbound insulin and metabolite diffusion from the inner cellular zone of the device. In contrast, a particularly large pore size may be unable to prevent immunoglobulins entry, which would lead to immune-mediated islet injury. This parameter is thus the single most important determinant of biomaterial device composition and design. Colton *et al.* observed that when mouse insulinoma cell clusters (MIN6) – encapsulated in 1 % agarose, 0.005 % HEMA, and 0.15 mg ml⁻¹ collagen and seeded into planar nuclepore membrane devices with various pore sizes (100–600 µm) – were transplanted into diabetic Wistar rats, sustained reversal of hyperglycemia was only seen at small pore-sizes (100–200 µm); with larger pore sizes (> 200 µm) hyperglycemia reversal was partial and short-lived [133-135].

Studies have also demonstrated that exposure to host defenses significantly alters membrane permeability and nutrient diffusion across the device-host interface. Kessler *et al.* devised experiments where they applied a protein coat to the encapsulation device, evaluated membrane permeability to glucose and insulin during *in vitro* culture and implanted the devices into rats for a one

week period. While no adherent, inflammatory or necrotic cells or fibrin deposits were noted in uncoated devices implanted *in vivo* (controls), protein adsorption onto the devices after implantation was greater than that noted during *in vitro* culture [136]. Glucose and insulin diffusion rates across the capsule surface were significantly lower in the implanted capsules, indicating that protein adsorption onto the capsule surface negatively impacts capsule permeability *in vivo*.

14.5. ADVANCES AND RECENT UPDATES IN ENCAPSULATION TECHNOLOGIES

Although encapsulation should theoretically ensure total isolation from the host's immune system and unfettered oxygen and nutrient influx across the host-device interface, in reality, graft rejection and hypoxia-induced injury are routinely observed [137,138]; this has led researchers to postulate that hypoxic-injury and cytokine-induced apoptosis are to blame. Some researchers have addressed these issues by employing layer-by-layer (LBL) nanoencapsulation, which involves the generation of several nano-scale coats by layering them over previous coats, thus enveloping the stem cells to achieve adequate immunoisolation while maintaining the diffusion distance to a minimum. LBL nanoencapsulation with multiple layers of polyelectrolyte [139] or PVA conjugated to a single layer of PEG-phospholipid [140] is also being investigated. LBL encapsulation has also been attempted in novel areas such as delayed-release pharmacotherapy and antioxidant drug delivery.

Incorporation of biological factors like fibroblast growth factor 1 (FGF-1) [141], vascular endothelial growth factor (VEGF) [142], anti-coagulants [143], anti-inflammatory molecules [144-148], immunoregulatory biomolecules like CXCL12 [149] and co-encapsulation with tolerogenic mesenchymal stem cells [150] are all currently being investigated to improve bioencapsulation device outcomes.

Several novel strategies to ameliorate hypoxia in encapsulated cells including the use of oxygen-generating chemicals [polydimethylsiloxane (PDMS)-encapsulated solid calcium peroxide] [151], co-encapsulation with photosynthetically-active microorganisms (*Synechococcus lividus*) [152] and implantable devices fitted with access ports to enable direct O₂ delivery – the β -Air device [153] – are all being evaluated.

14.5.1. Theracyte™

The Theracyte device (Theracyte Inc., San Clemente, CA) is a durable and retrievable planar macroencapsulation device that consists of a biocompatible bilayer polymer membrane – a 5 μ m PTFE outer layer with a polyester mesh attached, laminated onto a 0.45 μ m inner PTFE layer [154]. At one end of the

device, a polyethylene port provides access to the lumen for islet seeding. When implanted subcutaneously, the outer membranes induce neovascularization while the cell-impermeable inner membranes contain the insulin-releasing cells within [155]; this arrangement avoids contact with the host immune cells while improving oxygen and nutrient diffusion into the device.

Kumagai-Braesch *et al.* demonstrated that islet-containing Theracyte™ devices restored and sustained euglycemia in non-immunosuppressed alloimmunized diabetic rats after transplantation for a 6 month period [156]. Lee *et al.* demonstrated for the first time that human fetal pancreatic islet-like cell clusters could be matured *in vivo* after encapsulation within Theracyte™ devices. The encapsulated clusters demonstrated amelioration of hyperglycemia in diabetic immunodeficient and non-obese diabetic (NOD) mice [157]. Motte *et al.* reported that hESCs encapsulated within TheraCyte™ demonstrated enrichment in β -cell content as opposed to alginate microcapsules which demonstrated increased α -cell content [158]. Kirk *et al.* demonstrated reversal of hyperglycemia in diabetic mice 20 weeks after transplantation of hESCs encapsulated within the devices [159].

14.5.2. Recent developments in stem cell therapy for T1D

Stem cells are an attractive alternative to human islets, as the scarcity of transplant-quality islets from human cadaver donors, the need for several donors to fulfil the islet requirements of each recipient, and inconsistent and unreliable islet yields with human donor pancreases preclude the application of human islet allotransplantation to all patients diagnosed and currently living with T1D. Pancreatic stem cells [160,161], hESCs [162-164], induced pluripotent stem cells (iPSCs) [165-169], mesenchymal [170-174] and adipose-derived stem cells (ADSCs) [175,176] have all been used to derive islet-like cell clusters or insulin-producing cells (IPCs) that are viable, express markers similar to terminally differentiated β -cells (Insulin, GLUT2) and are able to respond to a glucose challenge. These cells have all demonstrated good function after encapsulation within bioencapsulation devices and several *in vivo* studies have also been conducted. Some research groups that were able to observe favorable outcomes have also applied and secured approval for phase I clinical trials in humans [166].

Mason *et al.* reported that embryonic pancreatic precursor cells that were dissociated, cultured *in vitro*, and photoencapsulated, within a synthetic PEG hydrogel preferentially differentiated into insulin-secreting β -cells. This seems to suggest that embryonic pancreatic precursor cells could be induced to proliferate and differentiate along the endodermal lineage to generate a population of glucose-responsive β -cells if exposed to specific chemical environments [177].

14.5.2.1. Pancreatic progenitor stem cells

In 2005, Todorov *et al.* reported the existence of multipotent pancreatic progenitor cells that could be successfully differentiated into insulin-producing cells [160]. A year later, Noguchi *et al.* reported the induction of pancreatic progenitor cells into insulin-producing cells, albeit with the use of adenoviral gene transfer [161]. While this was a significant breakthrough, the need for transgenic vectors to achieve this transition was a significant drawback.

14.5.2.2. Human embryonic stem cells (hESCs)

In 2012, Schulz *et al.* reported that a scalable manufacturing process for producing pancreatic progenitor cells which could reverse STZ-induced hyperglycemia in mice 4–5 months post-transplant had been developed [162]. In 2014, Pagliuca *et al.*, and Rezania *et al.* both reported successful validation of a seven-stage protocol to generate insulin-secreting cells that were able to reverse hyperglycemia in diabetic mice within months after transplantation [163,164]. These recent developments represent significant breakthroughs in developing a cure for T1D using hESC transplantation.

14.5.2.3. Induced pluripotent stem cells (iPSCs)

iPSCs have demonstrated the ability to differentiate into functional β -cells, *in vitro* [165]. iPSCs derived from mice and rhesus monkeys have demonstrated the ability to differentiate into glucose-responsive insulin-positive cells, resulting in a complete reversal of experimentally induced hyperglycemia after transplantation in diabetic mice (type 1 and 2) [166]. Jeon *et al.* reported successful generation of iPSCs from NOD-mouse fibroblasts and differentiation of these iPSCs into glucose-responsive insulin-producing cells which when transplanted into diabetic mice resulted in engraftment and reversal of hyperglycemia [167]. Recently, Shahjalal *et al.* and Kudva *et al.* reported successful differentiation of human-derived iPSCs into insulin-producing cells without using xenogeneic products or viral transgenic vectors respectively [168]. However, Thatava *et al.* suggested that translation of results observed in mice might face significant challenges before translation to humans when he reported that iPSCs isolated from T1D patients exhibited significant variations in their ability to differentiate into insulin-expressing islet-like cells [169].

14.5.2.4. Mesenchymal stem cells (MSCs)

MSCs have long been reported to have immunomodulatory properties. Yeung *et al.* reported that culture of MSCs with human islets could protect islets from pro-inflammatory cytokine-induced β -cell apoptosis [170]. Liu *et al.* reported that co-transplantation of islets with MSCs could potentially control graft inflammation as MSCs are known to secrete anti-inflammatory cytokines and also induce tolerance by suppressing Th1 lymphocytes and enhancing

regulatory T cell differentiation [171]. Their immunoregulatory properties are partly due to the fact that they secrete interleukin 10 (IL-10), leukemia inhibitory factor (LIF) and other immunomodulatory biomolecules. Carlsson *et al.* reported that new-onset T1D patients treated with autologous MSCs showed preserved or even increased C-peptide peak and area under the curve (AUC) values. MSCs may thus play a role in preservation of β -cell mass and function and halting disease progression in T1D [172].

14.5.2.5. Adipose-derived stem cells (ADSCs)

Jun *et al.* reported that co-culturing ADSCs with islets may protect the islets from hypoxic injury during culture, and that this strategy could be employed to improve islet cell survival and function prior to transplantation. This hypothesis was based on the observation that mice transplanted with ADSC co-cultured islets remained normoglycemic longer and required a lower islet mass to reverse diabetes [173]. Chandra *et al.* reported that ADSC-derived islet-like cell aggregates (ICAs) release human C-peptide in a glucose-responsive manner and transplantation ICAs into STZ-diabetic mice restored normoglycemia within a 4 week period [174].

14.5.2.6. Other cell sources

In addition to all the aforementioned cell types, pancreatic epithelial cells, ductal cells [175,176], exocrine cells [177], α -cells (transdifferentiation) [178], and even skin fibroblasts [179] have been demonstrated to be able to differentiate into β -cells under appropriate conditions.

14.5.3. Bioencapsulation technologies in islet and stem cell transplant clinical trials

14.5.3.1. Viacyte

In 2006, D'Amour *et al.* (Novocell Inc. San Diego, CA) reported development of a differentiation process that converted hESCs to endocrine cells which expressed insulin, but were only minimally responsive to glucose [180]. In 2008, the same group reported the development of glucose-responsive insulin secreting cells generated from hESCs [181]. In 2012, Schulz *et al.* reported that their team at Viacyte LLC (San Diego, CA), had developed a scalable manufacturing process for producing pancreatic progenitor cells which could reverse STZ-induced hyperglycemia in mice 4–5 months post-transplant [182]. In 2014, Viacyte secured approval to conduct the first ever phase I safety, tolerability and efficacy study on T1D patients in the USA using hESCs encapsulated within a macroencapsulation device (VC-01™) [183].

14.5.3.2. Biohub – DRI

In August 2014, the Diabetes Research Institute in Miami, Florida obtained FDA approval to conduct Phase I/II clinical trials to evaluate the safety and efficacy of transplanting human islets within a 'biodegradable scaffold' that can be safely transplanted in the *omentum* – a flap of adipose tissue found in the abdomen. The scaffold is created using the patient's plasma. After the islets are seeded in the scaffold, it will then be attached to the *omentum* using thrombin surrounding which an omental 'pouch' will be created to secure the implant. However, it is worth noting that this 'scaffold' is not immunoprotective, and the patient will require conventional immunosuppression to prevent graft rejection [184].

14.5.3.3. Beta-O₂ (β -air bio-artificial pancreas)

In 2010, Ludwig *et al.* reported sustained viability, function and immunoprotection after allogeneic subcutaneous transplantation in non-diabetic Göttingen minipigs [185]. In 2013, the same group reported normalization of blood glucose values in diabetic rodents over a 6 month period post transplantation [186] and reversal of hyperglycemia in diabetic Sinclair pigs for a 90 day period [187]. A pilot clinical trial performed in a 63 year old T1D patient demonstrated persistent islet function 10 months post transplantation without any immunosuppressive therapy [188].

14.5.3.4. Sernova corp (Cell Pouch™)

The Cell Pouch™ is currently undergoing an open label, non-randomized, Phase I/II safety and efficacy study of up to 20 patients with T1D undergoing allograft pancreatic islet transplantation in Canada. In 2013, Shapiro *et al.* reported that the first group of patients implanted with the Cell Pouch™ and transplanted with insulin-producing islets showed longer-term safety and biocompatibility with one patient beyond the 180 day time point [189].

Despite all exciting developments until multi-center, multidisciplinary, randomized, controlled, clinical trials report favorable outcomes, treatment modalities for patients with T1D remain limited.

The two principal issues of concern are:

1. The inherent propensity of hESC / iPSCs to proliferate in an unpredictable manner and undergoing malignant transformation.
2. Immune-mediated allogenic sensitization and subsequent graft rejection.

However, if these stem cell-derived insulin- producing cells are enclosed within protective, bioinert devices and implanted at appropriate sites, teratoma formation can be prevented and long-term maintenance of euglycemia can be achieved [190].

14.6. CONCLUSIONS

The management of T1D in most patients currently only involves regular blood glucose monitoring and insulin self-administration by patients either by means of injections or through an implanted insulin pump. Cell encapsulation within bioengineered devices and the insulin-producing cells derived from stem cells are two cutting-edge strategies that challenge such traditional treatment paradigms in the management of T1D and aim to revolutionize clinical islet transplantation [191]. Bioencapsulation devices may either be macroscopic as in the case with Viacyte® and β -air® or microscopic as in the case of Diabecell®. Future devices may be implanted under the skin, into the *omentum* or dispersed into the abdominal cavity. They might be implanted after a period of pre-vascularization to improve transplant outcomes or might concurrently allow for the delivery of angiogenic and immunomodulatory biomolecules, generate oxygen locally so as to augment tissue oxygen delivery and allow for easy retrieval of the device. They might contain mesenchymal or ADSCs in addition to insulin-producing cells to support engraftment and prolong cell survival. While encapsulation technologies aim for cure, strategies to reduce insulin dependence, hypoglycemia unawareness and eliminate episodes of hypoglycemic attacks while retarding end organ damage are also key determinants in deciding which bioencapsulation technologies are best suited for clinical translation.

Stem cell therapy has been repeatedly shown to possess limitless potential as it eliminates the primary impediment to widespread application of curative strategies for T1D – a lack of healthy, viable and functional donor islets. Several novel strategies to prevent recognition and ultimate destruction by the host immune system have been designed and are currently being evaluated. However, several challenges still remain unaddressed. Advances in bioencapsulation technologies and biomaterial manufacturing, and improvements in islet and stem cell yields and insulin release parameters, along with the development of standard operating procedures (SOP), quality control measures and stringent guidelines and are vital to achieve seamless translation of these promising therapies to clinical practice with the ultimate goal of reduced patient morbidity, mortality, better quality of life and long-lasting insulin independence [192].

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REFERENCES

1. American Diabetes Association. *Diabetes Care* **27**(Suppl 1) (2004) S5–S10.
2. R.M. Shenkman, J.J. Chalmers, B.J. Hering, N. Kirchhof, K.K. Papas. *Tissue Eng. Part C Methods* **15** (2009) 147–156.
3. V. Bisceglie. *Ztschr. F. Krebsforsch* **40** (1933) 122–140.
4. E.S. O'Sullivan, A.S. Johnson, A. Omer, J. Hollister-Lock, S. Bonner-Weir, C.K. Colton, G.C. Weir. *Diabetologia* **53** (2010) 937–945.
5. A.R. Kim, J.H. Hwang, H.M. Kim, H.N. Kim, J.E. Song, Y.I. Yang, K.H. Yoon, D. Lee, G. Khang. *J. Biomater. Sci. Polym. Ed.* **24** (2013) 1084–1098.
6. G. Langlois, J. Dusseault, S. Bilodeau, S.K. Tam, D. Magassouba, J.P. Halle. *Acta Biomater.* **5** (2009) 3433–3440.
7. F. Lim, A.M. Sun. *Science* **210** (1980) 908–910.
8. W. Cui, G. Barr, K.M. Faucher, X.L. Sun, S.A. Safley, C.J. Weber, E.L. Chaikof. *Transplant. Proc.* **36** (2004) 1206–1208.
9. M. Lamb, R. Storrs, S. Li, O. Liang, K. Laugenour, R. Dorian, D. Chapman, H. Ichii, D. Imagawa, C. Foster S. King, J.R.T. Lakey. *Transplant. Proc.* **43** (2011) 3265–3266.
10. Z.P. Lum, I.T. Tai, M. Krestow, J. Norton, I. Vacek, A.M. Sun. *Diabetes* **40** (1991) 1511–1516.
11. S. Veriter, J. Mergen, R.M. Goebbels, N. Aouassar, C. Gregoire, B. Jordan, P. Leveque, B. Gallez, P. Gianello, D. Dufrane. *Tissue Eng. Part A* **16** (2010) 1503–1513.
12. H. Yang, W. O'Hali, H. Kearns, J.R. Wright. *Transplantation* **64** (1997) 28–32.
13. Z.L. Zhi, A. Kerby, A.J. King, P.M. Jones, J.C. Pickup. *Diabetologia* **55** (2012) 1081–1090.
14. D. Dufrane, R.M. Goebbels, P. Gianello. *Transplantation* **90** (2010) 1054–1062.
15. D. Dufrane, R.M. Goebbels, A. Saliez, Y. Guiot, P. Gianello. *Transplantation* **81** (2006) 1345–1353.
16. R.B. Elliott, L. Escobar, P.L. Tan, O. Garkavenko, R. Calafiore, P. Basta, A.V. Vasconcellos, D.F. Emerich, C. Thanos, C. Bamba. *Transplant. Proc.* **37** (2005) 3505–3508.
17. P. Gianello, D. Dufrane. *Bull. Mem. Acad. R. Med. Belg.* **162** (2007) 439–449.
18. G. Basta, P. Montanucci, G. Luca, C. Boselli, G. Noya, B. Barbaro, M. Qi, K.P. Kinzer, J. Oberholzer, R. Calafiore. *Diabetes Care* **34** (2011) 2406–2409.
19. R. Calafiore, G. Basta, G. Luca, A. Lemmi, M.P. Montanucci, G. Calabrese, L. Racanicchi, F. Mancuso, P. Brunetti. *Diabetes Care* **29** (2006) 137–138.
20. R. Calafiore, G. Basta, G. Luca, A. Lemmi, L. Racanicchi, F. Mancuso, M.P. Montanucci, P. Brunetti. *Transplant. Proc.* **38** (2006) 1156–1157.
21. B.E. Tuch, G.W. Keogh, L.J. Williams, W. Wu, J.L. Foster, V. Vaithilingam, R. Philips. *Diabetes Care* **32** (2009) 1887–1889.
22. T. Kobayashi, Y. Aomatsu, H. Iwata, T. Kin, H. Kanehiro, M. Hisanaga, S. Ko, M. Nagao, Y. Nakajima. *Transplantation* **75** (2003) 619–625.
23. D.Y. Lee, S.J. Park, S. Lee, J.H. Nam, Y. Byun. *Tissue Eng.* **13** (2007) 2133–2141.
24. H. Dong, T.M. Fahmy, S.M. Metcalfe, S.L. Morton, X. Dong, L. Inverardi, D.B. Adams, W. Gao, H. Wang. *Plos One* **7** (2012) e50265.
25. P. Soon-Shiong, E. Feldman, R. Nelson, R. Heintz, Q. Yao, Z. Yao, T. Zheng, N. Merideth, G. Skjak-Braek, T. Espevik. *Proc. Natl. Acad. Sci. USA* **90** (1993) 5843–5847.

26. P. Soon-Shiong, R.E. Heintz, N. Merideth, Q.X. Yao, Z. Yao, T. Zheng, M. Murphy, M.K. Moloney, M. Schmehl, M. Harris, R. Mendez, R. Mendez, P.A. Sandford. *Lancet* **343** (1994) 950–951.
27. R.B. Elliot, O. Garkavenko, P. Tan, N.N. Skaletsky, A. Guliev, B. Draznin, *70th Scientific Sessions, American Diabetes Association*. Orlando, Florida, USA, 2010.
28. R.B. Elliott, L. Escobar, P.L. Tan, M. Muzina, S. Zwain, C. Buchanan. *Xenotransplantation* **14** (2007) 157–161.
29. V.F. Duvivier-Kali, A. Omer, M.D. Lopez-Avalos, J.J. O’Neil, G.C. Weir. *Am. J. Transplant.* **4** (2004) 1991–2000.
30. K. Suzuki, S. Bonner-Weir, N. Trivedi, K.H. Yoon, J. Hollister-Lock, C.K. Colton, G.C. Weir. *Transplantation* **66** (1998) 21–28.
31. W.J. Tze, S.C. Cheung, J. Tai, H. Ye. *Transplant. Proc.* **30** (1998) 477–478.
32. A. King, J. Lau, A. Nordin, S. Sandler, A. Andersson. *Diabetes Technol. Ther.* **5** (2003) 653–663.
33. T. Espevik, M. Otterlei, G. Skjak-Braek, L. Ryan, S.D. Wright, A. Sundan. *Eur. J. Immunol.* **23** (1993) 255–261.
34. M. Otterlei, K. Ostgaard, G. Skjak-Braek, O. Smidsrod, P. Soon-Shiong, T. Espevik. *J. Immunother.* **10** (1991) 286–291.
35. R.P. Lanza, R. Jackson, A. Sullivan, J. Ringeling, C. McGrath, W. Kuhlreiber, W.L. Chick. *Transplantation* **67** (1999) 1105–1111.
36. U. Zimmermann, F. Thurmer, A. Jork, M. Weber, S. Mimietz, M. Hillgartner, F. Brunnenmeier, H. Zimmermann, I. Westphal, G. Fuhr, U. Nöth, A. Haase, A. Steinert, C. Hehdreich. *Ann. NY Acad. Sci.* **944** (2001) 199–215.
37. S. Sakai, T. Ono, H. Ijima, K. Kawakami. *J. Biomater. Sci. Polym. Ed.* **14** (2003) 643–652.
38. H. Zimmermann, D. Zimmermann, R. Reuss, P.J. Feilen, B. Manz, A. Katsen, M. Weber, F.R. Ihmig, F. Ehrhart, P. Gessner, M. Behringer, A. Steinbach, L.H. Wegner, V.L. Sukhorukov, J.A. Vásquez, S. Schneider, M.M. Weber, F. Volke, R. Wolf, U. Zimmermann. *J. Mater. Sci. Mater. Med.* **16** (2005) 491–501.
39. B. Kulseng, B. Thu, T. Espevik, G. Skjak-Braek. *Cell Transplant.* **6** (1997) 387–394.
40. Y.A. Morch, I. Donati, B.L. Strand, G. Skjak-Braek. *Biomacromolecules* **7** (2006) 1471–1480.
41. A. King, S. Sandler, A. Andersson. *J. Biomed. Mater. Res.* **57** (2001) 374–383.
42. A.M. Rokstad, O.L. Brekke, B. Steinkjer, L. Ryan, G. Kollarikova, B.L. Strand, G. Skjak-Braek, I. Lacik, T. Espevik, T.E. Mollnes. *Acta Biomater.* **7** (2011) 2566–2578.
43. G.M. Vandenbossche, M.E. Bracke, C.A. Cuvelier, H.E. Bortier, M.M. Mareel, J.P. Remon. *J. Pharm. Pharmacol.* **45** (1993) 121–125.
44. S. Juste, M. Lessard, N. Henley, M. Menard, J.P. Halle. *J. Biomed. Mater. Res. A* **72** (2005) 389–398.
45. M.E. Pueyo, S. Darquy, F. Capron, G. Reach. *J. Biomater. Sci. Polym. Ed.* **5** (1993) 197–203.
46. B.L. Strand, T.L. Ryan, P. In’t Veld, B. Kulseng, A.M. Rokstad, G. Skjak-Brek, T. Espevik. *Cell Transplant.* **10** (2001) 263–275.
47. A. Omer, V. Duvivier-Kali, J. Fernandes, V. Tchipashvili, C.K. Colton, G.C. Weir. *Transplantation* **79** (2005) 52–58.

48. B. Kulseng, G. Skjak-Braek, L. Ryan, A. Andersson, A. King, A. Faxvaag, T. Espevik. *Transplantation* **67** (1999) 978–984.
49. P. De Vos, B.J. De Haan, G.H. Wolters, J.H. Strubbe, R. Van Schilfgaarde. *Diabetologia* **40** (1997) 262–270.
50. K. Jain, S. Asina, H. Yang, E.D. Blount, B.H. Smith, C.H. Diehl, A.L. Rubin. *Transplantation* **68** (1999) 1693–1700.
51. H. Iwata, T. Takagi, H. Amemiya. *Transplant. Proc.* **24** (1992) 52.
52. K.C. Yang, Z. Qi, C.C. Wu, Y. Shirouza, F.H. Lin, G. Yanai, S. Sumi. *Biochem. Biophys. Res Commun.* **393** (2010) 818–823.
53. I.S. Isayeva, B.T. Kasibhatla, K.S. Rosenthal, J.P. Kennedy. *Biomaterials* **24** (2003) 3483–3491.
54. K.S. Jones, M.V. Sefton, R.M. Gorczynski. *Transplantation* **78** (2004) 1454–1462.
55. C. Lhommeau, S. Toillon, T. Pith, L. Kessler, C. Jesser, M. Pinget. *J. Mater. Sci. Mater. Med.* **8** (1997) 163–174.
56. Z. Qi, C. Yamamoto, N. Imori, A. Kinukawa, K.C. Yang, G. Yanai, E. Ikenoue, Y. Shen, Y. Shirouzu, A. Hiura. *Cell Transplant.* **21** (2012) 525–534.
57. V. Nadithe, D. Mishra, Y.H. Bae. *Biotechnol. Bioeng.* **109** (2012) 2392–2401.
58. H. Aghajani-Lazarjani, E. Vasheghani-Farahani, S.A. Shojaosadati, S. Hashemi-Najafabadi, S. Zahediasl, T. Tiraihi, F. Atyabi. *J. Artif. Organs* **13** (2010) 218–224.
59. M.V. Sefton. *Biomaterials* **14** (1993) 1127–1134.
60. G.F. Klomp, S.H. Ronel, H. Hashiguchi, M. D'Andrea, W.H. Dobelle. *Trans. Am. Soc. Artif. Intern. Organs* **25** (1979) 74–76.
61. L. Kessler, M. Pinget, M. Aprahamian, P. Dejardin, C. Dange. *Horm. Metab. Res.* **23** (1991) 312–317.
62. T. Maki, J.P. Lodge, M. Carretta, H. Ohzato, K.M. Borland, S.J. Sullivan, J. Staruk, T.E. Muller, B.A. Solomon, W.L. Chick, A.P. Monaco. *Transplantation* **55** (1993) 713–717.
63. I.S. Sun, W. Parisius, G.M. Healy, I. Vacek, H.G. Macmorine. *Diabetes* **26** (1977) 1136–1139.
64. S.H. Lee, E. Hao, A.Y. Savinov, I. Geron, A.Y. Strongin, P. Itkin-Ansari. *Transplantation* **87** (2009) 983–991.
65. A.K. Sorenby, M. Kumagai-Braesch, A. Sharma, K.R. Hultenby, A.M. Wernerson, A.B. Tibell. *Transplantation* **86** (2008) 364–366.
66. R.L. Geller, T. Loudovaris, S. Neuenfeldt, R.C. Johnson, J.H. Brauker. *Ann. NY Acad. Sci.* **831** (1997) 438–451.
67. J. Brauker, L.A. Martinson, S.K. Young, R.C. Johnson. *Transplantation* **61** (1996) 1671–1677.
68. J.H. Brauker, V.E. Carr-Brendel, L.A. Martinson, J. Crudele, W.D. Johnston, R.C. Johnson. *J. Biomed. Mater. Res.* **29** (1995) 1517–1524.
69. C.K. Colton. *Cell Transplant.* **4** (1995) 415–436.
70. D.W. Scharp, C.J. Swanson, B.J. Olack, P.P. Latta, O.D. Hegre, E.J. Doherty, F.T. Gentile, K.S. Flavin, M.F. Ansara, P.E. Lacy. *Diabetes* **43** (1994) 1167–1170.
71. D.W. Scharp, N.S. Mason, R.E. Sparks. *World J. Surg.* **8** (1984) 221–229.
72. C.A. Hoesli, M. Luu, J.M. Piret. *Biotechnol. Prog.* **25** (2009) 1740–1751.
73. J.L. Dulong, C. Legallais, S. Darquy, G. Reach. *Biotechnol. Bioeng.* **78** (2002) 576–582.

74. T. Zekorn, M. Renardy, H. Planck, P. Zschocke, R.G. Bretzel, U. Siebers, K. Federlin. *Horm. Metab. Res. Suppl.* **25** (1990) 202–206.
75. J.J. Altmah. *J. Diabet. Complications* **2** (1988) 68–74.
76. P. Icard, F. Penfornis, C. Gotheil, J. Boillot, C. Cornec, F. Barrat, J.J. Altman. *Transplant. Proc.* **22** (1990) 724–726.
77. R.P. Lanza, K.M. Borland, J.E. Staruk, M.C. Appel, B.A. Solomon, W.L. Chick. *Endocrinology* **131** (1992) 637–642.
78. R.P. Lanza, K.M. Borland, P. Lodge, M. Carretta, S.J. Sullivan, T.E. Muller, B.A. Solomon, T. Maki, A.P. Monaco, W.L. Chick. *Diabetes* **41** (1992) 886–889.
79. R.P. Lanza, D.H. Butler, K.M. Borland, J.M. Harvey, D.L. Faustman, B.A. Solomon, T.E. Muller, R.G. Rupp, T. Maki, A.P. Monaco. *Transplant. Proc.* **24** (1992) 669–671.
80. P.E. Lacy, O.D. Hegre, A. Gerasimidi-Vazeou, F.T. Gentile, K.E. Dionne. *Science* **254** (1991) 1782–1784.
81. P. Prevost, S. Flori, C. Collier, E. Muscat, E. Rolland. *Transplant. Proc.* **27** (1995) 3393–3395.
82. A.S. Rivereau, S. Darquy, L. Chaillous, S. Maugendre, E. Gouin, G. Reach, P. Sai. *Diabetes. Metab.* **23** (1997) 205–212.
83. J. Schrezenmeir, L. Gero, C. Laue, J. Kirchgessner, A. Muller, A. Huls, R. Passmann, H.J. Hahn, L. Kunz, W. Mueller-Klieser. *Transplant. Proc.* **24** (1992) 2925–2929.
84. R. Cornolti, M. Figliuzzi, A. Remuzzi. *Cell Transplant.* **18** (2009) 195–201.
85. W.L. Chick, J.J. Perna, V. Lauris, D. Low, P.M. Galletti, G. Panol, A.D. Whittemore, A.A. Like, C.K. Colton, M.J. Lysaght. *Science* **197** (1977) 780–782.
86. A.D. Whittemore, W.L. Chick, P.M. Galletti, J.A. Mannick. *Surg. Forum.* **28** (1977) 93–97.
87. K. Tatarkiewicz, E. Sitarek, M. Sabat, T. Orlowski. *Transplant. Proc.* **28** (1996) 831–832.
88. H. Ohgawara, J. Miyazaki, S. Karibe, N. Katagiri, F. Tashiro, T. Akaike. *Transplant. Proc.* **27** (1995) 3319–3320.
89. L. Kessler, M. Aprahamian, M. Keipes, C. Damge, M. Pinget, D. Poinot. *Biomaterials* **13** (1992) 44–49.
90. A.D. Whittemore, W.L. Chick, P.M. Galletti, A.A. Like, C.K. Colton, M.J. Lysaght, P.D. Richardson. *Trans. Am. Soc. Artif. Intern. Organs* **23** (1977) 336–341.
91. P. De Vos, J.L. Hillebrands, B.J. De Haan, J.H. Strubbe, R. Van Schilfgaarde. *Transplantation* **63** (1997) 824–830.
92. J.H. Juang, S. Bonner-Weir, Y. Ogawa, J.P. Vacanti, G.C. Weir. *Transplantation* **61** (1996) 1557–1561.
93. A. Pileggi, R.D. Molano, C. Ricordi, E. Zahr, J. Collins, R. Valdes, L. Inverardi. *Transplantation* **81** (2006) 1318–1324.
94. R. Valdes, S. Martin, A. Cravioto, J. Tenopala. *Transplant. Proc.* **30** (1998) 481.
95. C.K. Colton, E.S. Avgoustiniatos. *J. Biomech. Eng.* **113** (1991) 152–170.
96. S. Hirofani, H. Ohgawara, T. Agishi, T. Akaike, S. Miyazaki. *Transplant. Proc.* **30** (1998) 485–489.
97. A. Andersson, D.L. Eizirik, C. Hellerstrom, R.C. Johnson, D.G. Pipeleers. *Transplant. Proc.* **26** (1994) 802–803.
98. E. Rafael, A. Wernerson, P. Arner, G.S. Wu, A. Tibell. *Cell Transplant.* **8** (1999) 317–326.

99. E. Rafael, G.S. Wu, K. Hultenby, A. Tibell, A. Wernerson. *Cell Transplant.* **12** (2003) 407–412.
100. I. Yakhnenko, W.K. Wong, I.I. Katkov, P. Itkin-Ansari. *Cryo. Letters* **33** (2012) 518–531.
101. M. Kumagai-Braesch, S. Jacobson, H. Mori, X. Jia, T. Takahashi, A. Wernerson, M. Flodstrom-Tullberg, A. Tibell. *Cell Transplant.* **22** (2013) 1137–1146.
102. D.J. Borg, E. Bonifacio. *Curr. Diab. Rep.* **11** (2011) 434–444.
103. N.V. Krishnamurthy, B. Gimi. *Crit. Rev. Biomed. Eng.* **39** (2011) 473–491.
104. O. Khanna, J.C. Larson, M.L. Moya, E.C. Opara, E.M. Brey. *J. Vis. Exp.* **66** (2012) pii3388.
105. H. Zimmermann, S.G. Shirley, U. Zimmermann. *Curr. Diab. Rep.* **7** (2007) 314–320.
106. G.L. Fiszman, A.L. Karara, L.M. Finocchiaro, G.C. Glikin. *Electron. J. Biotechnol.* **5** (2002) 23–24.
107. A.M. Sun. *Methods Enzymol.* **137** (1988) 575–580.
108. V.F. Duvivier-Kali, A. Omer, R.J. Parent, J.J. O'Neil, G.C. Weir. *Diabetes* **50** (2001) 1698–1705.
109. M.K. Nguyen, D.S. Lee. *Macromol. Biosci.* **10** (2010) 563–579.
110. K.Y. Lee, D.J. Mooney. *Chem. Rev.* **101** (2001) 1869–1879.
111. G.D. Nicodemus, S.J. Bryant. *Tissue Eng. Part B Rev.* **14** (2008) 149–165.
112. P. De Vos, J.F. Van Straaten, A.G. Nieuwenhuizen, M.de Groot, R.J. Ploeg, B.J. De Haan, R. Van Schilfgaarde. *Diabetes* **48** (1999) 1381–1388.
113. Z.L. Xin, S.L. Ge, X.K. Wu, Y.J. Jia, H.T. Hu. *World J. Gastroenterol.* **11** (2005) 5714–5717.
114. J.T. Wilson, E.L. Chaikof. *Adv. Drug Deliv. Rev.* **60** (2008) 124–145.
115. D.Y. Lee, K. Yang, S. Lee, S.Y. Chae, K.W. Kim, M.K. Lee, D.J. Han, Y. Byun. *J. Biomed. Mater. Res.* **62** (2002) 372–377.
116. J.Y. Jang, D.Y. Lee, S.J. Park, Y. Byun. *Biomaterials* **25** (2004) 3663–3669.
117. S. Krol, S. del Guerra, M. Grupillo, A. Diaspro, A. Gliozzi, P. Marchetti. *Nano Lett.* **6** (2006) 1933–1939.
118. Y. Teramura, Y. Kaneda, H. Iwata. *Biomaterials* **28** (2007) 4818–4825.
119. O. Khanna, M.L. Moya, E.C. Opara, E.M. Brey. *J. Biomed. Mater. Res. A* **95** (2010) 632–640.
120. Y. Teramura, H. Iwata. *Transplantation* **91** (2011) 271–278.
121. L. Baruch, O. Benny, A. Gilert, M. Ukobnik, O. Ben Itzhak, M. Machluf. *Biomed. Microdevices* **11** (2009) 1103–1113.
122. G.M. Campo, A. Avenoso, S. Campo, A. D'Ascola, P. Traina, D. Sama, A. Calatroni. *J. Cell. Biochem.* **106** (2009) 83–92.
123. A. Leung, G. Lawrie, L.K. Nielsen, M. Trau. *J. Microencapsul.* **25** (2008) 387–398.
124. M. Senzolo, J. Coppel, E. Cholongitas, A. Riddell, C.K. Triantos, D. Perry, A.K. Burroughs. *Blood Coagul. Fibrinolysis* **18** (2007) 227–236.
125. C.M. Bunker, B. Tiefenbach, A. Jahnke, C. Gerlach, T. Freier, K.P. Schmitz, U.T. Hopt, W. Schareck, E. Klar, P. de Vos. *Biomaterials* **26** (2005) 2353–2360.
126. E. Pedraza, M.M. Coronel, C.A. Fraker, C. Ricordi, C.L. Stabler. *Proc. Natl. Acad. Sci. USA* **109** (2012) 4245–4250.
127. S. Mansouri, Y. Merhi, F.M. Winnik, M. Tabrizian. *Biomacromolecules* **12** (2011) 585–592.
128. J.C. Pickup, Z.L. Zhi, F. Khan, T. Saxl, D.J. Birch. *Diabetes Metab. Res. Rev.* **24** (2008) 604–610.

129. J.T. Wilson, W. Cui, E.L. Chaikof. *Nano Lett.* **8** (2008) 1940–1948.
130. N.M. Luan, Y. Teramura, H. Iwata. *Biomaterials* **32** (2011) 6487–6492.
131. K. Tatsumi, K. Ohashi, Y. Teramura, R. Utoh, K. Kanegae, N. Watanabe, S. Mukobata, M. Nakayama, H. Iwata, T. Okano. *Biomaterials* **33** (2012) 821–828.
132. E. Kroon, L.A. Martinson, K. Kadoya, A.G. Bang, O.G. Kelly, S. Eliazzer, H. Young, M. Richardson, N.G. Smart, J. Cunningham, A.D. Agulnick, K.A. D'Amour, M.K. Carpenter, E.E. Baetge. *Nat. Biotechnol.* **26** (2008) 443–452.
133. A.V. Matveyenko, S. Georgia, A. Bhushan, P.C. Butler. *Am. J. Physiol. Endocrinol. Metab.* **299** (2010) E713–E720.
134. J. Candiello, S.S. Singh, K. Task, P.N. Kumta, I. Banerjee. *J. Biol. Eng.* **7** (2013) 9.
135. S. Kadam, S. Muthyala, P. Nair, R. Bhonde. *Rev. Diabet. Stud.* **7** (2010) 168–182.
136. P.K. Ngoc, P.V. Phuc, T.H. Nhung, D.T. Thuy, N.T. Nguyet. *Hum. Cell.* **24** (2011) 86–95.
137. N.E. Davis, L.N. Beenken-Rothkopf, A. Mirsoian, N. Kojic, D.L. Kaplan, A.E. Barron, M.J. Fontaine. *Biomaterials* **33** (2012) 6691–6697.
138. V. Chandra, G. Swetha, S. Muthyala, A.K. Jaiswal, J.R. Bellare, P.D. Nair, R.R. Bhonde. *Plos One* **6** (2011) e20615.
139. M.N. Mason, M.J. Mahoney. *Tissue Eng. Part A* **15** (2009) 1343–1352.
140. F.F. Zhu, P.B. Zhang, D.H. Zhang, X. Sui, M. Yin, T.T. Xiang, Y. Shi, M.X. Ding, H. Deng. *Diabetologia* **54** (2011) 2325–2336.
141. R. Maehr, S. Chen, M. Snitow, T. Ludwig, L. Yagasaki, R. Goland, R.L. Leibel, D.A. Melton. *Proc. Natl. Acad. Sci. USA* **106** (2009) 15768–15773.
142. K. Tateishi, J. He, O. Taranova, G. Liang, A.C. D'Alessio, Y. Zhang. *J. Biol. Chem.* **283** (2008) 31601–31607.
143. P.A. Lysy, G.C. Weir, S. Bonner-Weir. *Stem Cells Transl. Med.* **1** (2012) 150–159.
144. P. Blyszczuk, J. Czyz, G. Kania, M. Wagner, U. Roll, L. St-Onge, A.M. Wobus. *Proc. Natl. Acad. Sci. USA* **100** (2003) 998–1003.
145. O. Korsgren, B. Nilsson. *Curr. Opin. Organ. Transplant.* **14** (2009) 683–687.
146. A.C. Gruessner. *Rev. Diabet. Stud.* **8**(1) (2011) 6–16.
147. J.A. Shapiro. *Rev. Diabet. Stud.* **9**(4) (2012) 385–406.
148. V. Vaithilingam, J. Oberholzer, G.J. Guillemin, B.E. Tuch. *Rev. Diabet. Stud.* **7**(1) (2010) 62–73.
149. V. Vaithilingam, I.S. Tuch. *Rev. Diabet. Stud.* **8**(1) (2011) 51–67.
150. J. Parkinson, Developing a comprehensive system to protect islet cells and deliver transplants to all with insulin dependence, 2012.
http://www.diabetescare.net/content/upclose_detail.asp?id=446538
(Accessed July 30, 2013.)
151. G.H. Mao, G.A. Chen, H.Y. Bai, T.R. Song, Y.X. Wang. *Biomaterials* **30** (2009) 1706–1714.
152. M. Riopel, R. Wang. *Front. Biosci. (Landmark Ed.)* **19** (2014) 77–90.
153. V.K. Ramiya, M. Maraist, K.E. Arfors, D.A. Schatz, A.B. Peck, J.G. Cornelius. *Nat. Med.* **6**(3) (2000) 278–282.
154. Y. Evron, B. Zimmermann, B. Ludwig, U. Barkai, C.K. Colton, G.C. Weir, B. Arieli, S. Maimon, N. Shalev, K. Yavriyants, T. Goldman, Z. Gendler, L. Eizen, P. Vardi, K. Bloch, A. Barthel, S.R. Bornstein, A. Rotem. *Horm. Metab. Res.* **47**(1) (2015) 24–30.
155. F.W. Pagliuca, J.R. Millman, M. Gürtler, M. Segel, A. Van Dervort, J.H. Ryu, Q.P. Peterson, D. Greiner, D.A. Melton. *Cell.* **159**(2) (2014) 428–439.

156. B. Ludwig, A. Reichel, A. Steffen, B. Zimerman, A.V. Schally, N.L. Block, C.K. Colton, S. Ludwig, S. Kersting, E. Bonifacio, M. Solimena, Z. Gendler, A. Rotem, U. Barkai, S.R. Bornstein. *Proc. Natl. Acad. Sci. USA* **110**(47) (2013) 19054–19058.
157. U. Barkai, G.C. Weir, C.K. Colton, B. Ludwig, S.R. Bornstein, M.D. Brendel, T. Neufeld, C. Bremer, A. Leon, Y. Evron, K. Yavriyants, D. Azarov, B. Zimmermann, S. Maimon, N. Shabtay, M. Balyura, T. Rozenshtein, P. Vardi, K. Bloch, P. de Vos, A. Rotem. *Cell Transplant.* **22**(8) (2013) 1463–1476.
158. F.Y. McWhorter, C.T. Davis, W.F. Liu. *Cell. Mol. Life. Sci.* **72**(7) (2015) 1303–1316.
159. A. Rezanian, J.E. Bruin, P. Arora, A. Rubin, I. Batushansky, A. Asadi, S. O'Dwyer, N. Quiskamp, M. Mojibian, T. Albrecht, Y.H. Yang, J.D. Johnson, T.J. Kieffer. *Nat. Biotechnol.* **32**(11) (2014) 1121–1133.
160. E. Motté, E. Szepessy, K. Suenens, G. Stangé, M. Bomans, D. Jacobs-Tulleneers-Thevissen, Z. Ling, E. Kroon, D. Pipeleers. *Am. J. Physiol. Endocrinol. Metab.* **307**(9) (2014) E838–E846.
161. K. Kirk, E. Hao, R. Lahmy, P. Itkin-Ansari. *Stem Cell Res.* **12**(3) (2014) 807–814.
162. S.H. Lee, E. Hao, A.Y. Savinov, I. Geron, A.Y. Strongin, P. Itkin-Ansari. *Transplantation* **87**(7) (2009) 983–991.
163. E. Pedraza, A.C. Brady, C.A. Fraker, R.D. Molano, S. Sukert, D.M. Berman, N.S. Kenyon, A. Pileggi, C. Ricordi, C.L. Stabler. *Cell Transplant.* **22**(7) (2013) 1123–1135.
164. B. Ludwig, B. Zimerman, A. Steffen, K. Yavriyants, D. Azarov, A. Reichel, P. Vardi, T. German, N. Shabtay, A. Rotem, Y. Evron, T. Neufeld, S. Mimon, S. Ludwig, M.D. Brendel, S.R. Bornstein, U. Barkai. *Horm. Metab. Res.* **42**(13) (2010) 918–922.
165. B. Ludwig, A. Rotem, J. Schmid, G.C. Weir, C.K. Colton, M.D. Brendel, T. Neufeld, N.L. Block, K. Yavriyants, A. Steffen, S. Ludwig, T. Chavakis, A. Reichel, D. Azarov, B. Zimmermann, S. Maimon, M. Balyura, T. Rozenshtein, N. Shabtay, P. Vardi, K. Bloch, P. de Vos, A.V. Schally, S.R. Bornstein, U. Barkai. *Proc. Natl. Acad. Sci. USA* **109**(13) (2012) 5022–5027.
166. T. Neufeld, B. Ludwig, U. Barkai, G.C. Weir, C.K. Colton, Y. Evron, M. Balyura, K. Yavriyants, B. Zimmermann, D. Azarov, S. Maimon, N. Shabtay, T. Rozenshtein, D. Lorber, A. Steffen, U. Willenz, K. Bloch, P. Vardi, R. Taube, P. de Vos, E.C. Lewis, S.R. Bornstein, A. Rotem. *PLoS One* **8**(8) (2013) e70150.
167. U. Barkai, G.C. Weir, C.K. Colton, B. Ludwig, S.R. Bornstein, M.D. Brendel, T. Neufeld, C. Bremer, A. Leon, Y. Evron, K. Yavriyants, D. Azarov, B. Zimmermann, S. Maimon, N. Shabtay, M. Balyura, T. Rozenshtein, P. Vardi, K. Bloch, P. de Vos, A. Rotem. *Cell Transplant.* **22**(8) (2013) 1463–1476.
168. B. Ludwig, A. Reichel, A. Steffen, B. Zimerman, A.V. Schally, N.L. Block, C.K. Colton, S. Ludwig, S. Kersting, E. Bonifacio, M. Solimena, Z. Gendler, A. Rotem, U. Barkai, S.R. Bornstein. *Proc. Natl. Acad. Sci. USA* **110**(47) (2013) 19054–19058.
169. R. Pareta, J.P. McQuilling, S. Sittadjody, R. Jenkins, S. Bowden, G. Orlando, A.C. Farney, E.M. Brey, E.C. Opara. *Pancreas* **43**(4) (2014) 605–613.
170. O. Khanna, J.J. Huang, M.L. Moya, C.W. Wu, M.H. Cheng, E.C. Opara, E.M. Brey. *Microvasc. Res.* **90** (2013) 23–29.
171. M.L. Moya, M.R. Garfinkel, X. Liu, S. Lucas, E.C. Opara, H.P. Greisler, E.M. Brey. *J. Surg. Res.* **160**(2) (2010) 208–212.

172. M. Kumagai-Braesch, S. Jacobson, H. Mori, X. Jia, T. Takahashi, A. Wernerson, M. Flodström-Tullberg, A. Tibell. *Cell Transplant.* **22**(7) (2013) 1137–1146.
173. T.C. Schulz, H.Y. Young, A.D. Agulnick, M.J. Babin, E.E. Baetge, A.G. Bang, A. Bhoomik, I. Cepa, R.M. Cesario, C. Haakmeester, K. Kadoya, J.R. Kelly, J. Kerr, L.A. Martinson, A.B. McLean, M.A. Moorman, J.K. Payne, M. Richardson, K.G. Ross, E.S. Sherrer, X. Song, A.Z. Wilson, E.P. Brandon, C.E. Green, E.J. Kroon, O.G. Kelly, K.A. D'Amour, A.J. Robins. *PLoS One* **7**(5) (2012) e37004.
174. M. Chayosumrit, B. Tuch, K. Sidhu. *Biomaterials* **31**(3) (2010) 505–514.
175. X. Liu, X. Li, N. Zhang, X. Wen. *Med. Hypotheses* **85**(1) (2015) 82–84.
176. P.O. Carlsson, E. Schwarcz, O. Korsgren, K. Le Blanc. *Diabetes* **64**(2) (2015) 587–592.
177. Y. Jun, A.R. Kang, J.S. Lee, S.J. Park, D.Y. Lee, S.H. Moon, S.H. Lee. *Biomaterials* **35**(17) (2014) 4815–4826.
178. V. Chandra, G. Swetha, S. Muthyala, A.K. Jaiswal, J.R. Bellare, P.D. Nair, R.R. Bhonde. *PLoS One.* **6**(6) (2011) e20615.
179. J.L. Wilson, M.A. Najia, R. Saeed, T.C. McDevitt. *Biotechnol. Bioeng.* **111**(3) (2014) 618–631.
180. R. Krishnan, M. Alexander, L. Robles, C.E. Foste, J.R. Lakey. *Rev. Diabet. Stud.* **11**(1) (2014) 84–101.
181. S. Bonner-Weir, A. Inada, S. Yatoh, W.C. Li, T. Aye, E. Toschi, A. Sharma, *Biochem. Soc. Trans.* **36**(Pt 3) (2008) 353–356.
182. K. Jeon, H. Lim, J.H. Kim, N.V. Thuan, S.H. Park, Y.M. Lim, H.Y. Choi, E.R. Lee, J.H. Kim, M.S. Lee, S.G. Cho. *Stem Cells Dev.* **21**(14) (2012) 2642–2655.
183. J.F. Habener, V. Stanojevic, *Islets* **4**(3) (2012) 188–198.
184. T. Thatava, Y.C. Kudva, R. Edukulla, K. Squillace, J.G. De Lamo, Y.K. Khan, T. Sakuma, S. Ohmine, A. Terzic, Y. Ikeda. *Mol. Ther.* **21**(1) (2013) 228–239.
185. Y.C. Kudva, S. Ohmine, L.V. Greder, J.R. Dutton, A. Armstrong, J.G. De Lamo, Y.K. Khan, T. Thatava, M. Hasegawa, N. Fusaki, J.M. Slack, Y. Ikeda. *Stem Cells Transl. Med.* **1**(6) (2012) 451–461.
186. F. Pereyra-Bonnet, M.L. Gimeno, N.R. Argumedo, M. Ielpi, J.A. Cardozo, C.A. Giménez, S.H. Hyon, M. Balzaretti, M. Loresi, P. Fainstein-Day, L.E. Litwak, P.F. Argibay. *PLoS One* **9**(6) (2014) e100369.
187. H.M. Shahjalal, N. Shiraki, D. Sakano, K. Kikawa, S. Ogaki, H. Baba, K. Kume, S. Kume. *J. Mol. Cell Biol.* **6**(5) (2014) 394–408.
188. M. Lemper, G. Leuck, Y. Heremans, M.S. German, H. Heimberg, L. Bouwens, L. Baeyens. *Cell Death Differ.* **22** (2014) 1117–1130.
189. I. Todorov, I. Nair, K. Ferreri, J. Rawson, A. Kuroda, M. Pascual, K. Omori, L. Valiente, C. Orr, I. Al-Abdullah, A. Riggs, F. Kandeel, Y. Mullen. *Transplant. Proc.* **37**(8) (2005) 3420–3421.
190. H. Noguchi, G. Xu, S. Matsumoto, H. Kaneto, N. Kobayashi, S. Bonner-Weir, S. Hayashi. *Cell Transplant.* **15**(10) (2006) 929–938.
191. F.W. Pagliuca, J.R. Millman, M. Gürtler, M. Segel, A. Van Dervort, J.H. Ryu, Q.P. Peterson, D. Greiner, D.A. Melton. *Cell* **159**(2) (2014) 428–439.
192. D.M. Berman, J.J. O'Neil, L.C. Coffey, P.C. Chaffanjon, N.M. Kenyon, P. Ruiz Jr, A. Pileggi, C. Ricordi, N.S. Kenyon. *Am. J. Transplant.* **9**(1) (2009) 91–104.

**APPLICATIONS OF NANOMATERIALS IN
MECHANO-SENSITIVE TISSUES**

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15.1. INTRODUCTION

Bone, cartilage and tendon defects have specific structural, chemical and biological compositions. The extracellular matrix (ECM) of these musculoskeletal tissues interacts with cells to guide tissue formation. Since the ECM of such tissues is at the nanoscale, scaffolds in development are being developed which can mimic these structure. The structural features of the scaffolds affect cell adhesion, proliferation and differentiation.

In order to repair musculoskeletal defects of bone, cartilage or tendon, synthetic materials are widely used. It is important that such materials must mimic the natural environment and provide an optimal matrix environment, biological properties including appropriate chemical cues such as growth factors and optimal mechanical properties to guide tissue regeneration. This chapter views the properties of nanomaterials for bone, cartilage and tendon regeneration. It covers the aspects of incorporating nanoparticles (NPs) with scaffolds to improve mechanical properties and the biocompatibility of polymers. The utilisation of the fabricating inert materials by electrospinning to create fibrous scaffolds that mimic the natural environment of tissues will be discussed. The design, fabrication, challenges and success of incorporating of growth factors, genetic cues and drugs to enhance bone, cartilage and tendon formation will also be evaluated. An overview of the future challenges and directions of nanomaterials for musculoskeletal tissues will be provided.

15.2. APPLICATIONS OF NANOMATERIALS IN MECHANO-SENSITIVE TISSUES

Tissue engineering and regenerative medicine is a rapidly growing area and aims to find alternatives for transplanting tissues to restore damaged or non-functioning tissues [1,2]. The musculoskeletal tissues bone, cartilage and ligament / tendon are highly structured composite tissues consisting of fibres within a matrix [1,2]. Musculoskeletal defects often heal after anatomical correction and correct wound management [1,2]. However, when the distance is too large to bridge, current surgical techniques to restore such defects are inadequate, which leads to poor outcomes for the patient [1,2]. With most bone lesions, artificial biomaterials are used to fill the gaps aiming to cause bone repair and regeneration [1,2]. To repair ligament and tendon injuries, the injured tissues are aligned and bridged with a biomaterial support. With cartilaginous defects natural or synthetic materials aims to support the tissue regeneration and restoration of function [3]. However, despite the improvements in surgical techniques and biomaterial characteristics such restorative strategies often do not lead to full repair of the damaged

musculoskeletal tissue. Due to the inadequate surgical treatment options available there is a large clinical demand to create novel materials that have improved mechanical properties, enhanced material-tissue interaction that mimic the natural environment and ability to support tissue growth and organ formation [4,5].

The main strategy for tissue engineering research is restoring tissue by implanting scaffold with cells. One of the major challenges in regenerative medicine is to manufacture suitable scaffolds, which elicit the correct response from the local cells to form functional organ replacements [6,7]. ECM plays an important role in these interactions with the cell surface receptors, growth factors, which provide instrumental cues for cell survival and cell fate [6,7]. Scientific research carried out in last few decades has shown that the interaction of cells with nanotopographical features is important in engineering complex tissues, as cells respond to various topographical features at nanoscale such as nanopores, ridges, grooves and fibres within their environment [6,7].

Over the last decade, the use of nanotechnology has further accelerated the growth in regenerative medicine. Nanomaterials comprises of scaffolds with structural dimensions of less than 100 nm [8,9]. As biological tissues contain components, which are of this scale including collagen fibrils and hydroxyapatite (HA) crystals, cartilage and bone could also be referred to as bionano composite materials [8,9]. Furthermore, as the cell-cell interaction is at the nanoscale level, using nanotechnology provides a significant advantage in the ability to mimic the native tissue or organ [8,9].

Nanocomposite scaffolds containing biocompatible polymers and bioactive NPs have been investigated with particular interest for bone tissue engineering due to their optimal mechanical properties and suitable biocompatibility [10,11]. Among the scaffolds evaluated, there are specific groups of nanomaterials tested for regenerative medicine including NPs, carbon nanotubes, nanofibres and composite nanomaterials. Among these electrospun nanofibres meshes have been used extensively due to their ability to mimic the collagen and elastin of the natural ECM [10,11]. In addition, bioactive factors can be loaded directly into the nanofibres produced *via* electrospinning. The bioactive molecules can be released at a controlled rate and used to stimulate the proliferation and differentiation of adhered cells. One of the biggest challenges is how to preserve the bioactivity of the growth factors within the nanoscaffolds [12,13]. Several techniques have been utilised to absorb bioactive molecules within scaffolds for musculoskeletal applications [12,13].

This chapter provides the most updated overview on various types of nanomaterials, which are being investigated and are in use for mechano-sensitive tissues including bone, cartilage, ligament and tendon regeneration. Special focus is given to the use of NPs, nanofibres, nanotubes and composite nanomaterials. The success and challenges of the incorporation of drugs,

proteins, growth factors with nanomaterials will also be discussed to regenerate musculoskeletal tissues.

15.3. NANOMATERIALS IN MECHANO-SENSITIVE TISSUES

Nanotechnology has become a major frontier in advancing the field of musculoskeletal tissue engineering including the regeneration of bone, cartilage, tendon and ligament. The structure of bone, cartilage and ligament/tendon is very similar, they all have a hierarchical structure and in simple terms consists of a network of collagen fibrils with diameters of approximately 100 nm, which is encased in a tissue specific matrix [10,11]. It is this structural design and the interactions of the fibres with the matrix that defines the mechanical and biological characteristics of each tissue type. In general the repair of a skeletal defect requires the insertion of an autologous material or synthetic material to bridge the gap, which may remain or resorb with time as the defect heals. Several materials have been investigated over the years for musculoskeletal regeneration but one of the main factors preventing the development of successful materials is the failure to mimic the natural hierarchy of the native tissue structure [10,11]. Thus, it is not surprising that many groups have tried to utilise nanotechnology to incorporate nano-sized components into the materials to improve the success of the regeneration of tissue [10,11]. There are two main approaches by which this has been researched to date. Firstly, the native nanostructured hierarchy of the tissues can be mimicked to improve the invasion of appropriate cells to create the required tissue. Secondly, nanosized components can be incorporated to ensure the mechanical properties are like those of native tissue. In the field of nanotechnology, there are four main types of nanomaterials that can be nanofibres, nanotubes and nano-structured composites, of which will be detailed below (Figure 1).

Figure 1. Diagram to illustrate the different nanomaterials used in the regeneration of mechano-sensitive tissues.

15.4. BONE TISSUE REGENERATION

Bone tissue is a hierarchical structure consisting of both organic and inorganic components [14]. The organic collagen type I fibres and inorganic HA crystals create a mineralized collagen based-ECM [14]. The complex structure creates a cellular organization, which has an optimal mechanical stiffness and architecture for molecular cues to drive gene expression and protein production [14].

15.4.1. Clinical need

There are a number of bone graft substitutes used surgically to repair osseous defects including synthetic, autografts and allografts [15,16]. Autografts are the gold standard for the surgical repair of bone defects due to their low immunogenicity and contain all the properties to support good bone regeneration effect [15,16]. Autografts contain the ability to induce bone formation including growth factors, osteoprogenitor cells and three-dimensional porous matrix [15,16]. However, traditionally auto-graft bone grafts are taken from the iliac crest, which causes donor site morbidity, risk of

scarring, deformity and has several surgical risks including bleeding and infection [15,16]. Furthermore, large defects often require more than is available [15,16]. Therefore, to overcome such complications synthetic alternative are being investigated and this has initiated the field of bone tissue-engineering.

15.4.2. Evolution of nanomaterials for bone tissue engineering

Various nanomaterials have been developed for bone regeneration over the decade including metals, ceramics and polymeric materials [17,18]. An ideal bone scaffold material must have good mechanical properties, demonstrate adequate biocompatibility, show low patient morbidity, be easily available to surgeons, cost effective and support bone regeneration [19]. The size of pore and porosity are also important for bone tissue formation [20]. An ideal nanomaterial is aimed to mimic the natural hierarchical structure of bone, while maintaining the mechanical properties [20].

15.4.3. Nanoparticles (NPs)

The first type of nanomaterials that have been researched within bone regeneration are NPs [21]. NPs exist in the nanosize range, usually less than < 100 nm and due to their size can be used to deliver drugs, growth factors and genetic cues [21]. Nanoparticles have been commonly used in regenerative medicine and for bone tissue engineering [22]. The applications of NPs in bone spreads from drug delivery for cancer therapy to treatment of systemic bone diseases, cell labelling and controlling cells responses for bone differentiation [21,22]. Ideal properties which need to be incorporated in the design of NPs are to be nontoxic, bio-inert and they should invoke minimal side effects after implantation [23].

The main application of NPs for bone tissue engineering is mimicking the natural hierarchical structure of bone [21,22]. If tissue engineered scaffolds can mimic the native extracellular matrix, cell will be able to adhere, move and support tissue formation. NPs are promising candidates for bone engineering as they can mimic the same size as the HA crystals of the bone ECM and hence can provide these cellular cues [21,22]. Nanoparticle modified composite scaffolds offer far more promises to facilitate bone formation [21,22].

Site specific tissue formation has been a dream of every biomaterial scientist. To realize this ambition various bone inducing growth factors, proteins and drugs has been incorporated with NPs. To enhance the osteogenic differentiation of mesenchymal stem cells (MSCs) several growth factors have been combined with NPs [24]. The enhancement of osteogenic differentiation has shown to be supported by bone morphogenetic proteins (BMPs) in several studies to date [25-27]. Bone-morphogenetic protein-2 (BMP-2) incorporated on NPs has shown to support *in vitro* osteogenic differentiation and promote bone formation *in vivo* [28]. BMP-2 and bone-morphogenetic protein-7

(BMP-7) loaded NPs incorporated onto three-dimensional (3D)-fiber meshes, showed a synergistic effect as a bone forming scaffold [28].

Synthetic stimulators of bone formation have also been incorporated with NPs to promote bone formation which include NPs containing genetic cues [29-31]. Bioglass is a glass that contains biological and physiological functions suitable for the regeneration of osteochondral restoration. A bioactive glass nanoparticle (nBG) poly(lactic acid) (PCL) coating on HA/ β -tricalcium phosphate (HA/bTCP) scaffold demonstrated upregulation of osteogenic gene expression (Runx-2, collagen I, osteopontin and bone sialoprotein) and alkaline phosphatase activity in adipose derived stem cells (ADSCs) when used in combination with BMP-2 treatment [29]. As the delivery of proteins and growth factors poses challenges in terms of optimization of the release of the bioactive factors, NPs have been researched to act as gene carriers. The transfection approach is a promising strategy for regenerative medicine as it allows long-term expression, causing a potential for a longer therapeutic effect. Several NPs have been used to carry plasmids encoding transcription factors, growth factors and hormones. For example, a BMP-2 encoding plasmid was incorporated into calcium phosphate NPs on alginate hydrogels [30]. As NPs have can have controlled degradability, safety and ability to target the tissue of interest, gene therapy may show a promising future for bone regeneration [31].

Emerging area where NPs are investigated widely is stem cell therapy. It is hoped that stem cells therapy can promote tissue regeneration due to their self-renewal capacity. However, differentiation of stem cells in specific cell type *in vivo* still remains a challenge. Stem cell tracking by using various NPs such as quantum dots, fluorescence silica NPs and superparamagnetic iron oxide (SPIO) NPs is being used as a tool to evaluate stem cell migration and their phenotypes [32,33]. However, there are challenges to be faced once these techniques are optimized for bone tissue engineering. It is important that nanoparticle does not affect the MSCs potential for bone differentiation. Despite gold NPs and SPIO showing the ability to track MSC *in vitro* and *in vivo*, some studies have found the NPs to alter osteogenic differentiation, which warrants further investigation [34].

15.4.4. Nanocomposites for bone tissue engineering

Native bone consists of nanocomposite structure, ceramic HA particles and a collagen fiber matrix [35]. Hence, nanocomposite scaffolds made of biocompatible polymer and bioactive inorganic NPs have been extensively researched for bone tissue regeneration due to their ability to mimic the natural bone structure, whilst providing excellent mechanical properties and osteoinductive and osteoconductive properties [35-37]. Since natural bone matrix is a composite of biological ceramics (HA) and polymer (collagen) several synthetic and natural biomaterials hybrids have been developed to

mimic the natural bone structure and provide suitable scaffolds for bone tissue engineering [35-37]. Several bone graft materials have been researched to serve as a carrier to deliver the cells to the injured bone acting as a support to regenerate bone tissue including bioceramic materials, polymers, natural and metallic materials. Bioceramics, including calcium phosphate, bTCP, HA and calcium sulphate have excellent osteoconductive properties [35]. However, their brittle nature often causes such materials to fail as a bone substitutes [35,36]. To overcome this problem polymers have been combined with a ceramic material to obtain suitable scaffolds [35,37]. Combining bioactive ceramic particles to polymer materials improves the mechanical properties of the polymeric scaffolds and enhances their bone inductive behaviour [38,39]. Calcium phosphate NPs within PLGA microspheres showed proliferation of osteoblast cells, having a mechanical stiffness that was similar to bone tissue [40].

As bone contains HA particles, many studies have tried to incorporate nano-HA into the polymer matrix to improve the scaffold biological properties and support bone formation *in vivo*. For example, PCL and nano-HA have shown to improve the mechanical strength of PCL alone and stimulate osteoblast proliferation [41]. After the incorporation of n-HA into poly(lactic-co-glycolic acid) (PLGA) salt leached scaffolds, *in vivo* mineralisation was formed and osteogenesis was found in the radius defect of rabbits after nine weeks of implantation [42].

Natural polymers have also shown promise as bone graft scaffolds including collagen, silk, hyaluronic acid, gelatin, chitosan (CS) and alginate. Combining bioceramic and natural polymer to create nanocomposites has also showed to support osteogenesis. For example HA, bTCP and calcium phosphate have been incorporated into natural polymers to improve their mechanical properties and osteogenesis capacity [43-45]. Gelatin with HA nanocomposite materials demonstrated good cell adhesion and proliferation of human MSCs as well as good compressive strength [46]. CS has also showed promise as a bone graft substitute due its ability to support cell ingrowth and antibacterial properties showing bone formation when combined with HA [47].

15.4.5. Nanofibres in bone tissue engineering

The goal of the scaffold is to provide a 3D environment for cells to attach and form tissue [9]. One particular type of scaffold manufacture that has obtained a great research interest is nanofibres [9]. The development of nanofibres is expanding due to their potential ability to mimic the architecture of extracellular matrix (ECM) of the native tissues [9]. The major protein of the ECM is collagen which is arranged in nanofibres ranging from 50–500 nm in diameters which controls cell behaviour and in turn bone formation [9]. In bone, the ECM consists of the mineralised collagen type I fibres, which consists of 90 % collagen [9]. The mineralised collagen fibrils align and arrange in ways

to form a hierarchical structure and form full bone [9]. Therefore, developing nanofibrous scaffolds to mimic the natural ECM will allow for cell adhesion, proliferation and differentiation and in turn bone formation [9]. Extensive research is being carried out to understand the potential of nanofibrous scaffold for bone tissue engineering [9]. Nanofibres permit ingrowth, proliferation and differentiation of human MSCs downwards osteoblast like cells [9]. Nanofibres ability to promote cell adhesion, proliferation and cell migration is due to their high surface area to volume ratio [9].

There are several methods to generate nanofibres including electrospinning, phase separation and self-assembly [9]. However, electrospinning has dominated the literature showing to be the most effective way to create nanofibres and stimulate bone formation. Electrospinning allows the ability to control the thickness and composition of the nanofibres, porosity of the scaffold simply and effectively, which in turn enables the ability to control bone regeneration [48,49]. This technique creates polymer nanofibres by applying a high voltage to a syringe filled with polymer solution. The polymer solution is held at the end of the syringe tube due to surface tension and the electrical potential provides charge to the polymer solution [48,49]. The charge repulsion of the polymer solution induces a force that is opposite to the surface tension of the polymer solution [48,49]. As the electrical potential increases the surface of the polymer solution elongates to form a conical tube known as the Taylor Cone [48,49]. The critical electrical potential to overcome the surface tension causes the formation of a jet to be ejected from the Taylor Cone and eventually forms orientated nanofibres that are collected on a stationary or rotatory metallic collector. Several materials have found to be suitable for electrospinning including natural and synthetic polymer for bone tissue engineering [48,49].

Various natural polymers and proteins have been electrospun to biomimic ECM matrix for bone regeneration. Collagen has become a very well researched protein and has shown the ability to support several cell types including osteoblasts, chondrocytes, tenocytes and MSCs [50]. CS is another natural biomaterial, which has shown to be a suitable scaffold for musculoskeletal regeneration [51]. CS/poly(ethylene oxide) (PEO) nanofibres demonstrated the ability to support the attachment of osteoblasts and chondrocytes whilst sustaining adequate fibre mechanical and morphology [51]. Gelatin has shown to support the attachment of cells and mimic the ECM, proving to be a candidate for tissue engineering scaffolds. Gelatin has been electrospun with PCL polymer fibrous scaffolds and shown to improve mechanical strength and surface wettability of PCL or gelatin fibres [52]. Fibroin is another well-researched natural biomaterial for nanofibrous scaffolds due its biocompatibility [50]. Fibroin has shown to have adequate fibres length and surface area proving to be suitable for tissue engineering purposes [53].

Electrospun silk fibroin scaffolds which showed high surface area, porosity and interconnectivity, promoted adhesion and proliferation of cells for bone tissue

engineering applications [54]. Kim *et al.* investigated the hybrid electrospinning of silk fibroin (SF)/PCL nano /microfibrous composite scaffolds with various compositions [54]. The increments of SF nanofibre content in the composite scaffolds led to a decrease in pore size but did not have an effect of human mesenchymal stem cell (hMSC) differentiation [54]. However, composite scaffolds were better than PCL nanofibres alone and stimulated new bone formation in a rabbit calvarial defect model (Figure 2) [54].

Figure 2. **A** Diagram of the hybrid electrospinning system and an SF / PCL nano / microfibrous composite scaffold. **B** Changes in morphology of the PCL microfibrous scaffold and SF / PCL nano / microfibrous composite scaffolds consisting of varying SF-nanofiber content. **C** Masson's trichrome-stained histological section after implantation. **D** Quantitative analysis of new bone area at 8 weeks. Taken with permission from [54].

However, electrospun silk scaffolds are limited due to their low mechanical strength [55]. One strategy to overcome this problem was to incorporate HA NPs (HAp) within the nanofibrous scaffolds produced by electrospinning [55]. Kim *et al.* illustrated that it is possible to enhance the mechanical properties of the nanofibres by uniformly dispersing HAp within SF nanofibres [55]. The nanofibres showed peak strengths at the HAp content of 20 wt % [56]. The

success of incorporating HA with nanofibres was illustrated by Lai *et al.* demonstrating that the incorporation of nano HA (nHAp) within a CS/SF scaffold creates favourable microenvironment for bone tissue formation [56]. The osteogenic differentiation of hMSCs was observed during *in vivo* experiments. The authors demonstrated that histological and immunohistochemical analysis of the retrieved hMSCs/nanofibrous membrane scaffold (NMS) construct 1 and 2 months post implantation indicating that NMS had the potential for bone regeneration and suggested the scaffolds were promising scaffolds for bone tissue engineering [56].

Natural polymers have been found to be difficult to electrospun due to their high viscosity and low solubility [55]. To overcome this, synthetic polymers have been added to the natural polymers to create more compatible electrospun scaffolds including collagen and silk for tissue engineering purposes [57]. For example, PLGA nanofibres with gelatin and nHAP have shown to form biometric scaffolds suitable for bone formation [57]. In addition to natural fibrous scaffolds several synthetic polymers have been electrospun to create suitable scaffolds. A simple and compatible synthetic material PCL has been studied extensively for bone tissue engineering and regenerative medicine as a nanofibrous scaffold. The polymer PCL has been combined with several materials to create biomimetic nanofibres. Biomimetic 3D thick nanofibrous PCL scaffolds demonstrated bone formation *in vivo* showing the ability to mimic the fibrillar organization of the bone ECM [5]. The combination of PCL/aloevera/silk fibroin-HA nanofibrous scaffolds showed enhanced osteogenic differentiation and mineralisation in comparison to PCL nanofibres alone using adipose stem cells [7]. In addition, PCL nanofibres with phlorotannin, the main component of the brown alga *Ecklonia cava* has also showed to simulate bone formation of osteoblast like cells (MH63) [58]. Lou *et al.* demonstrated that nanofibrous poly(L-lactic acid) (PLLA) fibres with diameter of 70–300 nm with β -TCP improved the mechanical properties and the bioactivity of the PLLA [59]. The modified fibres supported MG-63 osteoblast proliferation, penetration and ECM deposition [59]. Titanium dioxide (TiO₂) nanofibrous scaffolds demonstrated better cell adhesion and proliferation compared to the flat control surfaces using cell cycle analysis [60]. Expression of stemness markers genes including Nanog3, Rex-1, SOX-2 and nestin of the ADSCs cultured on the control and TiO₂ nanofibrous surfaces was also higher on the fibrous surfaces than controls surfaces [60].

To support tissue formation, it has been extensively popular to load nanofibres with bioactive factors to modify the cell environment and mimic the natural ECM niche. Biofactors can be loaded onto nanofibres using several techniques including blending, coaxial spinning and immobilization [60–63]. Blending of the desired factors during electrospinning can lead to aggregation homogenous dispersion along the fibres [60–63]. Proteins, molecules and nucleic acid have shown to be blended due nanofibres using these methods. Coaxial electrospinning causes the factor to localize to the center of the fibre. This is

where two solutions are pumped through needles or cylinders and then electrospun to create a fibre [60-63]. Two solutions have different solubility, which prevents the mixing of the fibres during the electrospinning process. Lastly, the factor of interest can be adhered or immobilised onto the nanofibre surface using techniques such as chemical functionalization or self assembly [60-63].

Growing evidence of attaching growth factors to nanofibres *in vivo* to support bone formation has been reported. BMP-2 release from poly(ethylene glycol) (PEG) / PCL showed calvarial bone regeneration after 24 days in a rabbit model [64]. Further support illustrated that BMP-2 could regenerate bone formation on PLGA / HAp nanofibres in the bone defects of tibia in nude mice [65]. Eap *et al.* illustrated that PCL nanofibrous implant (from 700 μm – 1 cm thick) was functionalized with CS and BMP-7 growth factor using layer-by-layer technology, producing fish scale-like CS/BMP-7 nanoreservoirs [5]. The scaffolds were assessed using *in vivo* implantation, showing significantly more newly mineralized ECM in the functionalized implant compared to a bare scaffold after 30 days implantation, as shown by histological scanning electron microscopy / energy dispersive X-ray microscopy study and calcein injection [5]. Li *et al.* demonstrated that nanoparticle-embedded electrospun nanofibre scaffold controlled the dual delivery of BMP-2 and dexamethasone (DEX) and showed that *in vivo* bone formation (Figure 3) [66]. However, it has become a challenge to finely tune the release of the growth factors to match the physical needs of the *in vivo* environment to form bone tissue [66].

Figure 3. A. Schematic illustration of the fabrication of BMP-2-loaded BSA NPs stabilized with CS (A) and electrospinning of nanoparticle-embedded PCE copolymer nanofibers (B). B. Masson trichrome staining after implanted *in situ* for 4 and 12 weeks respectively for Control group without material (a), PCE (b), NPs / PCE (c), BNPs / PCE (d), DEX / PCE (e), BNPs / DEX / PCE (f), the red panels are the magnified regions in calvarial panorama. The statistical evaluations calculated from the histological staining pictures of 12 weeks ($n = 3$), in which negative is refer to the blank implanted group, NB represents the newly formed bone area, CB represents the calcified bone area, M stands for the bone marrow area and UM stands for the undegraded material area. Asterisk mark represents the significant difference between each other, $n = 3$ for each group, $p < 0.05$. C. Radiograms of the X-ray detection after implanted *in vivo* for 4, 8, 12 weeks respectively (A), and the rightmost disk is a calvarial defect template with a diameter of 8 mm. The statistical evaluations of apparent repair area (B), mean gray value (C) and bone repair level (D) were calculated from the radiograms ($n = 3$) for Control group without material (a), PCE (b), NPs / PCE (c), BNPs / PCE (d), DEX / PCE (e), BNPs / DEX / PCE (f). The normal calvaria mean gray value was calculated from an undamaged calvarial bone. Asterisk mark represents the significant difference between each other, $n = 3$ for each group, $p < 0.05$. Taken with permission from [66].

Biomaterials derived from the decellularisation of mature tissue can also contain the topographical and structural features and influence cellular activity [67]. Gibson *et al.* maximized this effect and incorporated the present decellularized extracellular matrix (DECM) particles in combination with synthetic nanofibers and examined the ability of these materials to influence

stem cell differentiation [67]. Nanofibre scaffolds containing up to 10 % particles showed differentiation compared to controls scaffolds after 3 weeks [67].

Extensive research has been carried out into the incorporation of HAp within nanofibres for bone tissue engineering [68]. Poly(L-lactic acid)-*co*-poly(ϵ -caprolactone) / silk fibroin / ascorbic acid / tetracycline hydrochloride (PLACL/SF/AA/TC) and nano-hydroxyapatite (nHA) demonstrated osteogenic differentiation of MSCs, demonstrated using alkaline phosphatase activity, mineralization and double immunofluorescence staining of both CD90 and osteocalcin [68]. Collagen nanofibres with nHA agglomerated by electrospraying improved the adhesion and metabolic activity of MC3T3-E1 osteoblasts [69].

Nanofibres can also act as drug-delivery system for musculoskeletal engineering [77]. Infection of implantable materials remains a long time surgical challenge, with extensive innovation been used to modify materials to prevent bacterial colonisation. It is now possible to include antimicrobial drugs into electrosupn nanofibres. Rapid release of rifampicin from PCL / nanofibres meshes illustrated good antimicrobial resistance to *Staphylococcus epidermidis* and prevented biofilm formation. The RF-incorporation nanofibre meshes did not sacrifice the osteogenic properties of the scaffolds showing up regulation of gene expression for preosteoblastic collagen type I and alkaline phosphatase (ALP) [70]. Strontium phosphate incorporation into nanofibres has also shown a potential material for bone tissue engineering. Su *et al.* utilised PCL nanofibres coated or blended with strontium phosphate and found it supported the osteogenic differentiation of human exfoliated deciduous teeth [71]. Alendronate (ALN) loaded PCL nanofibres scaffolds demonstrated ALP activity, calcium content and osteogenic differentiation of ADSCs genes *in vitro*. Furthermore, the ALN / PCL scaffolds had a positive effect on bone formation at 8 week after implantation [72]. Further understanding of bioceramics is important for developing smart bone tissue engineering strategies [71].

Carbon nanotubes (CNTs) have excellent mechanical properties, high surface area and light-weight making them ideal scaffold material to be investigated for tissue engineering purposes [73]. CNTs have been incorporated into several polymer materials including PCL, PLA and PLGA to form biomimetic scaffold for bone tissue engineering [74-76]. CNTs have also demonstrated the ability to support osteogenesis when incorporated with several scaffolds materials. Duan *et al.* illustrated that CNTs incorporated into PLA scaffolds by thermal induced phase separation promoted the osteogenic differentiation of bone marrow mesenchymal stem cells (BMMSCs) [73]. The CNTs enhanced the expression of osteogenesis related proteins as well as the formation of type I collagen [73]. However, the toxicity of CNTs, is still unclear and requires further exploration into the long-term effect on bone remodelling.

15.5. CARTILAGE TISSUE REGENERATION

Cartilage is an avascular tissue that lacks the capacity for repair [77]. Cartilage tissue contains chondrocytes entrapped in an ECM rich in proteoglycan macromolecules and collagen fibrils. Similarly to bone tissue, the hierarchical network structure of cartilage tissue is responsible for its mechanical properties [78]. Tissue engineering approaches have been mainly focused on seeding chondrocytes or MSCs on biomimetic scaffolds for cartilage tissue formation [77,78]. Designing suitable scaffolds for cartilage tissue engineering using nanomaterials requires the ability to mimic the native cartilage tissue architecture [77,78]. The scaffolds should have similar mechanical properties, biochemical composition as well their physical structure to native cartilage [77,78].

15.5.1. Clinical need

Creating biomaterials for cartilage engineering is driven from the thousands of joint procedures including knees and hips that are undertaken each year. Articular cartilage has a very poor ability to repair itself due the poor vascular supply [79]. Currently surgical techniques to restore failure of articular cartilage rely on the drilling or abrasion of the joint to simulate healing or by the delivery of growth factors at the injury site [79]. For larger defects, artificial hip and knee joints are created but these are not ideal for the young patients due to the limited lifespan of the joint [79]. Autografts are available to repair defects but are limited by donor site morbidity, complications from the surgical harvesting site and limited availability of tissue for large defects [79]. These imitations can be overcome by regenerating articular tissue by using tissue-engineering approaches [79].

15.5.2. Nanoparticles for cartilage tissue engineering

In a similar strategy to utilize NPs for bone formation, NPs have been incorporated into scaffolds to optimize cartilage formation. NPs can be combined into scaffold materials to provide nanoscale dimensions that mimic the natural ECM of cartilage, providing cellular cues and the ability to support cartilage formation. Furthermore, NPs can deliver growth factors, genetic material and drugs, which can support cartilage formation [81-82].

The incorporation of NPs to support cartilaginous differentiation of stem cells has been documented. For example, PLGA NPs were used to deliver SOX-9 to MSC and demonstrate cartilage formation [80]. Incorporation of aggrecan and cartilage oligomeric matrix protein (COMP) supported chondrogenic differentiation of hMSCs into chondrocytes [81]. Lu *et al.* similarly demonstrated that NPs could deliver genes to promote stem cells differentiation [82]. A CS scaffold combined with the design of a novel gene-activated matrix (GAM) embedded with hybrid hyaluronic acid (HYA) / CS / plasmid-DNA NPs encoding transforming growth factor

(TGF)- β 1 demonstrated an optimal environment for chondrocyte proliferation using histological and biochemical assays [82]. The double release of insulin growth factor-1 (IGF-1) and transforming growth factor-beta 1 (TGF- β 1) from NPs from PLGA scaffolds supported the cartilage formation of MSCs with the collagen type II and aggrecan expression higher than TGF-free scaffolds [83]. Shi *et al.* demonstrated that adipose derived stem cells transfected with bone morphogenetic protein-4 (BMP-4) showed expression of chondrogenesis-related genes and protein and promoted *in vivo* cartilage formation [84].

15.5.3. Nanocomposites for cartilage tissue engineering

The structural and mechanical properties of cartilage are due to the composition and architecture of the ECM. As highlighted earlier, the ECM consists of collagen fibrils and proteoglycan macromolecule clues and non-collagenous proteins to create a hierarchical network [85-86]. Therefore, in a similar approach to designing suitable bone scaffolds, hybrid combinations of biocompatible polymers and ceramic NPs have been investigated for cartilage tissue engineering to mimic the complex architecture of cartilage [85-86].

Numerous scaffold materials have been considered for cartilage repair to date. Both natural and synthetic polymers have been optimized for cartilage engineering. Natural nanoscaffolds have also illustrated to provide a compatible environment of cartilage formation due to the ability to interact with cells and provide optimal cues [85]. The combination of natural polymers and bioceramics has been investigated for cartilage formation. HA with polymer materials has supported cartilage formation [86]. Collagen type II and HA showed a complex three dimensional structure that had good physiochemical and biocompatibility showing histological and immunohistochemical staining for chondrocytes growth and essential protein matrix production [86].

Polymer-HAp composite have also been explored for cartilage repair, including nanofibrous scaffolds. For example, PLGA, poly(vinyl alcohol) (PVA) and PLLA have been electrospun with nHA and found to provide molecular signalling mechanisms ideal for cartilage formation [87-89]. nHA/collagen scaffolds demonstrated the ability to sustain chondrocyte cell attachment, abundant glycosaminoglycan (GAG) synthesis and natural morphology [90]. Collagen/alginate and HA scaffolds have also demonstrated to be promising candidates for osteochondral tissue engineering [91].

Metal and bioceramics materials hybrid combinations have also begun to be investigated for cartilaginous repair [92]. Certain metallic materials have shown to have osteoinductive characteristics and good biocompatibility and hence have been investigated for cartilage engineering. Nanostructured HA with zirconia has shown to repair the articular cartilage defects in rabbits [92]. Titanium has been used in surgical practice for a long-term to replace

cartilaginous defects. Work by Savaiano *et al.* showed that PLGA mixed with titanium NPs demonstrated chondrocyte adhesion was higher after the addition of the titanium NPs [93]. Gold NPs have also been incorporated into polymers and shown to improve cartilage formation [94].

Human adipose stem cells (hADSCs) have shown to be useful candidate for tissue engineering purposes due to their angiogenic and wound healing capabilities. Our group has developed polyhedral oligomeric silsesquioxane-poly(carbonate-urea) urethane (POSS-PCU), a novel nanocomposite polymer consisting of PCU with a nanoparticle, POSS incorporated within POSS-PCU supported ADSC survival and proliferation and differentiation into cartilage within the nanoscaffold [95].

15.5.4. Nanofibres for cartilage tissue engineering

Electrospinning of scaffolds has gained extensive attention in recent years being able to create suitable scaffolds for cartilage tissue engineering purposes. Nanofibres can facilitate cell behaviour to support cartilage formation due to the ability to create a multi-scale environment, which mimics the ECM of cartilaginous tissue [95-96]. Nanofibrous scaffolds have shown to promote human MSCs attachment, proliferation and also promote chondrogenic differentiation due to the ability to create the complex hierarchical architecture of natural cartilage [95-96].

The size of the nanofibres has shown to effect the attachment of cells and serum proteins [96]. Coburn *et al.* compared pellet and PVA nanofibres scaffolds cultures and demonstrated enhanced chondrogenic differentiation of mesenchymal stems cells as indicated by increased ECM production and cartilage specific gene expression while also permitting cell proliferation on the fibrous scaffolds [96]. Collagen and PLA nanofibres have also shown to synergistically promote the osteochondral regeneration of MSCs. Osteochondral defects were created in rabbits and repaired by implanting the collagen-nanofiber scaffold. There is an evidence of rapid subchondral bone emergence and cartilage formation using these scaffolds demonstrated by histological, biomechanical testing and microCT testing [97]. He *et al.* developed a biodegradable hybrid nanofibrous membrane of collagen and poly(L-lactic acid-co- ϵ -caprolactone) (PLCL, 75 : 25) for cartilage engineering. The scaffold demonstrated cartilage like tissue after 12 weeks of implantation in mice [49]. Shafiee *et al.* illustrated the effects of PLLA / PCL nanofibres on the cartilaginous capacity of nasal septum derived progenitors *in vitro* [98]. Aligned nanofibres illustrated better chondrogenic differentiation than randomly orientated PLLA / PCL scaffolds as shown by the upregulation of collagen II and aggrecan [98]. In addition, PCL electrospun nanofibres supported the MSC cartilaginous differentiation in a perfusion bioreactor including the cartilage-related genes such as aggrecan, collagen type II, and SOX9 [99].

To overcome poor cellular infiltration into nanofibres scaffolds, combination of nano- and microfibrinous architecture has been investigated. When used in combination these scaffolds showed cartilage regeneration [100-102]. Poly(D,L-lactide) (PDLLA) nanofibres coated with PDLLA microfibers showed better chondrocyte adhesion than PDLLA films and PDLLA microfibers alone [102].

The incorporation of cartilage specific cues into nanofibres has shown a promising strategy to improving tissue regeneration. Itani *et al.* investigated PLGA nanofibres with basic fibroblast growth (bFGF) factor-laden particles and an exterior fibrin sealant illustrated optimal cell maintenance and neocartilage formation in mice using nanofibres of 0.8–3.0 μm (Figure 4) [103].

Figure 4. **A.** Steps taken for the *in vivo* experiments, from chondrocyte harvest / digestion; to construction with incorporation of seeded cells from female beagles, basic fibroblast growth factor (bFGF) particles, and fibrin sealant; to autogenous implantation in a second surgical procedure 20 h after the chondrocyte harvest. **B.** Auricle-type scaffold 20 weeks after implantation, showing gross morphology (top) and safranin O staining (center and below) for the (left) 0.5-, (center) 3.0-, and (right) 20- μm groups. **C.** Quantitation of safranin O-positive areas in representative cross-sections of samples from the 0.5-, 3.0-, and 20- μm (above and center) groups. The total areas (above) were compared with the positive areas (neocartilage) identified by image analysis (center) to yield the percentage of neocartilage in the sample. (Below) Bar graph of the percentage of neocartilage at 20 weeks. Bars with error bars are average and standard errors, respectively (* $p < 0.01$; $n = 6$ in each group; values given as mean $\pm$ SE). Taken with permission from [103].

Similarly, PCL nanofibres functionalised with CS and HYA nanofibres cross-linked using carbodiimide chemistry illustrated that the rat bone marrow derived stem cells (rBMSCs) showed differentiation and production of hyaline cartilage proteins including collagen type II and aggrecan [104]. Further support, demonstrated that PCL scaffolds combined with 1 % high-molecular-weight sodium hyaluronate (NHA / MHA), 1 % hyaluronan and 200 μg TGF- β 1; NTGF/MTGF, or 0.1 % bovine serum albumin (BSA) also showed augmentation of MSCs and enabled chondrogenic differentiation [105].

Composite scaffolds have been used to obtain the mechanical properties and the biochemical composition. Zheug *et al.* demonstrated that natural nanofibrous articular cartilage extracellular matrix (ACECM) can mimic the biochemical composition but be improved when synthetic PLGA is combined to enhance the mechanical strength of ACECM. The composite scaffolds showed better cell proliferation and cell viability than PLGA alone due to the better mechanical properties, making it suitable for cartilage tissue engineering [106]. Nanofibres have also shown to reinforce hydrogels due to their poor mechanical properties and support cartilage formation. The incorporation of electrospun PLCL scaffold with gelatin demonstrated enhanced expression of chondrogenic genes and production of GAGs than those prepared without the hydrogel [107]. Similarly electrospun nanofibres combined with CS / glycerophosphate hydrogels illustrated hyaline cartilage regeneration [107]. Mechanical properties after reinforcement of the hydrogel with silk nanofibres demonstrated significant enhancement of the chondrogenic phenotype, illustrated by elevated GAG content [108]. Fibroin microfibers and collagen hydrogel were demonstrated suitable scaffolds after combined with a pure collagen hydrogel for vascular tissue engineering [109].

CNTs mimic the dimensions of the tissues where cells obtain cues to form cartilage *in vivo* and hence found to be a good candidate for tissue engineering. CNTs have excellent mechanical properties being suitable for load bearing tissues such as cartilage and bone. Several studies have tried to combine CNTs with nanofibres to create effect scaffolds for musculoskeletal engineering. Chahine *et al.* illustrated that single walled nanotubes (SWNT) nanocomposite scaffolds could be used in cartilage tissue engineering providing a structural reinforcement of the scaffolds mechanical properties [110]. Chondrocytes tolerated functionalized SWNT with COOH or -PEG with minimal toxicity of cells and increased collagen II (Col2a1) and fibronectin (Fn) gene expression throughout the culture in nanocomposite constructs, indicative of increased chondrocyte metabolic activity [110]. CNTs incorporated into alginate scaffolds have showed the chondrogenic differentiation of adipose stem cells showing expression of SOX-9, type II collagen [111].

Chondrocytes illustrated enhanced function on electrically highly dispersed CNTs in polycarbonate urethane compared to stimulated pure PCU. Higher amount of Fn was adsorbed on CNT / PCU composite compared PCU alone due

to increases hydrophilicity and increased nanometer scale roughness [112]. Furthermore, after the incorporation of multi-walled carbon tubes (MWCNTS) into PLLA nanofibres, a drastic mechanical strength and a compression Young's Modulus matching to natural cartilage was observed, which supported cartilage formation [113]. After 2 weeks of culture PLLA scaffolds with MWCNTs the scaffold showed cartilage formation and promising candidate for further exploration for cartilage regeneration [113].

Osteoarthritis is a degenerating disease of the articular joint. Intra-articular injection of pharmacologic agents into the cartilage resistant chondrocytes has left a problem to developed anti-osteoarthritis (anti-OA) medications. Sacchetti *et al.* described an efficient intra-articular delivery system based on SWCNTs [114]. They showed that PEG-SWCNTS were able to persist in a joint cavity for a suitable amount of time, enter the cartilage matrix and deliver gene inhibitors to the chondrocytes of both healthy and OA mice without causing any systemic side effects [114].

15.6. TENDON / LIGAMENT REGENERATION

Tendon and ligament injuries are a common musculoskeletal clinical problem for the young and active patients [114-119]. The economical and social burden that these injuries can cause makes these medical conditions a compelling area of research to date [114-119]. Tendon and ligament have a similar composition and structure *in vivo*, connecting skeletal muscle to the bone and bone to bone respectively. The ECM, mainly composed of collagen type I of both structures has uniaxial aligned structure leading to anisotropic mechanical properties [114-119]. The tendon matrix is composed of tenocytes with continuous matrix remodelling, whilst the ligament matrix composes of fibroblasts / fibrocytes [114-119].

15.6.1. Clinical need

Tendon and ligament injuries are caused by trauma, tumour resection and atrophy. Flexor tendon lacerations, of the hand are commonly caused by trauma and pose a significant challenge for the hand surgeon [114-119]. The Achilles tendon, the largest tendon of the body, is often damaged by Achilles tendinosis leading to rupture and a debilitating condition [114-119]. The most severe ligament injury is the Achilles ligament injury, which causes severe impediment to knee function [114-119]. Tendon and ligaments has a low regeneration for self-renewal due its poor vascularity often leading to a scarring mediated healing response and does not restore the biomechanical properties of the native tendon [114-119]. Several surgical techniques are used to restore severe damage. Biological grafts are used to repair damaged tendons but often are unable to provide the optimal mechanical strength [114-119]. Along with the morbidity and functional disability at the donor site tendon, autografts are

also limited by their availability [114-119]. Allografts are available to improve upon these complications but are limited to tissue rejection and disease transmission. To overcome these problems for tissue replacement synthetic grafts have been researched for tendon engineering [114-119].

Tissue-engineering aims to develop ideal substitutes to restore tendon / ligament function [114-119]. In a similar strategy to bone and cartilage engineering, the ideal scaffold for tendon regeneration must meet the physiological demands of the native tendon but also direct a cellular response to achieve healing [114-119]. Synthetic and biological scaffolds have been investigated for tendon / ligament repair [114-119]. Nanofibres have gained popularity in the field of tendon / ligament due to the ability to biological mimic the *in vivo* environment. The collagen-rich ECM can be resembled with nanofiber scaffolds due to high surface area-volume ratio, variable fibres and pore size allowing optimal matrix production [118-119].

15.6.2. Nanofibres for tendon / ligament tissue engineering

Due to fibrous nature of these tissues, the most common structure, which is used for tissue engineering strategy is fibrous scaffolds. As already highlighted nanofibres ranging from 1–100 nm closely match the size of the ECM fibres making them suitable to produce cellular cues [121-122]. Fibrous scaffolds have gained extensive interest in tendon / ligament regeneration due the high porosity allowing for high cell infiltration rate and the ability to create uniaxial alignment, which mimics the anisotropic structure of the native tissues. Aligned nanofibres have shown to be a suitable scaffold for engineering ligaments and tendon as it mimics the anisotropy of the native tissue [121-122].

Several studies have shown synthetic nanofibres supporting cell adhesion and promising candidates as scaffolds for tendon regeneration [120]. For example, PLGA nanofibres allowed the differentiation of porcine MSCs towards the tenogenic lineage. Silk and PCL nanofibres showed an optimal structure allowing the good fibroblast attachment and a promising scaffold for ligament engineering [121]. Similarly, PCL with random and aligned silk fibres showed large porosity and pore sizes by scanning electron microscopy (SEM), which favoured BMSCs proliferation whilst sustaining satisfactory mechanical properties for tendon repair [122].

Compared to bone marrow stem cells, ADSCs can be harvested using a minimally invasive procedure, are in abundant supply in current populations, have the comparable ability to differentiate into mesenchymal lineages and immunosuppressive capabilities and thus are an attractive stem cell source for the translational point of view for tendon engineering [123]. One study included ADSCs on PLGA, a biodegradable scaffold, with appropriate mechanical properties was tested for its ability to promote tendon healing. The *in vitro* studies demonstrated that cells remained viable within the scaffold and

growth factor release was sustained. The *in vivo* data supported that the cells remained at the injury site 9 days postoperatively and supported tendon healing [123].

Flexor tendon healing remains problematic due to the ability to surrounding cells to create an appropriate healing response. To provide adequate healing, tendon cells at the tendon stumps must migrate, proliferate and then produce ECM. There are many factors to determine optimal tendon behaviour. One factor to consider when designing an optimal fiber for tendon engineering is the orientation of the fibres. Ouyang *et al.* showed that human tendon progenitor cells (hTSPCs) were spindle shaped and oriented to the direction of the PLLA fibres, however showed tendon specific genes on aligned rather than random fibres [124]. The authors concluded that the orientation of the fibres can affect the hTSPCs differentiation. Lu *et al.* also showed similar results finding that integrin expression profiles for tenocytes was higher on aligned nanofibres than non-aligned nanofibres [125].

A few biological materials have been utilised as nanofibres to promote tendon regeneration. A recent study, illustrated that collagen and cellulose nanofibres dispersed in a collagen matrix supported human ligament cells and human endothelial cell function [126]. However, the mechanical properties of biological scaffolds have also caused further investigation of hybrid scaffolds [126]. It is also important to highlight the crimped nature of collagen fibrils in tendons and ligaments. The crimped nature of native tendon and ligaments can be mimicked using nanofibres by inducing a crimp when removing the mandrel when rotated at high speed. The resulting crimp structures have shown the same characteristics of the ECM of the tendon and ligament [127].

One strategy that has been implemented is that the ideal scaffold should contain growth factors and thus regulate cellular behaviour and support the formation of the target tissue [128]. Sahoo *et al.* demonstrated that bFGF releasing nanofibrous scaffolds facilitated BMSC proliferation, upregulated gene expression of tendon / ligament-specific ECM proteins, increased production and deposition of collagen and tenascin-C, reduced multi-potency of the BMSCs and induced tendon / ligament-like fibroblastic differentiation, indicating their potential in tendon / ligament tissue engineering applications [128]. Platelet derived growth factor (PDGF) releasing nanofibres affected the cellular activity of ADSCs. It was observed that the aligned nanofibres significantly enhanced the tenogenic differentiation of ADSCs comparing to the randomly aligned tendon markers such as tenomodulin and scleraxis [129]. Similarly, Liu *et al.* observed that electrospun PLLA copolymer fibres with bFGF enhanced cell proliferation as well as tendon healing [130]. A very successful example of loading NPs with growth factors for tendon healing was demonstrated using dextran glass NPs. Liu *et al.* trialled bFGF loaded dextran glass nanoparticle on PLLA fibres and found evidence of tendon healing *in vivo* (Figure 5) [130].

Figure 5. **A.** After incorporation of bFGF into DGNs by a freeze-induced particle-forming process, a bFGF / DGNs-loaded PLLA fibrous membrane was fabricated by electrospinning, producing a membrane which can release bFGF sustainably and has the ability to secure the biological activity of bFGF. **B.** SEM images of electrospun PLLA (A), bFGF-PLLA (B) and bFGF / DGNs-PLLA (C) fibers. TEM images of electrospun PLLA (D), bFGF-PLLA (E) and bFGF / DGNs-PLLA (F) fibers. Yellow arrows indicate the DGNs embedded into fibers with thickened regions of PLLA visible around them. **C.** Masson's trichrome staining of an untreated repair site (A, B, C) and a repair site wrapped with the PLLA membrane (D, E, F), bFGF-PLLA membrane (G, H, I) and bFGF / DGNs-PLLA membrane (J, K, L). Sutured site (S) and materials (M) could be detected. Regional magnification of the repair sites (A, D, G, J) showed the repaired sites of the tendon (B, E, H, K) while regional magnification of the repaired sites (B, E, H, K) revealed the arrangement of the collagen fibers (C, F, I, L). Arrows indicate the capillary vessels, and the square is the area of magnification of the subsequent photo. **D.** Gross evaluation of a rat model of Achilles tendon surgery after 21 days. (A) Untreated control group; (B) group treated with the PLLA membrane; (C) group treated with the bFGF-PLLA membrane; (D) group treated with the bFGF / DGNs-PLLA membrane. Tendon (T) was indicated in the pictures. Taken with permission from [130].

The incorporation of NPs to nanofibres has also been investigated for tendon tissue engineering. Infection is surgical complications, which can prevent tendon healing. Silk fibres impregnated with NPs have shown to improve the sterility against multiple microbes. Silk fibres were coated with CS impregnated with silver NPs (Ag-C-SF), which supported anti-microbial

activity and improved thermal stability, showing potential to be explored as a wound dressing and for tendon reconstruction [131].

Flexor tendon repair is often complicated by the formation of adhesions. Zhou *et al.* demonstrated a sophisticated *in vitro* and *in vivo* transfection experiments demonstrated using NPs / plasmid complex caused less severe adhesions, but did lower the tendon strength of the tendon healing [132]. They used micro RNA (miRNA) plasmids complexed with PLGA NPs to form nanoparticle / TGF- β 1 miRNA plasmid (nanoparticle / plasmid) complexes [132]. The repair of Achilles tendon healing has also been investigated using mesoporous silica NPs *in vivo* using a rat's Achilles tendon [133].

Another important element of tendon engineering is the ability to engineer complex tissue such as the tendon-bone insertion site that required strong materials with mineralised and unmineralised tissues with varied strength and stiffness. Kolluru *et al.* demonstrated that PLGA nanofibres which incorporated HA material demonstrated the highly desirable hardening behaviour even in the presence of surface mineralization [134]. The nanofibres provided a 200 % increase in fiber strength and 100 % elongation for thin and strongly bound mineral coatings [134]. An important feature of electrospinning is that the scaffolds can have graded physical and chemical properties. For the tendon-bone interface this is vital as this allows the mechanical and structural properties to be graded and mimic the *in vivo* tissue insertion site. The ability to control the mineral deposition of the scaffold may be another technique to recapitulate the tendon-bone interface. A few studies have aimed to grade the mineralisation along the nanofibres, which has resulted in good cell attachment [135-137].

15.7. SUMMARY OF DIFFERENT APPLICATIONS

15.7.1. Future challenges and prospectives

Mechano sensitive and load bearing tissues such as bone, cartilage, tendon or ligament are natural nanocomposite with an hieratical structure at nanoscale. Thus, nanomaterials are being thoroughly investigated as scaffolds for tissue engineering these musculoskeletal tissues. For bone tissue engineering, a number of synthetic and natural polymers have been identified as biomaterials for consideration. The use of polymer-ceramic materials remains a very interesting area of research as bone is composed of inorganic HA crystal and organic materials. However, the optimal polymer-ceramic hybrid is unknown and deserves further research interest for bone regeneration. Several polymers have been considered for cartilage, with hybrid materials being the likely candidate to support regeneration. Natural-based and synthetic based blends are being trialed to optimize the mechanical properties and cell interactions required for regeneration. However, despite many composite

materials being considered, many are far away from being used in clinical trials and require further optimisation.

In particular, electrospun nanofibres have shown the ability to support cell adhesion and differentiation for bone, cartilage and tendon engineering. The similarities to the natural ECM of nanofibres show that the high surface area and porosity is a favorable environment to support tissue formation for regenerative engineering purposes. The exact fiber diameter and orientation to match the ECM is still not clearly understood, as well as the correct combination of micro and nanofibres. As one material type cannot provide all the required characteristics of the ideal scaffold, it is likely hybrid scaffolds will be further explored for musculoskeletal applications. Electrospinning is also a promising candidate for regenerative scaffolds due to ability to combine the appropriate growth factor. However, the most appropriate growth factor, peptide or protein to support MSC differentiation for cartilaginous and bone formation is also a need to be further explored.

It is likely in the near future to have multi-component scaffolds, which mimic tendon, cartilage and bone tissue in regards to structure and functionality. Further research, to optimise fabrication of porous scaffolds with tailored properties will be explored to achieve smart materials suitable for musculoskeletal regeneration.

REFERENCES

1. A.S. Brydone, D. Meek, S. MacLaine. *Proc. Inst. Mech. Eng.* **224** (2020) 1329–1343.
2. R. James, M. Deng, C. Laurencin, S. Kumbar. *Front. Mater. Sci.* **5** (2011) 342–357.
3. A.P. Mathew, K. Oksman, D. Pierron, M.F. Harmand. *Macromol. Biosci.* **13** (2013) 289–298.
4. M. Kim, G. Kim. *Carbohydr Polym.* **90** (2012) 592–601.
5. S. Eap, D. Morand, F. Clauss, O. Huck, J.F. Stoltz, J.C. Lutz, J.E. Gottenberg, N. Benkirane-Jessel, L. Keller, F. Fioretti. *Biomed. Mater. Eng.* **25** (1 Suppl) (2015) 79–85.
6. P. Bhattacharjee, B. Kundu, D. Naskar, T.K. Maiti, D. Bhattacharya, S.C. Kundu. *Biopolymers.* **103** (2015) 271–84.
7. S. Shanmugavel, V.J. Reddy, S. Ramakrishna, B. Lakshmi, V.G. Dev. *J. Biomater. Appl.* **29** (2013) 46–58.
8. A. Ardeshtyrlajimi, M. Mossahebi-Mohammadi, S. Vakilian, L. Langroudi, E. Seyedjafari, A. Atashi, M. Soleimani. *Cell Prolif.* **48** (2015) 47–58.
9. L. Ghasemi-Mobarakeh, M.P. Prabhakaran, P. Balasubramanian, G. Jin, A. Valipouri, S. Ramakrishna. *J. Nanosci. Nanotechnol.* **13** (2013) 4656–4657.
10. T.G. Scott, G. Blackburn, M. Ashley, I.S. Bayer, A. Ghosh, A.S. Biris, A. Biswas. *J. Nanosci. Nanotechnol.* **13** (2013) 1–22.
11. N.G. Sahoo, Y.Z. Pan, L. Li, C.B. He. *Nanomedicine (Lond.)* **8** (2013) 639–653.

12. M.H. Hettiaratchi, T. Miller, J.S. Temenoff, R.E. Guldberg, T.C. McDevitt. *Biomaterials* **35**(25) (2014) 7228–7238.
13. G. Gainza, S. Villullas, J.L. Pedraz, R.M. Hernandez, M. Igartua. *Nanomedicine* **11**(6) (2015) 1551–1573.
14. G.G. Walmsley, A. McArdle, R. Tevlin, A. Momeni, D. Atashroo, M.S. Hu, A.H. Feroze, V.W. Wong, P.H. Lorenz, M.T. Longaker, D.C. Wan. *J. Biomed. Nanotechnol.* **10** (2014) 959–969.
15. M. Hosseinkhani, D. Mehrabani, M.H. Karimfar, S. Bakhtiyari, A. Manafi, R. Shirazi. *World J. Plast. Surg.* **3** (2014) 3–7.
16. M. Mravic, B. Péault, A.W. James. *Biomed. Res. Int.* **2014** (2014) 865270.
17. S.M. Zakaria, S.H. Sharif Zein, M.R. Othman, F. Yang, J.A. Jansen. *Tissue Eng. Part B Rev.* **19** (2013) 431–441.
18. I. Martin, D. Wendt, M. Heberer. *Trends Biotechnol.* **22** (2004) 80–86.
19. F.J. O'Brien. *Mater. Today* **14** (2011) 88–95.
20. A.C. Jones, C.H. Arns, A.P. Sheppard, D.W. Huttmacher, B.K. Milthorpe, M.A. Knackstedt. *Biomaterials* **28** (2007) 2491–2504.
21. T.J. Webster, E.S. Ahn. *Adv. Biochem. Eng. Biotechnol.* **103** (2007) 275–308.
22. A. Tautzenberger, A. Kovtun, A. Ignatius. *Int. J. Nanomed.* **7** (2012) 4545–4557.
23. A. Bhirde, J. Xie, M. Swierczewska, X. Chen. *Nanoscale* **3** (2011) 142–153.
24. S.E. Kim, O. Jeon, J.B. Lee, M.S. Bae, H.J. Chun, S.H. Moon, I.K. Kwon. *J. Biomed. Sci.* **15** (2008) 771–777.
25. J. Guan, J. Zhang, Z. Zhu, X. Niu, S. Guo, Y. Wang, C. Zhang. *Stem Cell. Res. Ther.* **6** (2015) 5.
26. H. Lysdahl, A. Baatrup, C.B. Foldager, C. Bünger. *Biores. Open Access.* **3** (2014) 278–285.
27. Y. Açil, A.A. Ghoniem, J. Wiltfang, M. Gierloff. *J. Craniomaxillofac. Surg.* **42** (2014) 2002–2009.
28. P. Yilgor, K. Tuzlakoglu, R.L. Reis, N. Hasirci, V. Hasirci. *Biomaterials* **30** (2009) 3551–3559.
29. Z. Lu, S.I. Roohani-Esfahani, J. Li, H. Zreiqat. *Nanomedicine* **11** (2015) 219–228.
30. M.D. Krebs, E. Salter, E. Chen, K.A. Sutter, E. Alsberg. *J. Biomed. Mater. Res. A* **92** (2010) 1131–1138.
31. O.C. Farokhzad. *Expert Opin. Drug Deliv.* **5** (2008) 927–929.
32. R.J. Byers, E.R. Hitchman. *Prog Histochem Cytochem.* **45** (2011) 201–237.
33. B.J. Muller-Borer, M.C. Collins, P.R. Gunst, W.E. Cascio, A.P. Kypson. *J. Nanobiotechnology* **5** (2007) 9.
34. Y.C. Chen, J.K. Hsiao, H.M. Liu, I.Y. Lai, M. Yao, S.C. Hsu, B.S. Ko, Y.C. Chen, C.S. Yang, D.M. Huang. *Toxicol. Appl. Pharmacol.* **245** (2010) 272–279.
35. T.L. Arinzeh, T. Tran, J. Mcalary, G. Daculsi. *Biomaterials* **26** (2005) 3631–3638.
36. C. Wang, Y. Xue, K.L. Lin, J.X. Lu, J. Chang. *J. Sun. Acta Biomater.* **8** (2012) 350–360.
37. D.M. Luo, L. Sang, X.L. Wang, S.M. Xu, X.D. Li. *Mater. Lett.* **65** (2011) 2395–2397.
38. D.T.J. Barone, J.M. Raquez, P. Dubois. *Polym. Adv. Technol.* **22** (2011) 463–475.
39. T. Serizawa, T. Tateishi, M. Akashi. *J. Biomater. Sci. Polym. Ed.* **14** (2003) 653–663.
40. J.R. Popp, K.E. Laflin, B.J. Love, A.S. Goldstein. *J. Tissue Eng. Regen. Med.* **6** (2012) 12–20.

41. S.J. Heo, S.E. Kim, J. Wei, Y.T. Hyun, H.S. Yun, D.H. Kim, J.W. Shin, J.W. Shin. *J. Biomed. Mater. Res. A* **89** (2009) 108–116.
42. D.X. Wang, Y. He, L. Bi, Z.H. Qu, J.W. Zou, Z. Pan, J.J. Fan, L. Chen, X. Dong, X.N. Liu, G.X. Pei, J.D. Ding. *Int. J. Nanomedicine* **8** (2013) 1855–1865.
43. R.J. Kane, H.E. Weiss-Bilka, M.J. Meagher, Y. Liu, J.A. Gargac, G.L. Niebur, D.R. Wagner, R.K. Roeder. *Acta Biomater.* (2015) 16–25.
44. Y.J. Hong, J.S. Chun, W.K. Lee. *Colloids Surf. B Biointerfaces* **83** (2011) 245–253.
45. J. Ramier, D. Grande, T. Boudierlique, O. Stoilova, N. Manolova, I. Rashkov, V. Langlois, P. Albanese, E. Renard. *J. Mater. Sci. Mater. Med.* **25** (2014) 1563–1575.
46. T.M. Mututuvvari, A.L. Harkins, C.D. Tran. *J. Biomed. Mater. Res. A* **101** (2013) 3266–3277.
47. R.L. Dahlin, F.K. Kasper, A.G. Mikos. *Tissue Eng. Part B Rev.* **17** (2011) 349–364.
48. M.P. Prabhakaran, L. Ghasemi-Mobarakeh, S. Ramakrishna. *J. Nanosci. Nanotechnol.* **11** (2011) 3039–3057.
49. X. He, W. Fu, B. Feng, H. Wang, Z. Liu, M. Yin, W. Wang, J. Zheng. *Regen Med.* **8** (2013) 425–436.
50. N. Bhattarai, D. Edmondson, O. Veis, F.A. Matsen, M. Zhang. *Biomaterials* **26** (2005) 6176–6184.
51. Y. Zhang, H. Ouyang, C.T. Lim, S. Ramakrishna, Z.-M. Huang. *J. Biomed. Mater. Res. Part B: Appl. Biomater.* **72B** (2005) 156–165.
52. H.J. Jin, J. Chen, V. Karageorgiou, G.H. Altman, D.L. Kaplan. *Biomaterials* **25** (2004) 1039–1047.
53. B.M. Min, G. Lee, S.H. Kim, Y.S. Nam, T.S. Lee, W.H. Park. *Biomaterials* **25** (2004) 1289–1297.
54. B.S. Kim, K.E. Park, M.H. Kim, H.K. You, J. Lee, W.H. Park. *Int. J. Nanomedicine* **9** (10) (2015) 485–502.
55. H. Kim, L. Che, Y. Ha, W. Ryu. *Mater. Sci. Eng. C Mater. Biol. Appl.* **40** (2014) 324–335.
56. G.J. Lai, K.T. Shalumon, J.P. Chen. *Int. J. Nanomedicine* **10** (2015) 567–584.
57. D. Li, H. Sun, L. Jiang, K. Zhang, W. Liu, Y. Zhu, J. Fangteng, C. Shi, L. Zhao, H. Sun, B. Yang. *ACS Appl. Mater. Interfaces.* **6** (2014) 9402–9410.
58. W. Ji, Y. Sun, F. Yang, J.J. van den Beucken, M. Fan, Z. Chen, J.A. Jansen. *Pharm. Res.* **28** (2011) 1259–1272.
59. T. Lou, X. Wang, G. Song, Z. Gu, Z. Yang. *Int. J. Biol. Macromol.* **69** (2014) 464–470.
60. A.W. Tan, L. Tay, K.H. Chua, R. Ahmad, S.A. Akbar, B. Pingguan-Murphy. *Int. J. Nanomedicine* **9** (2014) 5389–5401.
61. N.G. Rim, C.S. Shin, H. Shin. *Biomed. Mater.* **8** (2013) 014102.
62. D.R. Nisbet, J.S. Forsythe, W. Shen, D.I. Finkelstein, M.K. Horne. *J. Biomater. Appl.* **24** (2009) 7–29.
63. A. Ferrand, S. Eap, L. Richert, S. Lemoine, D. Kalaskar, S. Demoustier-Champagne, H. Atmani, Y. Mély, F. Fioretti, G. Schlatter, L. Kuhn, G. Ladam, N. Benkirane-Jessel. *Macromol. Biosci.* **14** (2014) 45–55.
64. H. Zhu, D. Yu, Y. Zhou, C. Wang, M. Gao, H. Jiang, H. Wang. *J. Biomed. Mater. Res. B Appl. Biomater.* **101** (2013) 541–52.
65. H. Nie, M.L. Ho, C.K. Wang, C.H. Wang, Y.C. Fu. *Biomaterials* **30** (2009) 892–901.

66. L. Li, G. Zhou, Y. Wang, G. Yang, S. Ding, S. Zhou. *Biomaterials* **37** (2015) 218–229.
67. M. Gibson, V. Beachley, J. Coburn, P.A. Bandinelli, H.Q. Mao, J. Elisseeff. *Biomed. Res. Int.* **2014** (2014) 469120.
68. C. Gandhimathi, J.R. Venugopal, A.Y. Tham, S. Ramakrishna, S.D. Kumar. *Mater. Sci. Eng. C Mater. Biol. Appl.* **49** (2015) 776–785.
69. N. Ribeiro, S.R. Sousa, C.A. van Blitterswijk, L. Moroni, F.J. Monteiro. *Biofabrication* **6** (2014) 035015.
70. X.N. Chen, Y.X. Gu, J.H. Lee, W.Y. Lee, H.J. Wang. *Eur. Cell. Mater.* **24** (2012) 237–248.
71. W.T. Su, P.S. Wu, T.Y. Huang. *Mater.Sci. Eng. C Mater. Biol. Appl.* **46** (2015) 427–434.
72. Y.P. Yun, S.J. Kim, Y.M. Lim, K. Park, H.J. Kim, S.I. Jeong, S.E. Kim, H.R. Song. *J. Biomed. Nanotechnol.* **10** (2014) 1080–1090.
73. S. Duan, X. Yang, F. Mei, Y. Tang, X. Li, Y. Shi, J. Mao, H. Zhang, Q. Cai. *J. Biomed. Mater. Res. A* **103** (2015) 1424–1435.
74. S.Y. Kim, J.Y. Hwang, J.W. Seo, U.S. Shin. *J. Colloid. Interface Sci.* **442** (2015) 147–153.
75. J. Alam, M. Alam, M. Raja, Z. Abduljaleel, L.A. Dass. *Int. J. Mol. Sci.* **15** (2014) 19924–3.
76. J. Xu, Y. Xie, H. Zhang, Z. Ye, W. Zhang. *Colloids Surf. B Biointerfaces* **123** (2014) 907–15.
77. S.W. O'Driscoll. *J. Bone Joint Surg. Am.* **80** (1998) 1795–1812.
78. J.S. Temenoff, A.G. Mikos. *Biomaterials.* **21** (2000) 431–440.
79. M. Falah, G. Nierenberg, M. Soudry, M. Hayden, G. Volpin. *nt Orthop.* **34** (2010) 621–630.
80. J.H. Kim, J.S. Park, H.N. Yang, D.G. Woo, S.Y. Jeon, H.-J. Do, H.-Y. Lim, J.M. Kim, K.-H. Park. *Biomaterials* **32** (2011) 268–278.
81. S.Y. Jeon, J.S. Park, H.N. Yang, D.G. Woo, K.H. Park. *Stem Cells Dev.* **23** (2014) 305–317.
82. H. Lu, L. Lv, Y. Dai, G. Wu, H. Zhao, F. Zhang. *PLoS One* **8** (7) (2013) e69950.
83. A.B. Ertan, P. Yilgor, B. Bayyurt, A.C. Calikoğlu, C. Kaspar, F.N. Kk, G.T. Kose, V. Hasirci. *J. Tissue Eng. Regen. Med.* **7** (2013) 149–160.
84. J. Shi, X. Zhang, J. Zhu, Y. Pi, X. Hu, C. Zhou, Y. Ao. *Arthroscopy* **29** (2013) 2001–2011.
85. S. Yamane, N. Iwasaki, Y. Kasahara, K. Harada, T. Majima, K. Monde, S. Nishimura, A. Minami. *J. Biomed. Mater. Res. A* **81** (2007) 586–593.
86. Z. Yang, Z. Chen, K. Liu, Y. Bai, T. Jiang, D. Feng, G. Feng. Experimental study on tissue engineered cartilage complex three-dimensional nano-scaffold with collagen type II and hyaluronic acid *in vitro*. *Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi* **27** (2013) 1240–1245.
87. L. Chen, W.M. Zhu, Z.Q. Fei, J.L. Chen, J.Y. Xiong, J.F. Zhang, L. Duan, J. Huang, Z. Liu, D. Wang, Y. Zeng. *BioMed. Res. Int.* **2013** (2013) 412745.
88. Y. Guo, J. Guo, D. Bai, H. Wang, X. Zheng, W. Guo, W. Tian. *Artif Organs* **38** (2014) 580–586.
89. W. Zhu, K. Chen, W. Lu, Q. Sun, L. Peng, W. Fen, H. Li, Y. Ou, H. Liu, D. Wang, Y. Zeng. *In Vitro Cell Dev. Biol. Anim.* **50** (2014) 214–220.
90. L. Jia, Z. Duan, D. Fan, Y. Mi, J. Hui, L. Chang. *Mater. Sci. Eng. C Mater. Biol. Appl.* **33** (2013) 727–734.

91. E. Quinlan, A. López-Noriega, E. Thompson, H.M. Kelly, S.A. Cryan, F.J. O'Brien. *J. Control Release* **198** (2015) 71–79.
92. A. Sotoudeh, A. Jahanshahi, M.A. Takhtfooladi, A. Bazazan, A. Ganjali, M.P. Harati. *Acta Cir. Bras.* **28** (2013) 340–345.
93. J.K. Savaiano, T.J. Webster. *Biomaterials* **25**(7-8) (2004) 1205–1213.
94. S.H. Hsu, H.J. Yen, C.L. Tsai. *Artif. Organs* **31** (2007) 854–868.
95. L. Guasti, B. Vagaska, N.W. Bulstrode, A.M. Seifalian, P. Ferretti. *Nanomedicine* **10** (2014) 279–289.
96. J.M. Coburn, M. Gibson, S. Monagle, Z. Patterson, J.H. Elisseeff. *Proc. Natl. Acad. Sci. U.S.A* **109** (2012) 10012–7.
97. S. Zhang, L. Chen, Y. Jiang, Y. Cai, G. Xu, T. Tong, W. Zhang, L. Wang, J. Ji, P. Shi, H.W. Ouyang. *Acta Biomater.* **9** (2013) 7236–7247.
98. A. Shafiee, E. Seyedjafari, E. Sadat Taherzadeh, P. Dinarvand, M. Soleimani. *J. Ai. Mater. Sci. Eng. C Mater. Biol. Appl.* **40** (2014) 445–454.
99. M.L. Alves da Silva, A. Martins, A.R. Costa-Pinto, P. Costa, S. Faria, M. Gomes, R.L. Reis, N.M. Neves. *Biomacromolecules* **13**(11) (2010) 3228–3236.
100. L. Koepsell, L. Zhang, D. Neufeld, H. Fong, Y. Deng. *Macromol. Biosci.* **11** (2011) 391–399.
101. E.J. Levorson, P. Raman Sreerekha, K.P. Chennazhi, F.K. Kasper, S.V. Nair, A.G. Mikos. *Biomed. Mater.* **8** (2013) 014103.
102. I. Wimpenny, N. Ashammakhi, Y. Yang. *J. Tissue Eng. Regen. Med.* **6** (2012) 536–549.
103. Y. Itani, S. Asamura, M. Matsui, Y. Tabata, N. Isogai. *Plast. Reconstr. Surg.* **133** (2014) 805e–813e.
104. P. Lee, K. Tran, W. Chang, N.B. Shelke, S.G. Kumbar, X. Yu. *J. Biomed. Nanotechnol.* **10** (2014) 1469–1479.
105. J.C. Schagemann, S. Paul, M.E. Casper, J. Rohwedel, J. Kramer, C. Kaps, H. Mittelstaedt, M. Fehr, G.G. Reinholz. *J. Biomed. Mater. Res. A* **101** (2013) 1620–1628.
106. X. Zheng, F. Yang, S. Wang, S. Lu, W. Zhang, S. Liu, J. Huang, A. Wang, B. Yin, N. Ma, L. Zhang, W. Xu, Q. Guo. *J. Mater. Sci. Mater. Med.* **22** (2011) 693–704.
107. M. Kim, B. Hong, J. Lee, S.E. Kim, S.S. Kang, Y.H. Kim, G. Tae. *Biomacromolecules* **13** (2012) 2287–2298.
108. F. Mirahmadi, M. Tafazzoli-Shadpour, M.A. Shokrgozar, S. Bonakdar. *Mater. Sci. Eng. C Mater. Biol. Appl.* **33** (2013) 4786–4794.
109. M.A. de Moraes, E. Paternotte, D. Mantovani, M.M. Beppu. *Macromol. Biosci.* **12** (2012) 1253–1264.
110. N.O. Chahine, N.M. Collette, C.B. Thomas, D.C. Genetos, G.G. Loots. *Tissue Eng. Part A* **20** (2014) 2305–2315.
111. A. Valiani, B. Hashemibeni, E. Esfandiary, M.M. Ansar, M. Kazemi, N. Esmaeili. *Int. J. Prev. Med.* **5** (2014) 825–834.
112. D. Khang, G.E. Park, T.J. Webster. *J. Biomed. Mater. Res. A* **86** (2008) 253–260.
113. B. Holmes, N.J. Castro, J. Li, M. Keidar, L.G. Zhang. *Nanotechnology* **24** (2013) 365102. 20.
114. C. Sacchetti, R. Liu-Bryan, A. Magrini, N. Rosato, N. Bottini, M. Bottini. *ACS Nano.* **8**(12) (2014) 2280–2291.
115. S. Font Tellado, E. Rosado Balmayor, M. Van Griensven. *Adv. Drug Deliv. Rev.* 2015 Mar 14.
116. B.D. Smith, D.A. Grande. *Nat. Rev. Rheumatol.* **11**(4) (2015) 213–222.

117. A.J. Lomas, C.N. Ryan, A. Sorushanova, N. Shologu, A.I. Sideri, V. Tsioli, G.C. Fthenakis, A. Tzora, I. Skoufos, L.R. Quinlan, G. O'Laighin, A.M. Mullen, J.L. Kelly, S. Kearns, M. Biggs, A. Pandit, D.I. Zeugolis. *Adv. Drug Deliv. Rev.* **84** (2014) 257–277.
118. R.J. Egli, R. Luginbuehl. *Swiss Med. Wkly.* **142** (2012) w13647.
119. T.K. Hiong Teh, J.C. Hong Goh, S.L. Toh. *Curr. Pharm. Des.* **21**(15) (2015) 1991–2005.
120. S. Sahoo, S.L. Toh, J.C. Goh. *Biomaterials* **31** (2010) 2990–2998.
121. E. Naghashzargar, S. Farè, V. Catto, S. Bertoldi, D. Semnani, S. Karbasi, M.C. Tanzi. *J. Appl. Biomater. Funct. Mater.* **13**(2) (2014).
122. C. Yang, G. Deng, W. Chen, X. Ye, X. Mo. *Colloids Surf. B Biointerfaces* **122** (2014) 270–276.
123. C.N. Manning, A.G. Schwartz, W. Liu, J. Xie, N. Havlioglu, S.E. Sakiyama-Elbert, M.J. Silva, Y. Xia, R.H. Gelberman, S. Thomopoulos. *Acta Biomater.* **9** (2013) 6905–6914.
124. Z. Yin, T.M.H. Nguyen, X. Chen, H.W. Ouyang. 13th International Conference on Biomedical Engineering IFMBE Proceedings Vol. 23, 2009, pp. 1397–1400.
125. K.L. Moffat, A.S. Kwei, J.P. Spalazzi, S.B. Doty, W.N. Levine, H.H. Lu. *Tissue Eng. Part A* **15** (2009) 115–126.
126. A.P. Mathew, K. Oksman, D. Pierron, M.F. Harmand. *Macromol. Biosci.* **13** (2013) 289–298.
127. D.C. Surrao, J.W. Hayami, S.D. Waldman, B.G. Amsden. *Biomacromolecules* **11** (2010) 3624–3629.
128. S. Sahoo, L.T. Ang, J. Cho-Hong Goh, S.L. Toh. *Differentiation* **79** (2010) 102–110.
129. X. Cheng, C. Tsao, V.L. Sylvia, D. Cornet, D.P. Nicolella, T.L. Bredbenner, R.J. Christy. *Acta Biomater.* **10** (2014) 1360–1369.
130. S. Liu, M. Qin, C. Hu, F. Wu, W. Cui, T. Jin, C. Fan. *Biomaterials* **34** (2013) 4690–4701.
131. K. Karthikeyan, S. Sekar, M.P. Devi, S. Inbasekaran, C.H. Lakshminarasiah, T.P. Sastry. *J. Mater. Sci. Mater. Med.* **22** (2011) 2721–2726.
132. Y. Zhou, L. Zhang, W. Zhao, Y. Wu, C. Zhu, Y. Yang. *Biomaterials* **34**(33) (2013) 8269–8278.
133. A. Suwalski, H. Dabboue, A. Delalande, S.F. Bensamoun, F. Canon, P. Midoux, G. Saillant, D. Klatzmann, J.P. Salvetat, C. Pichon. *Biomaterials* **31** (2010) 5237–5245.
134. P.V. Kolluru, J. Lipner, W. Liu, Y. Xia, S. Thomopoulos, G.M. Genin, I. Chasiotis. *Acta Biomater.* **9** (2013) 9442–9450.
135. W. Liu, Y.C. Yeh, J. Lipner, J. Xie, H.W. Sung, S. Thomopoulos, Y. Xia. *Langmuir* **27** (2011) 9088–9093.
136. J. Lipner, W. Liu, Y. Liu, J. Boyle, G.M. Genin, Y. Xia, S. Thomopoulos. *J. Mech. Behav. Biomed. Mater.* **40** (2014) 59–68.
137. J. Xie, B. Ma, P.L. Michael, F.D. Shuler. *Macromol. Biosci.* **12** (2012) 1336–4.

Chapter

16

NANOMATERIALS FOR CARTILAGE TISSUE ENGINEERING

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16.1. INTRODUCTION

Cartilage is a flexible connective tissue found in many areas of the body, such as within synovial joints (articular cartilage), the external ear (auricular cartilage), and the intervertebral disc (annulus fibrosis and nucleus pulposus). Classified into three distinct types (hyaline, elastic and fibrocartilage), this tissue is composed of specialized cells (chondrocytes) embedded in an extracellular matrix (ECM) primarily consisting of collagen fibres, proteoglycans, and elastic fibres (found only in elastic cartilage) – with the types and proportions of constituents unique for different cartilages. As all cartilages are avascular, cell growth and tissue regeneration is limited by the hypoxic environment, thus limiting the self-regenerative capacity of the damaged cartilage [1]. This motivates the search for tissue engineering strategies to repair cartilage defects caused by degenerative joint diseases, cancer or trauma.

Articular cartilage injuries are most common in the knee and can lead to premature arthritis if left untreated [2]. Repetitive stresses can cause irreparable damage in the joints, leading to the development of osteoarthritis. In osteoarthritis, the water content in the cartilage increases due to disruption of the matrix structure and increased permeability, thus leading to decreased stiffness and load bearing capacity. In the case of ear cartilage, external ear deformities are typically a result of both acquired and congenital defects. Acquired external ear deformities are caused by cancerous lesions that extend into the underlying cartilage and require amputation, as well as injuries due to animal bites and automobile accidents [3]. Congenital ear defects, such as external ear microtia and anotia, affect 129 out of ~700,000 newborns in Canada in 2006–2007 [4]. These deformities have significant psychosocial impact on individuals, thus motivating the need for surgical corrective procedures. Similar to the ear, the nose is highly susceptible to injury due to its exposed and unprotected position. Nasal fractures account for half of all facial fractures [5]. In addition, malformations of the nose and ear are found in congenital disorders that are characterized by facial malformations, such as Treacher Collins syndrome and Apert syndrome [6,7]. These malformations may also require cartilage reconstruction. Patients with congenital or benign stenosis of the trachea, resulting from trauma, inflammation or illness, require tracheal reconstruction [8]. Acquired tracheal stenosis occurs in 2–11 % of newborns [9]. In general, cancers in the nose, ear and trachea are increasing in numbers and often require resection, replacement and reconstruction.

Reconstructing or replacing the cartilage is challenging due to the complex geometry of cartilaginous structures in the facial region and the synovial joint surfaces as well as the required mechanical properties of the defect or repair site [1]. Advancements in tissue engineering have led to the development of

biomaterials and culturing methodologies for cartilage regeneration. This chapter will focus on the use of nanomaterials for cartilage tissue engineering and regeneration.

16.2. CARTILAGE TISSUE BIOLOGY

Cartilage, a flexible connective tissue found in the nose, ear, trachea, ribs, joints and intervertebral discs (Figure 1), plays various roles in the human body depending on their type and location. These include providing structural support, maintaining shape and absorbing shock.

Figure 1. Cartilaginous regions in the human body

In the embryo, the cartilage is formed when mesenchyme cells start to aggregate to form a blastema at the 5th week of gestation [2]. This is followed by the secretion of cartilaginous matrix by the cells of blastema, now called chondroblasts. The extracellular matrix produced pushes the cells apart, and the cells, now called chondrocytes, become encased in this specialized matrix. The mesenchymal tissue around the blastema develops into a membrane called the perichondrium.

As such, the cartilage is mainly composed of specialized cells called chondrocytes, which are situated in lacunae and secrete the extracellular

matrix consisting of collagens, elastin, glycoproteins and proteoglycans. Cartilages of different types and functions vary in amounts of these extracellular matrix components. There are three types of cartilage – hyaline, elastic and fibrocartilage (Figure 2).

Figure 2. Three different types of cartilage. Hematoxylin and eosin staining of cross sections of (A) hyaline cartilage in the human nasal septum, (B) elastic cartilage in the rabbit ear, and (C) fibrocartilage in the intervertebral disc. * indicates perichondrium surrounding the hyaline (A) and elastic cartilage tissues (B)

16.2.1. Hyaline cartilage

Hyaline cartilage is the predominant form of cartilage in the human body. It is present in the nasal septum, ribs, tracheal rings, and on the articular joint surfaces. The function of hyaline cartilage is primarily mechanical to provide resistance against bending, compression and impact [2]. This tissue is primarily composed of hydrated aggregates of proteoglycans within a collagen II meshwork. In most cases (with the exception of articular cartilage), hyaline cartilage is covered by an outer fibrous membrane called the perichondrium. Articular cartilage, however, has a specialized outer layer (adjacent to the synovial fluid) referred to as the superficial zone. This zone, which has some similarities to the perichondrium, provides a low-friction interface as well as contains anti-adhesive proteins (proteoglycan 4 or lubricin) to aid in joint motion [2].

Hyaline cartilage is generally composed of 1–5 % chondrocytes by volume, 65–80 % wet weight of water, 10–20 % wet weight of collagen, and 3–7 % wet weight of proteoglycans [2]. The collagen network provides tensile strength, and this macrofibrillar framework is primarily consisted of type II collagen (90–95 %). Other collagens in hyaline cartilage include collagen type VI, IX, X and XI, and have different functions including attaching chondrocytes to the matrix (collagen VI), contributing to tensile properties and inter-fibrillar connections (collagen IX), providing structural support and contributing to cartilage mineralization (collagen X in the case of articular cartilage), and

nucleating fibril formation (collagen XI) [2]. Proteoglycans are proteins with glycosaminoglycan side chains. These hydrophilic macromolecules provide compressive strength to the tissue, and are responsible for maintaining the fluid and electrolyte balance [10]. They have negatively charged sulphate and carboxylate groups, in turn attracting positively charged molecules and increasing the total concentration of inorganic ions and osmolarity in the extracellular matrix. These molecules are produced and secreted by the chondrocytes in the matrix, and can be divided into two types: 1) large aggregating proteoglycan monomers or aggregans and 2) small proteoglycans such as biglycan, decorin, fibromodulin and lumican [11]. Glycosaminoglycans (GAGs), the subunits of proteoglycans, are disaccharide molecules. The two main types of GAGs are chondroitin sulphate and keratin sulphate. They are bound to the protein core through sugar bonds to form aggrecan, and can retain water.

Nutrients diffuse from the synovial fluid to the articular tissue. Joint loading creates cyclical pressures necessary for the normal function of hyaline cartilage and the transport of water and nutrients between the cartilage and synovial fluid.

16.2.2. Elastic cartilage

Elastic cartilage is found in the ear, particularly in the pinna and Eustachian tube, as well as in the larynx and epiglottis. Its role is to provide structural support and flexibility in these regions. While structurally similar to hyaline cartilage, the flexibility of elastic cartilage is due to its high content of elastic fibers (elastin and associated microfibrils), which are woven into a cartilaginous matrix also consisting primarily of collagen II and aggregating proteoglycans. Similar to most hyaline cartilages, elastic cartilage is surrounded by the perichondrium.

16.2.3. Fibrocartilage

Fibrocartilage is found in the intervertebral discs, costochondral joints, sacroiliac joints and pubic symphysis as well as specialized structures such as the meniscus. This tissue differs from hyaline and elastic cartilages, as it is characterized by an abundance of both collagen I and II fibers. Depending on the particular fibrocartilage, these fibers may have distinct orientations, such as in the annulus fibrosus (of the intervertebral disc) and meniscus, which arrange into longitudinal and circumferential fibers [12]. This tissue can withstand high tensile stresses due to the arrangement of collagen fibers but generally does not perform well under compression [13].

16.3. STRATEGIES FOR CARTILAGE REPAIR

The goal of treating damaged or diseased cartilage, as with other damaged tissues, is to restore tissue structural integrity and function. Current clinical strategies include tissue implantation (Section 16.3.1.1.) and approaches to stimulate cartilage regeneration (Section 16.3.1.2.). In addition, a growing number of studies are performed to evaluate the use of engineered tissues to treat cartilage defects (Section 16.3.2.).

16.3.1. Current clinical strategies

For the repair of articular cartilage defects, the strategy used depends on the extent of damage and/or degeneration. Defects of the articular cartilage that do not penetrate to the subchondral bone cannot undergo intrinsic healing processes since the progenitors of the bone marrow space are not accessible. In contrast, defects that penetrate the subchondral bone have been shown to undergo intrinsic repair processes that usually result in a fibrocartilage tissue that possesses inferior properties compared to hyaline cartilage. Defects in joint cartilages are commonly treated with either implantation of tissues (Section 16.3.1.1.) or cartilage regeneration approaches (Section 16.3.1.2.).

Cartilaginous defects in the external ear, nose and trachea are commonly treated with surgical procedures, with or without the implantation of tissues or prosthetics (Section 16.3.1.1.). The surgical procedure may involve the resection of the damaged/diseased cartilage, which may be followed by anastomosis in the case of the trachea. While defects in facial cartilaginous features (*i.e.* ear and nose) may not cause pain as with defects in the joint region, the treatment of these defects can significantly improve the quality of life for patients. Besides correcting the appearance of these features, surgical treatment of the nasal cartilage defects may improve breathing problems. The need for clinical treatment of tracheal defects is clear as tracheal defects or abnormalities may cause severe breathing problems and eventually lead to premature death.

Different current clinical strategies for cartilage repair and reconstruction will be briefly described here. As this chapter focuses on nanomaterials used for cartilage tissue engineering, we refer readers to excellent reviews [2,14] for more details on these clinical approaches.

16.3.1.1. Tissue implantation

Tissue implantation is a widely used strategy for the repair and reconstruction of articular cartilage defects. One commonly used procedure is osteochondral transfer used for the repair of osteochondral defects [2,15,16]. This method involves the harvest of cylindrical osteochondral donor plugs from the low weight bearing areas within the knee joint. The defect site is first prepared followed by implantation of donor osteochondral plug(s) (one or more depending on the size of the defect) to fill the defect. This treatment needs to

be limited to 1–4 cm² so as to prevent excessive donor site morbidity [17]. While it was originally developed as an open procedure, it can now be done arthroscopically with current surgical instrumentation and techniques. However, this procedure remains to be technically difficult and can result in variable patient outcomes based on differences in the height of the defect cartilage and the surrounding native cartilage as well as the accumulation of fibrocartilage within the gaps regions between the transplanted plugs [17,18]. For the repair of chondral defects, other implantation treatment methods involve grafting strips of perichondrium [19] or periosteum [20–22] to the defect using fibrin glue. In the case of periosteal grafts, the problem of graft calcification can occur over time. However, periosteum is more available (than perichondrium) and is widely used for transplantation purposes. The periosteum is also of interest for cartilage repair as this tissue contains a population of chondrocyte precursor cells in its inner layer (referred to as the cambium) [14].

Approaches for reconstruction of the ear, nose and trachea include the surgical transplantation of autologous tissues, allografts, prosthetic materials, or a combination of these methods. One conventional method is to harvest autologous cartilage from the rib, which is subsequently shaped by the surgeon, for surgical replacement of the damaged tissue [23]. While this approach can be quite successful, this invasive procedure creates a donor site defect and may also require multiple revision surgeries. Importantly, the mechanical properties of the rib cartilage may not be suitable for reconstruction in all cases. Prostheses that may be used for reconstruction purposes are made from silicone or wax, but there have been increased reports of infections in patients.

While conventional treatment of tracheal disease can routinely be accomplished by a direct anastomosis [8], only half of the tracheal length for adult patients and one third for pediatric patients can be resected. Alternative approaches are then required for larger defects. Experimentally, tracheal conduits have been shaped from autografts such as bowel segments and jejunal flap with costal cartilage skeleton [24,25], allografts [26,27], prosthetic materials such as poly(dimethyl siloxane) (Silastic) covered with Dacron mesh at the ends for suturing purposes or a Marlex mesh reinforced with a Teflon spiral [28,29], or a combination such as combined jejunal tissue with a Dacron graft [30]. These replacements do not have the same properties of the native tissue and their implantation may result in inflammation and poor integration to the host tissue.

16.3.1.2. Cartilage regeneration

Cartilage regeneration techniques have primarily been explored for the repair of articular cartilage defects. Penetration of subchondral bone is the oldest and most commonly used method to induce cartilage regeneration through bone

marrow stimulation [2]. It is useful for a full thickness chondral defect containing exposed subchondral bone. The principle of this method is to disrupt the subchondral blood vessels by penetrating the subchondral bone plate, in turn forming a fibrin clot on the defect site. When protected from loading, the fibrin clot recruits bone marrow mesenchymal stem cells, which proliferate and differentiate into chondrocyte-like cells. The most common of these approaches is microfracture. Microfracture is a simple and common first-line arthroscopic treatment for symptomatic chondral defects [2]. First described by Steadman *et al.* [31], this procedure involves the removal of all unstable cartilage within the defect site followed by the creation of fracture holes (using an arthroscopic awl) to penetrate the subchondral bone. As a result, the defect becomes filled with a fibrin clot, which allows the differentiation of pluripotent marrow cells. Spongialization is a more radical version of marrow stimulation which involves the complete excision of the subchondral bone at the defect site [32].

Another type of articular cartilage regeneration approach is cell based therapy. Specifically, autologous chondrocyte implantation (ACI) or matrix-induced autologous chondrocyte implantation (MACI) therapies have been used for the repair of articular, chondral defects. This procedure involves enzymatically isolating and propagating autologous articular chondrocytes (obtained from the low weight bearing regions in the joint) and then implanting these cells into the defect site as a cell suspension (ACI) or in combination with a matrix material (MACI) [14,33,34]. The implanted cells produce their own extracellular matrix to repair the damage site. Clinical studies showed promising results for this approach. Brittberg *et al.* [35] showed that 11 out of 15 biopsies from treated femoral condyle lesions had hyaline-like appearance, and the treatment was durable up to 11 years [36]. However, the success of this approach is controversial. Knutsen *et al.* [37] found ACI and microfracture to be comparable treatment methods after 5 years, while a more recent study by Saris *et al.* [38] showed that ACI resulted in better tissue regeneration in chondral defects than microfracture despite having comparable clinical scores. Long term studies need to be performed in order to evaluate the survival of the ACI tissue. A modified version of the ACI method involves the injection of bone marrow mesenchymal stem cells rather than chondrocytes [39].

16.3.2. Tissue engineering approach

As few of the current treatment methods described in Section 16.3.1. result in successful repair or regeneration of damaged cartilage, the development of new procedures are warranted. One popular research area is tissue engineering, which combines principles of cell biology, medicine and engineering to create tissue constructs that possess the morphological and functional properties of native tissue. Important features that need to be achieved for development of implantable engineered cartilaginous tissues include: the creation of large sized implants and attainment of appropriate

mechanical properties, biochemical composition and cellular morphology. The classical tissue engineering approach is to seed isolated cells onto scaffolds and to cultivate the cell / scaffold constructs in a bioreactor (Figure 3) and / or in the presence of stimulatory factors. Thus far, engineered cartilage constructs have unsatisfactory mechanical properties and collagen contents much less than native tissue [40,41]. For example, the flexural modulus of engineered nasal constructs is typically less than half of that of the native nasal cartilage [40]. The proper stiffness is important for the engineered cartilaginous tissues to maintain their structure. Current developments in cartilage tissue engineering is focused on yielding engineered tissues that have improved structure and functionality.

Figure 3. The classical tissue engineering approach. (A) Cells, commonly chondrocytes or mesenchymal stem cells, are first isolated from the patient's tissues by an enzymatic digestion. The image here shows microscopy image of rabbit ear chondrocytes. The cells are then seeded into (B) a scaffold [122] or a mold [99], or (C) directly into a bioreactor. A bioreactor may be just a static dish or more sophisticated systems that can provide shear stress and control oxygen tension. The image here shows a bioreactor that provides intermittent shear stimulation to engineered cartilage tissues [123]. (D) The constructs are grown until reaching desired properties that mimic the native tissue. The image here shows an engineered ear using a mold placed in a continuous flow bioreactor [99]. (E) The engineered tissues can potentially be implanted into the patient.

16.3.2.1. Cells

Chondrocytes are specialized cells in the cartilage that synthesize and maintain the matrix structure [2]. They are spheroidal in shape and have no cell-to-cell contacts. These cells are sparsely arranged within the matrix and receive nutrition *via* diffusion through the matrix. They depend on an anaerobic metabolism and survive on low oxygen concentrations. Although they have high metabolic activity, the total activity is low due to a low cellular volume of the tissue. Chondrocytes are commonly used in cartilage tissue engineering because they are the cells resident in the native cartilage.

While full depth chondrocytes have been predominantly used for transplantation and tissue engineering, specific chondrocyte sub-populations can be used to improve the functional properties of tissue engineered cartilage. Waldman *et al.* [42] investigated the use of chondrocytes from different zones of the articular cartilage for tissue engineering, including full-thickness, mid-and-deep zone and deep zone chondrocytes. These different sub-populations were isolated and cultured on porous calcium phosphate substrates. While collagen synthesis was the highest with full-thickness chondrocytes, mid-and-deep zone chondrocytes yielded engineered tissues with improved mechanical properties and increased amount of proteoglycans. Dowthwaite *et al.* [43] isolated a chondrocyte sub-population from the superficial zone of the immature bovine articular cartilage. This sub-population has progenitor cell-like properties with high colony forming ability as well as high propagation capacity. Colony-derived populations of these progenitors could be engrafted into different connective tissue lineages.

The application of stem cell technology is gaining interest to be used for cartilage tissue engineering since stem cells have high proliferative capacity and multi-lineage differentiation potential. The precursor of chondrocytes is mesenchymal stem cells (MSCs). Traditionally, the source of these stem cells is the bone marrow. Various studies have been conducted to promote the propagation and differentiation of MSCs, using growth factors, biomaterials and different stimulation methods such as the application of mechanical forces or the control of oxygen tension in bioreactors. Adult bone marrow derived MSCs can give rise to chondrocytes when cultured in a 3D environment in the presence of transforming growth factor- β (TGF- β) family of growth factors [44]. In particular, TGF- β 1 has been shown to induce *in vitro* chondrogenesis of rabbit bone marrow derived MSCs [45]. Human bone marrow derived MSCs could also undergo chondrogenic differentiation under appropriate conditions [46,47]. These cells also increase osteochondral differentiation when implanted *in vivo* [48,49].

Recently, new sources of mesenchymal stem cells have been discovered, including adipose tissue, blood and the synovium. Mesenchymal stem cells isolated from human synovial membranes were shown to be capable of expansion into larger numbers and induction of chondrogenesis *in vitro* [50].

In addition, it has been reported that *in vitro* expanded human articular chondrocytes have characteristics similar to a progenitor cell, although it is not yet known the specific type of progenitor cells [51]. In another study, a MSC population was found in normal and osteoarthritic human articular cartilage [52]. There is a higher number of progenitor cells in osteoarthritic cartilage compared to normal cartilage. It is unclear whether this is due to the recruitment of these cells from the surrounding synovium or the conversion of MSCs in the defect into an immature phenotype.

Multiple cell types may be co-cultured in cartilage tissue engineering to mimic the complex composition and structure of each native cartilage tissue. While the cartilage is composed of a single cell type (chondrocytes), the adjacent cells play an important role in the functionality of the cartilaginous organs. For example, the auricular cartilage (elastic cartilage) is composed of a central chondrogenic layer sandwiched between two perichondrial layers. Giardini-Rosa *et al.* [53] developed a new method of engineering auricular cartilage constructs with structure similar to the native tissue, consisting of the cartilage zone with a single perichondrial layer on top. The lumens of trachea are lined with epithelial cells, and the lack of epithelial lining has been linked to tracheal stenosis in tissue-engineered trachea [41]. The co-culture of perichondrial and epithelial cells with chondrocytes may improve the functionality of engineered auricular and tracheal tissues.

16.3.2.2. Biomaterials

Scaffolds are important to provide a three-dimensional structure for supporting the synthesis of cartilage-specific extracellular matrix proteins, depending on the type (hyaline, elastic, fibrocartilage) of cartilage to be developed. Numerous scaffolds have been investigated for cartilage tissue engineering, including both natural and synthetic biomaterials. There are also different methodologies for the fabrication of scaffolds used in cartilage tissue engineering. Important factors need to be considered in scaffold development, such as biocompatibility, mechanical stability, pore size and porosity, shape, degradation, and ability to integrate with native tissue [1,14].

Natural biomaterials for cartilage tissue engineering include alginate, chitosan, collagen, fibrin and hyaluronan (Table 1). Alginate is commonly used to encapsulate and culture chondrocytes or to study the chondrogenic differentiation of cultivated mesenchymal stem cells. Chia *et al.* [54] showed that chondrocytes encapsulated in alginate produced higher levels of glycosaminoglycans compared to cells in monolayers, which dedifferentiate into a fibroblast-like phenotype. However, the alginate does not promote redifferentiation. Lin *et al.* [55] similarly showed that primary porcine chondrocytes could maintain their differentiated state for 4 weeks of culture in porous three-dimensional alginate scaffolds within a perfusion system. In another study, MSCs were encapsulated in alginate beads and treated with

TGF- β 3 for 3, 6 or 14 days, after which the cells were cultured without the growth factor for an additional 2 weeks [56]. Stimulation with TGF- β 3 within the first 3 days of cultivation appeared to be necessary for chondrogenesis. Xu *et al.* [57] developed a staging scheme for *in vitro* chondrogenic differentiation of human mesenchymal stem cells by growing the cells in three-dimensional alginate gels. The cells undergo four stages: 1) Stage 1 at days 0–6 expressing collagen I, VI, Sox 4 and BMP-2, 2) Stage 2 at days 6–12 expressing HAPLN1, collagen XI and Sox 9, 3) Stage 3 at days 12–18 expressing matrilin 3, Ihh, Homeobox 7, chondroadherin and WNT 11, and 4) Stage 4 at days 18–24 expressing aggrecan, collagen IX, II and X, osteocalcin, fibromodulin, PTHrP and alkaline phosphatase. Chondrocytes in alginate were also injected into mice *in vivo* [58]. Dobratz *et al.* [58] injected human nasal septal chondrocytes in sodium alginate subcutaneously on the back of mouse followed by injection with calcium chloride to crosslink the alginate. The engineered cartilage was harvested at different time points and most explants resembled the native cartilage in gross morphology. While many explants resembled native cartilage in histological morphology, there was presence of fibrous tissue areas in the engineered cartilage. When grown for more than 26 weeks, the engineered cartilage resembled native septal cartilage in terms of collagen content.

Table 1. Natural biomaterials used for cartilage tissue engineering

Biomaterial	Methods	Cells Used	Significant Results	Ref.
Alginate	<i>in vitro</i> cell encapsulation	human nasal septal chondrocytes	higher level of GAG compared to monolayers	[54]
		porcine chondrocytes	maintained differentiated state for 4 weeks	[55]
		human MSCs	stimulation with TGF- β 3 led to chondrogenesis	[56]
		human MSCs	identified 4 stages of <i>in vitro</i> chondrogenic differentiation	[57]
	<i>in vivo</i> cell injection into mice subcutaneously	human nasal septal chondrocytes	injection into mice led to growth of native-like cartilage	[58]
Chitosan	seeding of scaffolds with different fiber sizes	porcine chondrocytes	supported matrix production	[59]

Biomaterial	Methods	Cells Used	Significant Results	Ref.
	seeding of sponges with different pore sizes, cultivated in a rotating bioreactor	porcine chondrocytes	control polyglycolic acid (PGA) meshes led to DNA and GAG contents more similar to native cartilage	[60]
	loaded chitosan microspheres with TGF- β 1, incorporated microspheres into chitosan scaffolds and seeded cells	rabbit chondrocytes	release of TGF- β 1 in a multiphasic manner; stimulated cell proliferation and collagen II synthesis	[61]
	loaded BMP-6 onto scaffolds and seeded cells	mouse MC3T3-E1 cells	increased GAG, collagen II and DNA	[84]
Collagen	seeding of porous sponges	bovine articular chondrocytes	chondrogenesis with serum or with Nutridoma as serum replacement	[62]
	encapsulation of cells in hydrogels and implantation into rabbits subcutaneously	rabbit BMSCs	cells showed characteristics of chondrocytes	[63]
	seeding of hybrid scaffolds made of collagen I and PLGA and implantation into mice subcutaneously	bovine chondrocytes	deposition of cartilaginous extracellular matrix	[64]
Fibrin	delivery of chondrocytes to treat human articular cartilage defects	autologous chondrocytes	grafts filled and integrated in patients' defects after 24 months	[65]
Hyaluronan	cultured autologous chondrocytes on scaffolds and implanted constructs into human cartilage defects	autologous chondrocytes	hyaline-like cartilage formed in the grafted site after 17.5 months	[66]

Biomaterial	Methods	Cells Used	Significant Results	Ref.
	seeding of MSCs in photocrosslinked hydrogels; <i>in vitro</i> culture and <i>in vivo</i> subcutaneous culture in mice	human MSCs	higher expression of cartilage specific markers than in PEG hydrogels; incorporation of TGF- β 3 increased collagen II, aggrecan, Sox9 <i>in vivo</i>	[67]

Chitosan is another natural material used for cartilage tissue engineering due to its similarity with glycosaminoglycans present in the native cartilage. Ragetty *et al.* [59] fabricated chitosan nonwoven scaffolds with fibers of different sizes (4, 13 and 22 μm in width), using a replica molding technique. These scaffolds were seeded with porcine chondrocytes and supported matrix production (GAG and collagen II) per chondrocyte compared to the control polyglycolic acid mesh, especially those with smaller fibers. The increased matrix production is likely due to the chemical composition of the scaffolds. In another study, porcine chondrocytes were cultured on chitosan sponges with different pore sizes (10, 10–50, 70–120 μm in diameter) or polyglycolic acid mesh for 28 days in a rotating bioreactor [60]. While the proliferation and metabolic activity of chondrocytes improved with increasing pore size of chitosan scaffolds, polyglycolic acid meshes produced tissue constructs with DNA and GAG contents more closely resembling that of the native cartilage. Chitosan is a common biomaterial used to deliver bioactive molecules to cultured chondrocytes [61].

Yates *et al.* [62] cultured bovine articular chondrocytes on three-dimensional porous collagen sponges in both standard and serum-free conditions. Generally, the collagen sponges provided a favorable environment for chondrogenesis. At 4 weeks, chondrogenesis by cells cultured with Nutridoma as a serum replacement was equivalent or better compared to cells cultured with serum, while cells cultured with insulin-transferrin-selenium (ITS) serum replacement showed poor chondrogenesis due to decreased cell survival. Chondrogenesis of mesenchymal stem cells can also be induced by providing a chondrogenic environment. For example, bone marrow mesenchymal cells were encapsulated in a collagen hydrogel which was then placed in diffusion chambers that allow permeation of body fluids and infiltration of host cells [63]. These chambers with cell-encapsulated hydrogels were implanted subcutaneously in the back of rabbits. At 8 weeks, the mesenchymal cells grown in the hydrogels showed characteristics of chondrocytes. Tissues grown in collagen-alginate hydrogels expressed less collagen II than those in collagen-based hydrogels. In another study, bovine chondrocytes were cultured on hybrid scaffolds made of collagen I and PLGA, which were then

transplanted subcutaneously into mice [64]. The PLGA mesh acts as a skeleton and the collagen microsponges aided cell attachment and growth. Three hybrid scaffolds were investigated: (i) PLGA mesh with collagen microsphere formed in its interstices, (ii) PLGA mesh with collagen microsphere formed on one side, and (iii) PLGA mesh with collagen sponge formed on both sides. Transplants made of all three scaffolds led to deposition of cartilaginous extracellular matrix. The scaffolds made of PLGA mesh with collagen microsphere on one side or both sides showed increased GAG, collagen II and aggrecan compared to the scaffolds with PLGA mesh with collagen microsphere in the interstices.

Fibrin has been used as a biomaterial to deliver autologous chondrocytes to treat full thickness articular cartilage defects [65]. At 24 months post-surgery, 29 out of 30 patients showed fair to excellent results, with only one poor result. These grafts had good fill and integration in the patients' defects.

Clinical trials using Hyalograft CTM, a hyaluronan-based scaffold, have been performed to treat articular cartilage defects [66]. Pavesio *et al.* [66] cultured expanded autologous chondrocytes on hyaluronan scaffolds and implanted the constructs into the cartilage defects in patients. Most grafted sites showed hyaline-like cartilage after an average follow-up time from implantation of 17.5 months. MSCs can interact with hyaluronic acid through their cell surface receptors (*e.g.* CD44) as hyaluronic acid is a native component of cartilage. As such, hyaluronic acid hydrogels could play a role in the differentiation of these stem cells. In both *in vitro* and *in vivo* cultures, MSCs seeded in photocrosslinked hyaluronic acid hydrogels showed chondrogenesis and expressed higher levels of cartilage specific markers compared to using PEG hydrogels [67]. *In vivo*, the addition of TGF- β 3 enhanced the expression of collagen type II, aggrecan and Sox9.

Natural scaffolds can have problems with immunogenic incompatibility and batch inconsistency [14]. In contrast, synthetic scaffold properties can typically be better controlled. Synthetic biomaterials for cartilage tissue engineering include polyhydroxyacids, such as polylactide (PLA), polyglycolide (PGA), poly(ϵ -caprolactone) (PCL) and their copolymers, and polyethylene glycol (PEG) hydrogels (Table 2).

Table 2. Synthetic and composite biomaterials used for cartilage tissue engineering

Biomaterial	Methods	Cells Used	Significant Results	Ref.
poly(lactic-co-glycolic acid) (PLGA)	seeding of scaffolds and implantation into mice subcutaneously	rat chondrocytes	formation of neocartilage after 4 months	[69]
	seeding of microspheres and injection into mice subcutaneously	rabbit chondrocytes	good adhesion to microsphere surface; formation of solid white cartilaginous tissues in implantation site after 4 weeks	[70]
	seeding of MSCs onto scaffolds and transplantation into rabbit knee defects	rabbit MSCs	defects filled with smooth white hyaline-like tissues after 12 weeks	[71]
	seeding of cells in fibrin onto scaffolds	rabbit articular chondrocytes	fibrin aided cell attachment and distribution; led to more GAG production compared to PLGA only control	[79]
	seeding of cells onto scaffolds with immobilized hyaluronic acid	bovine articular chondrocytes	enhanced cell attachment as well as synthesis of GAG and collagen compared to PLGA only control	[80]
	seeding of cells onto hyaluronic acid modified scaffolds	human adipose derived stem cells	higher expression of chondrogenic marker genes and increased production of GAG and collagen II compared to PLGA only control	[81]

Biomaterial	Methods	Cells Used	Significant Results	Ref.
polyglycolic acid (PGA)	seeding of scaffolds and comparison to PLGA scaffolds	human chondrocytes	expression of aggrecan and collagen II higher in PGA scaffolds	[72]
polycaprolactone (PCL)	<i>in vitro</i> culture in porous scaffolds	human MSCs	cell-scaffold constructs became hard after 3 weeks; higher DNA and GAG contents when scaffolds were treated with Pluronic F127 and/or collagen	[73]
	culture in nanofibrous scaffolds	human bone marrow derived MSCs	multi-lineage differentiation of MSCs	[74]
	immobilized TGF- β 3 lentivirus to woven scaffolds and cultured cells	human MSCs	formation of cartilaginous extracellular matrix; induced chondrogenesis as effectively as with TGF- β 3 supplemented medium	[85]
poly(ethylene glycol) (PEG)	incorporated cleavable RGD peptide into hydrogels and encapsulated cells	human MSCs	RGD promoted chondrogenesis of MSCs, MMP-13 produced by cells led to the release of RGD as necessary for complete differentiation of cells as limited by the presence of RGD	[75]
	cell encapsulation in photopolymerized hydrogels	bovine condylar chondrocytes	cell viability was maintained and PEG hydrogels are suitable for cell encapsulation	[76]

Biomaterial	Methods	Cells Used	Significant Results	Ref.
composite polyglycolic acid/polyethylene	polyethylene cylindrical rods were wrapped with PGA fibres, seeded with cells, cultured with TGF- β 1 and IGF-1 and implanted into mice subcutaneously	porcine bone marrow MSCs	formation of mature cartilage after 8 weeks	[77]
composite polyglycolic acid/ hydroxyapatite	seeding onto scaffolds and implantation into full thickness cartilage defects	rabbit MSCs	formation of hyaline cartilage and subchondral bone	[78]

Polyglycolic and poly-L-lactic based scaffolds have been generally found to increase the presence of proteoglycans as well as the proliferation and differentiation of chondrocytes, as compared to collagen based scaffolds [68]. Baek *et al.* [69] seeded *in vitro* cultured chondrocytes onto PLGA scaffolds, and the tissue constructs were implanted in the subcutaneous pocket of nude mice. After 4 months of implantation, neocartilage was formed with the same dimensions of the scaffold. The explants showed chondrocytes within lacunae in a mature cartilaginous matrix that expressed collagen type II. Rabbit chondrocytes seeded on PLGA microspheres of 30–80 μ m in diameter showed good adhesion to the microsphere surface [70]. *In vivo*, chondrocytes mixed with these PLGA microspheres were injected into subcutaneous sites in mice, and solid white cartilaginous tissues were formed in the implantation site after 4 and 9 weeks. The resulting tissues appeared histologically mature and expressed high levels of sulfated GAGs and collagen. As well, PLGA scaffolds allow the differentiation of progenitor cells and provide structural support for chondrogenesis and articular cartilage repair [71]. Uematsu *et al.* [71] seeded MSCs onto 3D PLGA scaffolds and transplanted them into defects in rabbit knees. At 12 weeks after transplantation, the defects were filled with smooth white tissues that were hyaline-like. Zwingmann *et al.* [72] seeded human chondrocytes onto PGA and PLGA scaffolds. The synthesis of aggrecan and the mRNA gene expression for collagen II were higher in PGA groups.

Porous PCL scaffolds with pore size of 100–150 μ m were used to culture MSCs for 3 weeks *in vitro* [73]. These cell-scaffold complexes became harder and easily manipulated after 3 weeks. When the PCL scaffolds were treated with

Pluronic F127 and /or collagen, the resulting tissues had higher DNA and GAG contents and showed more chondrogenic differentiation. PCL was also used to fabricate 3D nanofibrous scaffolds for the culture and multi-lineage differentiation of human bone marrow derived MSCs [74].

PEG hydrogels are commonly used for cell encapsulation because their hydrolytic degradation can be tunable. Salinas *et al.* [75] incorporated cleavable arginylglycylaspartic acid (RGD) peptide sequence into PEG hydrogels by using a thiol-acrylate photo-polymerization. RGD has been shown to promote the survival and induce chondrogenesis of human MSCs in PEG hydrogels, but it limits complete differentiation of cells with its sustained presence. By including an MMP-13 specific cleavable linker to the peptide sequence, RGD could be released from the hydrogels with the production of MMP-13 by encapsulated cells. After 21 days, MSCs in RGD-releasing hydrogels produced more GAG and collagen II as compared with hydrogels with uncleavable RGD. In a separate study, Nicodemus *et al.* [76] encapsulated condylar chondrocytes in photopolymerized PEG hydrogels. PEG hydrogels were found suitable for cell encapsulation as the cell viability was maintained over the culture period.

Composite scaffolds can be fabricated using multiple biomaterials. Zhu *et al.* [77] created a PGA/polyethylene composite scaffold, with high density polyethylene carved into cylindrical rods and wrapped with PGA fibres. Porcine bone marrow MSCs were seeded into the scaffolds and cultured for 3 weeks in chondrogenic medium containing TGF- β 1 and insulin-like growth factor (IGF-1). The resulting tissue constructs were implanted subcutaneously into mice. At 8 weeks post-implantation, mature cartilage was formed around the polyethylene as well as inside the pores of the polyethylene scaffold. Zhou *et al.* [78] evaluated a composite scaffold made of PGA and hydroxyapatite. Rabbit MSCs were seeded onto PGA and hydroxyapatite scaffolds, and the cell-seeded PGA and hydroxyapatite constructs joined together after 72 h. The cell-PGA-hydroxyapatite composite constructs were implanted into full thickness cartilage defects in the intercondylar fossa of the femur, resulting in the formation of hyaline cartilage and a complete subchondral bone formation.

A combination of natural and synthetic biomaterials can also be used for cartilage tissue engineering to synergize the biomimetic properties of natural materials and the tunable mechanical properties of synthetic materials. Sha'ban *et al.* [79] seeded rabbit articular chondrocytes in fibrin onto PLGA scaffolds to minimize cell loss during seeding and to achieve homogeneous cell distribution. This hybrid scaffold was capable of promoting *in vitro* chondrogenesis and led to greater sulphated GAG production as compared with PLGA control. Yoo *et al.* [80] immobilized hyaluronic acid onto macroporous PLGA scaffolds to enhance the attachment of chondrocytes as well as the synthesis of GAG and collagen compared to unmodified PLGA scaffolds. Human adipose derived stem cells cultured on hyaluronic

acid-modified PLGA scaffolds expressed higher levels of chondrogenic marker genes and increased in the production of sulphated GAG and collagen II, as compared to those cultured in unmodified PLGA scaffolds at 4 weeks [81].

Bioactive molecules such as peptides and growth factors can be incorporated and delivered in biomaterials for cartilage tissue engineering. Growth factors such as FGF, TGF- β 1 and IGF-1 have been shown to enhance chondrogenesis [63,82,83]. Cai *et al.* [61] loaded chitosan microspheres with TGF- β 1, and rabbit chondrocytes were cultured on chitosan scaffolds with the TGF- β 1 microspheres for 3 weeks. TGF- β 1 could be released from the microspheres in a controlled multiphasic manner and stimulated cell proliferation and the synthesis of collagen type II. Another chondrogenic differentiation factor, bone morphogenetic protein-6 (BMP-6), was loaded onto chitosan scaffolds and increased the contents of GAG, collagen II and DNA in cultured cell-scaffold constructs [84].

Brunger *et al.* [85] created lentivirus-based cell-instructive scaffolds to mediate the differentiation of human mesenchymal stem cells and increase the formation of cartilaginous extracellular matrix. This was done by using poly-L-lysine to immobilize transforming growth factor β 3 (TGF- β 3) lentivirus to 3D woven PCL scaffolds. The gene delivery of TGF- β 3 using the scaffolds induced chondrogenesis as effectively as the conventional differentiation method with TGF- β 3 supplemented medium. Immobilization of the lentiviral vector to a biomechanically functional scaffold enabled sustained transgene expression and extracellular matrix formation.

There is increasing evidence that cell behavior can be influenced by changes in nanoscale topographical surfaces. Various studies have been performed to create and evaluate new biomaterials with such features. Nanomaterials used in cartilage tissue engineering will be further discussed in Section 16.4.

The use of scaffolds in conventional cartilage tissue engineering provides a structural support for the growth of cells and eases the shaping of the constructs, but can elicit immunological responses once implanted into the body. Whitney *et al.* [86] explored the scaffold-free method of cartilage tissue engineering, seeding high densities of cells (1–3 million cells cm⁻²) in a custom biochamber with two compartments separated by a porous polyester membrane. This method produces cartilage sheets of approximately 4 cm by 4 cm. The Waldman group developed a method to grow large articular and auricular cartilage constructs from a small cell population (7000–13000 cells cm⁻²) without the use of scaffolds [53,87]. In this method, cells are seeded directly into the wells of a chamber, which is connected to a pump that provides a continuous flow of culture medium. After 4 weeks, 3D tissues that take the shape of the well (3 cm²) and have a thickness of approximately 500 μ m are formed. This continuous flow bioreactor setup improves the transport of nutrients and metabolic waste to promote tissue growth.

16.3.2.3. Bioreactors

Cartilage tissue constructs can be grown in bioreactors that control the environmental conditions and provide specific factors for desired tissue growth. Bioreactors can maintain physiological conditions, improve transport of nutrients and wastes, and provide biomimetic stimulation.

The simplest form of the bioreactor is the cultivation of tissue constructs in a Petri dish or culture plate with or without placing it on a shaker [88,89]. Other bioreactors include spinner flasks, flasks with stirrers and rotating vessels [90-93]. These bioreactors provide shear stress to the tissue constructs to enhance nutrition by convection as opposed to diffusion alone. Rotating vessels are more advanced bioreactors that allow tissue constructs to float freely in the culture medium while the vessel rotates around an axis at a constant speed. It was previously shown that chondrocyte-seeded scaffolds cultured in rotating vessels for 8 weeks resulted in larger constructs than those cultured in static and stirred bioreactors [93]. Constructs grown in rotating bioreactors also had the highest concentrations of glycosaminoglycan and collagen and the best mechanical properties.

16.3.2.3.1. Control of oxygen tension

Cultured cells require nutrients and oxygen to proliferate. In static culture, oxygen and carbon dioxide diffuse into and out of the cells. As a result, the medium close to the cells has a decreasing oxygen tension and increasing carbon dioxide tension. If the medium is not circulated, the rate of diffusion into the cells decreases and eventually ceases due to the lack of concentration gradient of oxygen across the cell surface. This leads to cell death. Stirred flask bioreactors can provide shear stresses and enable the mixing of oxygen and nutrients throughout the culture medium during the cultivation of cells for cartilage tissue engineering. As chondrocytes are primarily anaerobic cells, several studies have investigated the effects of oxygen tension on tissue formation. The effects of hypoxia are variable, with some studies indicating that hypoxia leads to improvements in tissue formation [94], whereas others have shown no effects [95].

16.3.2.3.2. Transport of nutrients and metabolic waste

Continuous flow bioreactors can be used for the engineering of various cartilaginous tissues so as to eliminate the need for large numbers of cells as with other approaches and to accelerate the formation of engineered tissues. Previous approaches of cartilage tissue engineering required a large number of cells to generate tissues of physiologically relevant size and mechanical properties. For example, cell density of 10^6 cells cm^{-2} is necessary to engineer nasal cartilage tissues that can withstand surgical manipulations and support the structure of the nose [96]. A separate cell expansion stage is usually used to obtain a large number of cells for cartilage tissue engineering. This expansion

stage tends to lead to de-differentiation of cartilage cells. Brenner *et al.* [87] developed a novel strategy to engineer cartilage tissues of 3 cm² from a small population of cells (only 10⁴ cells cm⁻²) without the cell expansion step, by growing tissues in a continuous flow bioreactor. Also, the continuous flow bioreactor can accelerate matrix production compared to static growth such that no scaffold will be necessary. Using a bioreactor with media flow likely improves tissue growth due to better transport of nutrients and metabolic waste.

16.3.2.3.3. Growth factors

Bioreactors can provide more suitable culture conditions to tissue constructs by adding growth factors that support chondrogenesis. The application of growth factors such as bone morphogenetic protein-2 (BMP-2), IGF-1, and TGF- β (1 or 3) in the bioreactor increased the compressive and tensile properties of engineered cartilage tissues [97]. The proliferation of chondrocytes, when seeded at low densities, was increased by adding combinations of growth factors such as TGF- β 1, fibroblast growth factor 2 (FGF-2) and platelet-derived growth factor BB (PDGF-BB) into the bioreactor [98]. In a separate study, elastic cartilage constructs grown by culturing rabbit auricular chondrocytes in the continuous flow bioreactor showed increased deposition of cartilaginous extracellular matrix, improved expression and localization of elastin, improved mechanical properties and greater thickness when insulin or IGF-1 was added [99]. These constructs had properties comparable to the native auricular cartilage after a 4 week cultivation period. On the other hand, the inhibition of interleukin-6 may improve the growth of cartilage constructs in bioreactors [100].

16.3.2.3.4. Application of mechanical forces

The function of chondrocytes can also be influenced by mechanical signals. The application of mechanical forces can be transduced into biochemical signals leading to the stimulation of cartilaginous extracellular matrix synthesis (process of mechanotransduction). In addition, mechanical stimulation of tissue engineered cartilage can also result in the improvement of mechanical properties of the developed tissue constructs. These applied forces simulate loading that would be normally exerted during activities of daily movement. Specifically, cyclical loading of articular chondrocytes has been shown to increase extracellular matrix production and cell viability within engineered cartilage constructs [101,102]. Other bioreactors have been developed to provide dynamic changes in hydrostatic pressure, tension, shear, vibrations, *etc.* to articular cartilage *in vitro*, which are reviewed in [103]. For example, shear stresses can also be applied to cartilage tissue constructs using rotating vessels as described previously (Section 16.3.2.3.).

16.4. NANOMATERIALS FOR CARTILAGE TISSUE ENGINEERING

New methods in nanomaterial science, such as electrospinning and 3D fibre deposition, have been developed to improve the quality of the scaffolds on the nanoscale. Similarly, nanoparticles can also be incorporated into biomaterials. The addition of these nanoscale features can produce scaffolds that better mimic the extracellular matrix environment found in the native cartilage, which is believed to increase the interaction of the cells with the scaffold to improve the functionality of the resultant engineered tissue construct. It is believed that cell behavior is largely affected by nanoscale features, leading to changes in the cytoskeleton, cell morphology, focal adhesions, motility as well as gene expression. Studies have suggested that there may be a topography-dependent cell transduction that is independent of biochemical signals, and this is related to the pattern and spacing of adhesive ligands [104,105]. This type of cell transduction has been found to be similar to mechanotransduction [105,106].

16.4.1. Nanosurfaces

Both physical (*i.e.* lithography) and chemical (*i.e.* treatment with acids and bases) methods have been used to create geometrically defined nanopatterns on tissue engineering scaffolds (Figure 4). Balasundaram *et al.* [107] created highly porous surfaces with nanoscale roughness by nanoembossing polyurethane (PU) and PCL. The two polymers were casted over a crystalline titanium surface with plasma-deposited spiky nanofeatures, which can be modified using different parameters for the fabrication process. The resulting polyurethane and PCL surfaces have nanoscale surface roughness and high surface energy compared to surfaces casted over titanium without spikes. These surfaces increased chondrocyte numbers, intracellular protein production and collagen secretion by chondrocytes compared with smooth surfaces. Park *et al.* [108] modified PLGA scaffolds using a chemical etching technique with 1 N sodium hydroxide for 10 min. The treated PLGA scaffolds enhanced chondrocyte numbers as well as intracellular and extracellular protein content compared to untreated PLGA scaffolds. This may be due to increased hydrophilicity, porosity, surface area and degree of nanometer roughness. The authors suggest chemically treated PLGA may be suitable for articular cartilage repair.

Figure 4. Nanosurfaces created with physical and chemical methods for cartilage tissue engineering. (A-F) Nanoembossed polyurethane (NPU) and nanoembossed polycaprolactone (NPCL) [107]. (A-B) Scanning electron microscopy images of NPU at low (A) and high (B) magnifications. (C) Scanning electron microscopy image of NPCL. (D-E) Chondrocyte density was increased on NPU (D) and NPCL (E) compared to unmodified substrates (PU, PCL). (F) Intracellular protein production was increased on NPU and NPCL compared to unmodified substrates. (G-J) NaOH-treated PLGA scaffold [108]. (G-H) Scanning electron microscopy images of unmodified (G) and NaOH-treated (H) PLGA scaffolds. (I) Cell density was increased on NaOH-treated scaffolds compared to unmodified scaffolds. (J) GAG synthesis was increased on NaOH-treated scaffolds compared to unmodified scaffolds.

16.4.2. Nanofibres

Nanofibrous scaffolds are commonly prepared using electrospinning (Figure 5). Li *et al.* [44,74] fabricated electrospun, three-dimensional nanofibrous scaffolds from a synthetic biodegradable polymer, PCL. The resulting scaffolds had uniform, randomly oriented nanofibres of 700 nm in diameter. These scaffolds resemble collagen fibrils and supported the multi-lineage differentiation of bone marrow-derived human mesenchymal stem cells when cultured in specific differentiation media *in vitro*. Specifically the cartilage specific gene profile, including aggrecan, collagen II and collagen X, was expressed when cells were grown in chondrogenic medium with TGF- β 1.

Figure 5. Nanofibrous scaffolds used in cartilage tissue engineering. (A–B) Electrospun nanofibrous polycaprolactone scaffold [44]. (A) Scanning electron microscopy image of polycaprolactone scaffold with fibres of 500–900 nm in diameter. (B) GAG synthesis was increased when MSC cultures on the nanofibrous scaffolds were treated with TGF- β 1. (C–F) Aligned electrospun nanofibrous polycarbonate urethane scaffolds modified with an anionic dihydroxyl oligomer [110]. (C–D) Scanning electron microscopy images of scaffolds under continuous tension (monotonic) (C) or with tension only for 24 h (relaxed) (D). (E–F) Type I collagen (green) appeared more organized parallel to the cells (red, actin cytoskeleton; blue, cell nuclei) in monotonic scaffolds (E) than in relaxed scaffolds (F). (G–I) Electrospun nanofibrous poly(lactic acid) membranes modified by treatment with oxygen plasma and subsequent covalent grafting of cationized gelatin molecules [112]. (G) Bulk appearance of nanofibrous membrane before implantation into rabbits subcutaneously. (H) Scanning electron microscopy image of nanofibrous membrane. (I) Bulk appearance of tissue constructs 4 weeks after implantation into rabbits subcutaneously.

In vivo, electrospun PCL scaffolds with fibres of 400 nm in diameter, coated with chitosan or uncoated, were implanted under periosteum in rabbits and TGF- β 1 or vehicle was injected into the implant site [109]. The scaffolds were removed after 1, 3, 5 or 7 days and the scaffolds and periosteum were cultured separately for 6 weeks. The longer duration of implantation led to increased cartilage formation in the uncoated scaffolds. Cells in the uncoated scaffolds produced more GAG and cartilage compared to those in chitosan-coated scaffolds. Overall, these nanofibre scaffolds allowed infiltration of periosteal cells *in vivo*, which in turn produced engineered cartilage *in vitro*.

Turner *et al.* [110] cultured bovine annulus fibrosus cells on electrospun-aligned nanofibrous polycarbonate urethane scaffolds, and the constructs were strained under tension either throughout the entire culture duration (monotonic condition) or only for the first 24 h (relaxed condition). Cells on the relaxed scaffolds showed increased proliferation and collagen synthesis. On the other hand, the cells and matrix on the monotonic strained scaffolds

were more aligned, which in turn led to higher elastic modulus. Determining the optimal applied tensile forces and elastic modulus for the tissue engineering of the annulus fibrosus, a fibrocartilage within the intervertebral disc, may be necessary to better mimic the native tissue.

Besides electrospinning, thermally induced phase separation can also be used to fabricate nanofibrous scaffolds [111]. A poly(β -caprolactone)-*block*-poly(L-lactide) (PCL-b-PLLA) copolymer was used to fabricate nanofibrous scaffolds using this method. PCL-b-PLLA / tetrahydrofuran solution, with the addition of particles of 125–200 μm , was quenched to $-20\text{ }^{\circ}\text{C}$ or below and then gelled for 2 h due to the PCL and PLLA microcrystals. The gel was immersed in water for 2 days to leach out the particles and then lyophilized to obtain nanofibrous scaffolds. The nanofibrous scaffold had higher specific surface area and protein adsorption compared to a solid-walled scaffold. Chondrocytes cultured on the nanofibrous scaffold showed spherical chondrocyte-like phenotype, produced greater amounts of proteins and DNA, and expressed higher levels of collagen II and aggrecan mRNA.

Electrospun PLLA nanofibrous membranes were modified by treatment with oxygen plasma to introduce carboxylate groups on the surface, onto which cationized gelatin molecules were covalently grafted [112]. *In vitro*, the modified membranes enhanced the viability, proliferation and differentiation of rabbit articular chondrocytes compared to unmodified membranes. Cells had proper morphology and grew into the interior of the membranes, as shown by scanning electron microscopy. Cell differentiation was evident by the increased secretion of glycosaminoglycan and collagen and expression of characteristic markers of chondrocytes, such as collagen II, aggrecan and Sox9. *In vivo*, the subcutaneous implantation of cell-seeded membranes led to the formation of cartilage tissues after 28 days.

16.4.3. Nanocomposites

While polymer-hydroxyapatite nanoparticle composites are extensively explored for bone repair, they may also be used for cartilage regeneration (Figure 6). Spadaccio *et al.* [113] investigated the use of electrospun fibres of PLLA loaded with hydroxyapatite nanoparticles for the chondrogenic differentiation of human MSCs. These composite scaffolds induced the chondrogenic differentiation of MSCs, as evident in Sox9 expression and the presence of cartilage specific proteoglycans, compared to a PLLA control. Sox9 is a chondrogenic transcription factor that activates the production of collagen type II and aggrecan. Pan *et al.* [114] prepared nanohydroxyapatite reinforced poly(vinyl alcohol) (PVA) composite gels by an *in situ* synthesis of nanohydroxyapatite particles in PVA solution combined with a freeze / thaw technique. The mechanical properties of the hydrogel could be modified to mimic those of the articular cartilage by changing the nanohydroxyapatite content, PVA concentration and freeze / thaw cycle times.

Figure 6. The incorporation of nanoparticles and nanotubes into scaffolds for cartilage tissue engineering. (A-C) The incorporation of hydroxyapatite nanoparticles into poly(lactic acid) scaffolds [113]. (A-B) Scanning electron microscopy images of poly(lactic acid) membranes with hydroxyapatite nanoparticles at low (A) and high (B) magnifications (nanoparticles indicated with arrows). (C) The expression of chondrogenic specific marker Sox-9 in human MSCs was increased when cultured in basal medium on poly(lactic acid) scaffold with hydroxyapatite nanoparticles (PLLA-HA Basal) compared to poly(lactic acid) only control (PLLA Basal). (D-F) The incorporation of gold nanoparticles into type II collagen [115]. (D-E) Scanning electron microscopy images of surfaces of type II collagen only (D) and type II collagen with 2.5 % gold nanoparticles (E). (F) Expression of Sox-9 was increased with the addition of gold nanoparticles compared to type II collagen only (CII). (G-I) The incorporation of carbon nanotubes into polycarbonate urethane [119]. (G-H) Scanning electron microscopy images of bare polycarbonate urethane surface (PCU) (G) and PCU with carbon nanotubes (H). (I) Chondrocyte density was increased on electrically stimulated PCU with carbon nanotubes (CNT/PCU) compared to bare PCU.

Metallic nanoparticles, such as gold, have certain optical, electronic and biological properties beneficial for cartilage repair. Researchers have incorporated these nanoparticles into polymers to fabricate polymer-metal nanoparticle composites for cartilage tissue engineering. Hsu *et al.* [115] incorporated gold nanoparticles into hydrogels made of porcine collagen II. These nanoparticles increased mechanical properties. At 0.1 % gold

nanoparticles, the composite hydrogels promoted chondrocyte proliferation. An increased gold nanoparticle content led to increased expression of collagen I, aggrecan and Sox9, up to 2.5 % gold content.

The type and amount of nanoparticles incorporated in the nanocomposite scaffolds can be changed to modify surface properties such as surface area, roughness and charge, which may affect the adhesion and proliferation of chondrocytes. Compared with surfaces with conventional or microscale surface roughness, the adhesion of chondrocytes increased on PLGA/titanium dioxide (titania) composite scaffolds with a nanosurface [116]. The scaffolds were created by adding nanometer grain size titania to PLGA solution to create PLGA/titania composites and then chemically treating the composites with different concentrations of sodium hydroxide for different time periods to obtain surface features. Moreover, it was found that these PLGA/titania composites increased the intracellular synthesis of alkaline phosphatase and chondrocyte-expressed growth factors, compared to unmodified surfaces.

Carbon nanotubes have desirable mechanical and electrical properties for tissue engineering. The native extracellular matrix has a natural nanotopography that supports cell attachment and interactions, in turn affecting cell morphology and arrangement. This nanotopography could be mimicked using the structural arrangements of carbon nanotubes. In addition, it was previously shown that electric fields affect the growth and remodeling of cartilage [117,118]. Khang *et al.* [119] investigated the effect of surface roughness and electrical stimulation on the functions of chondrocytes using composite film made of carbon nanotubes and polycarbonate urethane. The composite film had higher nanometer surface roughness and is more hydrophilic compared to plain polymer, which enhanced chondrocyte adhesion and cell density. A lateral electrical stimulation of low voltage was applied across the composite films and this was shown to further enhance cell density. Carbon nanotubes can also be physically entrapped into collagen matrix to better mimic the natural extracellular matrix [120].

A nanoparticle/hydrogel system was developed for delivering two growth factors, BMP-7 and TGF- β 2, to promote the chondrogenesis of MSCs [121]. The alginate hydrogel contains BMP-7 and polyion complex nanoparticles with TGF- β 2, such that a fast release of BMP-7 and a slow release of TGF- β 2 occur.

16.5. CONCLUSIONS

Despite advancements in scaffold design and bioreactor conditions, engineered cartilage constructs remain mechanically inferior to native cartilage tissues. In addition, engineered tissues thus far showed poor integration as well as deterioration in its quality over time when evaluated *in vivo*. The development of new nanomaterials with desired properties may improve chondrogenesis, resulting in more suitable tissue grafts for cartilage repair and regeneration. Here, the recent progress in the development of these materials has been discussed. Some of the recent studies in cartilage tissue engineering involve nanosurfaces fabricated using lithography or acid/base treatments, nanofibrous scaffolds fabricated by electrospinning or thermally induced phase separation, and nanocomposites fabricated by adding nanoparticles or carbon nanotubes to scaffolds. The introduction of nanoscale features creates scaffolds that more closely resemble the native cartilage in its extracellular matrix structure. This improves the interaction of cells with the scaffold and in turn tissue growth. Nanomaterials have shown success in enhancing cell adhesion, morphology, growth, differentiation and synthesis of cartilaginous extracellular matrix. Future work in this field should continue to develop nanocomposites with both mechanical and biochemical properties that adequately mimic the human cartilage tissue such that the morphological and functional properties of the resultant engineered cartilage tissue can be suitable for eventual clinical translation.

REFERENCES

1. J. Raghunath, J. Rollo, K.M. Sales, P.E. Butler, A.M. Seifalian. *Biotechnol. Appl. Biochem.* **46** (2007) 73–84.
2. A.M. Bhosale, J.B. Richardson. *Br. Med. Bull.* **87** (2008) 77–95.
3. L. Nayyer, K.H. Patel, A. Esmaeili, R.A. Rippel, M. Birchall, G.O'Toole, P.E. Butler, A.M. Seifalian. *Plast. Reconstr. Surg.* **129** (2012) 1123–1137.
4. D.V. Luquetti, E. Leoncini, P. Mastroiacovo. *Birth Defects Res. A. Clin. Mol. Teratol.* **91** (2011) 813–822.
5. D. Erdmann, K.E. Follmar, M. Debruijn, A.D. Bruno, S.H. Jung, D. Edelman, S. Mukundan, J.R. Marcus. *Ann. Plast. Surg.* **60** (2008) 398–403.
6. P.D. Phelps, D. Poswillo, G.A. Lloyd. *Clin. Otolaryngol. Allied Sci.* **6** (1981) 15–28.
7. Y. Wang, R. Xiao, F. Yang, B.O. Karim, A.J. Iacovelli, J. Cai, C.P. Lerner, J.T. Richtsmeier, J.M. Leszl, C.A. Hill, K. Yu, D.M. Ornitz, J. Elisseeff, D.L. Huso, E.W. Jabs. *Development* **132** (2005) 3537–3548.
8. K. Kojima, L.J. Bonassar, A.K. Roy, H. Mizuno, J. Cortiella, C.A. Vacanti. *FASEB J.* **17** (2003) 823–828.
9. S.C. Elkerbout, R.A. van Lingen, J. Gerritsen, R.J. Roorda. *Arch. Dis. Child.* **68** (1993) 37–40.
10. J.A. Buckwalter, H.J. Mankin. *Instr. Course Lect.* **47** (1998) 477–486.
11. P.J. Roughley. *Eur. Cell Mater.* **12** (2006) 92–101.
12. M. Benjamin, E.J. Evans. *J. Anat.* **171** (1990) 1–15.
13. R.M. Aspden, Y.E. Yarker, D.W. Hukins. *J. Anat.* **140** (Pt 3) (1985) 371–380.
14. S.N. Redman, S.F. Oldfield, C.W. Archer. *Eur. Cell Mater.* **9** (2005) 23–32.
15. Y. Matsusue, T. Yamamuro, H. Hama. *Arthroscopy* **9** (1993) 318–321.
16. L. Hangody, G. Kish, Z. Karpati, I. Udvarhelyi, I. Szigeti, M. Bely. *Orthopedics* **21** (1998) 751–756.
17. L. Hangody, P. Fules. *J. Bone Joint Surg. Am.* **85A** (Suppl 2) (2003) 25–32.
18. R. Gudas. *Medicina (Kaunas)* **38** (2002) 52–57.
19. G.N. Homminga, S.K. Bulstra, P.S. Bouwmeester, A.J. van der Linden. *J. Bone Joint Surg. Br.* **72** (1990) 1003–1007.
20. V.A. Ritsila, S. Santavirta, S. Alhopuro, M. Poussa, H. Jaroma, J.M. Rubak, A. Eskola, V. Hoikka, O. Snellman, K. Osterman. *Clin. Orthop. Relat. Res.* **302** (1994) 259–265.
21. T.E. Orr, A.M. Patel, B. Wong, G.P. Hatzigiannis, T. Minas, M. Spector. *J. Biomed. Mater. Res.* **44** (1999) 308–313.
22. H. Alfredson, R. Lorentzon. *Ortop. Traumatol. Rehabil.* **3** (2001) 216–223.
23. A.O. Oseni, P.E. Butler, A.M. Seifalian. *Ann. Plast. Surg.* **71** (2013) 238–244.
24. E. Letang, J. Sanchez-Lloret, J.M. Gimferrer, J. Ramirez, A. Vicens. *Ann. Thorac. Surg.* **49** (1990) 955–958.
25. P.C. Cavadas. *Reconstr. Surg.* **101** (1998) 937–942.
26. A. Messineo, R.M. Filler, T. Joseph, A. Bahoric, C.R. Smith. *J. Pediatr. Surg.* **29** (1994) 697–700.
27. K. Kushibe, T. Tojo, H. Sakaguchi, M. Takahama, K. Nishizaki, K. Nezu, S. Taniguchi. *Ann. Thorac. Surg.* **70** (2000) 1876–1879.
28. J.L. Graziano, A. Spinazzola, W.E. Neville. *Ann. Thorac. Surg.* **4** (1967) 1–11.
29. N. Okumura, T. Nakamura, Y. Shimizu, T. Natsume, Y. Ikada. *ASAIO Trans.* **37** (1991) M317–319.

30. J.C. Banis Jr., K. Churukian, M. Kim, J.M. Gu, G.L. Anderson, S. Kaneko, T. Keelen, J.H. Barker. *Plast. Reconstr. Surg.* **98** (1996) 1046–1051.
31. J.R. Steadman, W.G. Rodkey, K.K. Briggs. *J. Knee Surg.* **15** (2002) 170–176.
32. R.P. Ficat, C. Ficat, P. Gedeon, J.B. Toussaint. *Clin. Orthop. Relat. Res.* **144** (1979) 74–83.
33. G. Bachmann, E. Basad, D. Lommel, J. Steinmeyer. *Radiologe* **44** (2004) 773–782.
34. M. Ronga, F.A. Grassi, P. Bulgheroni. *Arthroscopy* **20** (2004) 79–84.
35. M. Brittberg, A. Lindahl, A. Nilsson, C. Ohlsson, O. Isaksson, L. Peterson. *N. Engl. J. Med.* **331** (1994) 889–895.
36. M. Brittberg, L. Peterson, E. Sjogren-Jansson, T. Tallheden, A. Lindahl. *J. Bone Joint Surg. Am.* **85A** (Suppl 3) (2003) 109–115.
37. G. Knutsen, J.O. Drogset, L. Engebretsen, T. Grontvedt, V. Isaksen, T.C. Ludvigsen, S. Roberts, E. Solheim, T. Strand, O. Johansen. *J. Bone Joint Surg. Am.* **89** (2007) 2105–2112.
38. D.B. Saris, J. Vanlauwe, J. Victor, M. Haspl, M. Bohnsack, Y. Fortems, B. Vandekerckhove, K.F. Almqvist, T. Claes, F. Handelberg, K. Lagae, J. van der Bauwhede, H. Vandenuecker, K.G. Yang, M. Jelic, R. Verdonk, N. Veulemans, J. Bellemans, F.P. Luyten. *Am. J. Sports Med.* **36** (2008) 235–246.
39. S. Wakitani, T. Goto, S.J. Pineda, R.G. Young, J.M. Mansour, A.I. Caplan, V.M. Goldberg. *J. Bone Joint Surg. Am.* **76** (1994) 579–592.
40. J.P. Caffrey, A.M. Kushnaryov, M.S. Reuther, V.W. Wong, K.K. Briggs, K. Masuda, R.L. Sah, D. Watson. *Otolaryngol. Head Neck Surg.* **148** (2013) 576–581.
41. L.M. Ott, R.A. Weatherly, M.S. Detamore. *Ann. Biomed. Eng.* **39** (2011) 2091–2113.
42. S.D. Waldman, M.D. Gryn timer, R.M. Pilliar, R.A. Kandel. *J. Orthop. Res.* **21** (2003) 132–138.
43. G.P. Dowthwaite, J.C. Bishop, S.N. Redman, I.M. Khan, P. Rooney, D.J. Evans, L. Houghton, Z. Bayram, S. Boyer, B. Thomson, M.S. Wolfe, C.W. Archer. *J. Cell Sci.* **117** (2004) 889–897.
44. W.J. Li, R. Tuli, C. Okafor, A. Derfoul, K.G. Danielson, D.J. Hall, R.S. Tuan. *Biomaterials* **26** (2005) 599–609.
45. B. Johnstone, T.M. Hering, A.I. Caplan, V.M. Goldberg, J.U. Yoo. *Exp. Cell Res.* **238** (1998) 265–272.
46. M.F. Pittenger, A.M. Mackay, S.C. Beck, R.K. Jaiswal, R. Douglas, J.D. Mosca, M.A. Moorman, D.W. Simonetti, S. Craig, D.R. Marshak. *Science* **284** (1999) 143–147.
47. J.U. Yoo, T.S. Barthel, K. Nishimura, L. Solchaga, A.I. Caplan, V.M. Goldberg, B. Johnstone. *J. Bone Joint Surg. Am.* **80** (1998) 1745–1757.
48. D.E. Ashhurst, B.A. Ashton, M.E. Owen. *J. Orthop. Res.* **8** (1990) 741–749.
49. J. Goshima, V.M. Goldberg, A.I. Caplan. *Biomaterials* **12** (1991) 253–258.
50. C. De Bari, F. Dell'Accio, P. Tylzanowski, F.P. Luyten. *Arthritis Rheum.* **44** (2001) 1928–1942.
51. T. Tallheden, J.E. Dennis, D.P. Lennon, E. Sjogren-Jansson, A.I. Caplan, A. Lindahl. *J. Bone Joint Surg. Am.* **85A** (Suppl 2) (2003) 93–100.
52. S. Alsalamah, R. Amin, T. Gemba, M. Lotz. *Arthritis Rheum.* **50** (2004) 1522–1532.
53. R. Giardini-Rosa, P.P. Joazeiro, K. Thomas, K. Collavino, J. Weber, S.D. Waldman. *Tissue Eng. Part A* **20** (2014) 1012–1026.

54. S.H. Chia, M.R. Homicz, B.L. Schumacher, E.J. Thonar, K. Masuda, R.L. Sah, D. Watson. *J. Am. Coll. Surg.* **200** (2005) 691–704.
55. Y.J. Lin, C.N. Yen, Y.C. Hu, Y.C. Wu, C.J. Liao, I.M. Chu. *J. Biomed. Mater. Res. A* **88** (2009) 23–33.
56. A.T. Mehlhorn, H. Schmal, S. Kaiser, G. Lepski, G. Finkenzeller, G.B. Stark, N.P. Sudkamp. *Tissue Eng.* **12** (2006) 1393–1403.
57. J. Xu, W. Wang, M. Ludeman, K. Cheng, T. Hayami, J.C. Lotz, S. Kapila. *Tissue Eng. Part A* **14** (2008) 667–680.
58. E.J. Dobratz, S.W. Kim, A. Voglewede, S.S. Park. *Arch. Facial Plast. Surg.* **11** (2009) 40–47.
59. G.R. Ragetly, G.J. Slavik, B.T. Cunningham, D.J. Schaeffer, D.J. Griffon. *J. Biomed. Mater. Res. A* **93** (2010) 46–55.
60. D.J. Griffon, M.R. Sedighi, D.V. Schaeffer, J.A. Eurell, A.L. Johnson. *Acta Biomater.* **2** (2006) 313–320.
61. D.Z. Cai, C. Zeng, D.P. Quan, L.S. Bu, K. Wang, H.D. Lu, X.F. Li. *Chin. Med. J. (Engl.)* **120** (2007) 197–203.
62. K.E. Yates, F. Allemann, J. Glowacki. *Cell Tissue Bank.* **6** (2005) 45–54.
63. L. Zheng, H.S. Fan, J. Sun, X.N. Chen, G. Wang, L. Zhang, Y.J. Fan, X.D. Zhang, *J. Biomed. Mater. Res. A* **93** (2010) 783–792.
64. W. Dai, N. Kawazoe, X. Lin, J. Dong, G. Chen, *Biomaterials* **31** (2010) 2141–2152.
65. M.K. Kim, S.W. Choi, S.R. Kim, I.S. Oh, M.H. Won, *Knee Surg. Sports Traumatol. Arthrosc.* **18** (2010) 528–534.
66. A. Pavesio, G. Abatangelo, A. Borriore, D. Brocchetta, A.P. Hollander, E. Kon, F. Torasso, S. Zanasi, M. Marcacci, *Novartis Found. Symp.* **249** (2003) 203–217; discussion 229–233, 234–208, 239–241.
67. C. Chung, J.A. Burdick, *Tissue Eng. Part A* **15** (2009) 243–254.
68. D.A. Grande, C. Halberstadt, G. Naughton, R. Schwartz, R. Manji, *J. Biomed. Mater. Res.* **34** (1997) 211–220.
69. C.H. Baek, Y.J. Ko, *Laryngoscope* **116** (2006) 1829–1834.
70. S.W. Kang, O. Jeon, B.S. Kim, *Tissue Eng.* **11** (2005) 438–447.
71. K. Uematsu, K. Hattori, Y. Ishimoto, J. Yamauchi, T. Habata, Y. Takakura, H. Ohgushi, T. Fukuchi, M. Sato, *Biomaterials* **26** (2005) 4273–4279.
72. J. Zwingmann, A.T. Mehlhorn, N. Sudkamp, B. Stark, M. Dauner, H. Schmal, *Tissue Eng.* **13** (2007) 2335–2343.
73. H.J. Kim, J.H. Lee, G.I. Im, *J. Biomed. Mater. Res. A* **92** (2010) 659–666.
74. W.J. Li, R. Tuli, X. Huang, P. Laquerriere, R.S. Tuan, *Biomaterials* **26** (2005) 5158–5166.
75. C.N. Salinas, K.S. Anseth, *Biomaterials* **29** (2008) 2370–2377.
76. G.D. Nicodemus, I. Villanueva, S.J. Bryant, *J. Biomed. Mater. Res. A* **83** (2007) 323–331.
77. L. Zhu, Y. Wu, H. Jiang, W. Liu, Y. Cao, G. Zhou, *Br. J. Oral. Maxillofac. Surg.* **48** (2010) 462–465.
78. X.Z. Zhou, V.Y. Leung, Q.R. Dong, K.M. Cheung, D. Chan, W.W. Lu, *Int J Artif Organs* **31** (2008) 480–489.
79. M. Sha'ban, S.H. Kim, R.B. Idrus, G. Khang, *J Orthop Surg Res* **3** (2008) 17.
80. H.S. Yoo, E.A. Lee, J.J. Yoon, T.G. Park, *Biomaterials* **26** (2005) 1925–1933.
81. S.C. Wu, J.K. Chang, C.K. Wang, G.J. Wang, M.L. Ho, *Biomaterials* **31** (2010) 631–640.

82. A.A. Worster, B.D. Brower-Toland, L.A. Fortier, S.J. Bent, J. Williams, A.J. Nixon, *J Orthop. Res.* **19** (2001) 738–749.
83. A. Yamaguchi, *Semin Cell. Biol.* **6** (1995) 165–173.
84. A.C. Akman, R. Seda Tigli, M. Gumusderelioglu, R.M. Nohutcu, *Artif. Organs* **34** (2010) 65–74.
85. J.M. Brunger, N.P. Huynh, C.M. Guenther, P. Perez-Pinera, F.T. Moutos, J. Sanchez-Adams, C.A. Gersbach, F. Guilak, *Proc. Natl. Acad. Sci. U.S.A.* **111** (2014) E798–806.
86. G.A. Whitney, H. Mera, M. Weidenbecher, A. Awadallah, J.M. Mansour, J.E. Dennis, *Biores. Open Access* **1** (2012) 157–165.
87. J.M. Brenner, M. Kunz, M.Y. Tse, A. Winterborn, D.D. Bardana, S.C. Pang, S.D. Waldman, *Biotechnol. Prog.* **29** (2013) 213–221.
88. L.E. Freed, I. Martin, G. Vunjak-Novakovic, *Clin. Orthop. Relat. Res.* (1999) S46–58.
89. L.E. Freed, G. Vunjak-Novakovic, R. Langer, *J. Cell Biochem.* **51** (1993) 257–264.
90. B.Y. Bouchet, M. Colon, A. Polotsky, A.H. Shikani, D.S. Hungerford, C.G. Frondoza, *J. Biomed. Mater. Res.* **52** (2000) 716–724.
91. A.N. Brown, B.S. Kim, E. Alsberg, D.J. Mooney, *Tissue Eng.* **6** (2000) 297–305.
92. M. Stading, R. Langer, *Tissue Eng.* **5** (1999) 241–250.
93. G. Vunjak-Novakovic, I. Martin, B. Obradovic, S. Treppo, A.J. Grodzinsky, R. Langer, L.E. Freed, *J. Orthop. Res.* **17** (1999) 130–138.
94. R.H. Das, G.J. van Osch, M. Kreukniet, J. Oostra, H. Weinans, H. Jahr, *J. Orthop. Res.* **28** (2010) 537–545.
95. S. Saini, T.M. Wick, *Tissue Eng.* **10** (2004) 825–832.
96. M.S. Reuther, K.K. Briggs, M.K. Neuman, K. Masuda, R.L. Sah, D. Watson, *Otolaryngol. Head Neck Surg.* **148** (2013) 753–757.
97. B.D. Elder, K.A. Athanasiou, *Osteoarthritis Cartilage* **17** (2009) 114–123.
98. S.E. Francioli, I. Martin, C.P. Sie, R. Hagg, R. Tommasini, C. Candrian, M. Heberer, A. Barbero, *Tissue Eng.* **13** (2007) 1227–1234.
99. R.G. Rosa, P.P. Joazeiro, J. Bianco, M. Kunz, J.F. Weber, S.D. Waldman, *PLoS One* **9** (2014) e105170.
100. P. Wang, F. Zhu, N.H. Lee, K. Konstantopoulos, *J. Biol. Chem.* **285** (2010) 24793–24804.
101. B. Pingguan-Murphy, I. Nawi, *Clinics (Sao Paulo)* **67** (2012) 939–944.
102. C.J. Hunter, J.K. Mouw, M.E. Levenston, *Osteoarthritis Cartilage* **12** (2004) 117–130.
103. R.M. Schulz, A. Bader, *Eur. Biophys. J.* **36** (2007) 539–568.
104. A.S. Curtis, N. Gadegaard, M.J. Dalby, M.O. Riehle, C.D. Wilkinson, G. Aitchison, *IEEE Trans Nanobioscience* **3** (2004) 61–65.
105. M.J. Dalby, M.O. Riehle, D.S. Sutherland, H. Agheli, A.S. Curtis, *Eur. J. Cell. Biol.* **83** (2004) 159–169.
106. B.K. Teo, S.T. Wong, C.K. Lim, T.Y. Kung, C.H. Yap, Y. Ramagopal, L.H. Romer, E.K. Yim, *ACS Nano* **7** (2013) 4785–4798.
107. G. Balasundaram, D.M. Storey, T.J. Webster, *Int. J. Nanomedicine* **9** (2014) 1845–1853.
108. G.E. Park, M.A. Pattison, K. Park, T.J. Webster, *Biomaterials* **26** (2005) 3075–3082.
109. M.E. Casper, J.S. Fitzsimmons, J.J. Stone, A.O. Meza, Y. Huang, T.J. Ruesink, S.W. O'Driscoll, G.G. Reinholz, *Osteoarthritis Cartilage* **18** (2010) 981–991.

110. K.G. Turner, N. Ahmed, J.P. Santerre, R.A. Kandel. *Spine J.* **14** (2014) 424–434.
111. L. He, B. Liu, G. Xipeng, G. Xie, S. Liao, D. Quan, D. Cai, J. Lu, S. Ramakrishna. *Eur. Cell. Mater.* **18** (2009) 63–74.
112. J.P. Chen, C.H. Su. *Acta Biomater.* **7** (2011) 234–243.
113. C. Spadaccio, A. Rainer, M. Trombetta, G. Vadala, M. Chello, E. Covino, V. Denaro, Y. Toyoda, J.A. Genovese. *Ann. Biomed. Eng.* **37** (2009) 1376–1389.
114. Y. Pan, D. Xiong, F. Gao. *J. Mater. Sci. Mater. Med.* **19** (2008) 1963–1969.
115. S.H. Hsu, H.J. Yen, C.L. Tsai. *Artif. Organs* **31** (2007) 854–868.
116. S. Kay, A. Thapa, K.M. Haberstroh, T.J. Webster. *Tissue Eng.* **8** (2002) 753–761.
117. L.A. MacGinitie, Y.A. Gluzband, A.J. Grodzinsky. *J. Orthop. Res.* **12** (1994) 151–160.
118. W. Wang, Z. Wang, G. Zhang, C.C. Clark, C.T. Brighton. *Clin. Orthop. Relat. Res.* (2004) S163–173.
119. D. Khang, G.E. Park, T.J. Webster. *J. Biomed. Mater. Res. A* **86** (2008) 253–260.
120. R.A. MacDonald, B.F. Laurenzi, G. Viswanathan, P.M. Ajayan, J.P. Stegmann. *J. Biomed. Mater. Res. A* **74** (2005) 489–496.
121. S.M. Lim, S.H. Oh, H.H. Lee, S.H. Yuk, G.I. Im, J.H. Lee. *J. Mater. Sci. Mater. Med.* **21** (2010) 2593–2600.
122. D.C. Surrao, J.W. Hayami, S.D. Waldman, B.G. Amsden. *Biomacromolecules* **11** (2010) 3624–3629.
123. S.D. Waldman, C.G. Spiteri, M.D. Gryn timer, R.M. Pilliar, R.A. Kandel. *J. Orthop. Res.* **21** (2003) 590–596.

Chapter

17

EXPLORING TREATMENTS FOR OCULAR SURFACE DISEASES

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17.1. INTRODUCTION

Tissue engineering is a rapidly evolving field which can provide options to treat a wide range of diseases. Using tissue engineering, researchers have recently transplanted whole tissues such as the trachea [1] and the vagina [2] into patients and parts of damaged tissues, for example skin [3] (which has actually been in use since the early 1980s), urethra [4], cornea [5], and pancreatic islets [6].

Tissue engineering is based on growing cells on or within substrates commonly known as scaffolds. Scaffolds can be decellularised tissue (for example de-epidermised dermis [7] or decellularised pancreas [8]) or fabricated from either natural [9] or synthetic polymers [10] in the form of films [11], hydrogels [12] or electrospun scaffolds [13]. However, consideration must be given as to whether the technique developed is for laboratory based research purposes or for clinical translation. Using decellularised tissue for human transplantation requires tissues to be sourced with full ethical consent of the donors or relatives, which are handled through a tissue bank in order to minimise the risk of disease transmission [14]. This requires the establishment of well-run tissue banks which is a time consuming process and despite very best banking practices it is not possible to completely eliminate all risks of viral transmission. Accordingly, to avoid any risk of disease transmission using human tissue and delays in its use, ready-to-use synthetic alternatives for scaffolds may be better option.

A large amount of work has been carried out to investigate how to deliver cells to tissue surfaces, particularly for the cornea. This chapter describes the diseases that can affect the ocular surface, the current clinical treatments and the possible future treatments including the use of nanotechnology in the treatment of corneal diseases.

17.2. BACKGROUND

The eye consists of several different tissues, each playing a key role in vision (Figure 1). Damage to any of the tissues can adversely affect sight ranging from blurred vision to total blindness.

Figure 1. Schematic diagram of the cornea

The cornea is the first tissue that light passes through when entering the eye [15], after which it undergoes refraction several times as it moves through the different tissues of the eye until it eventually falls on to the retina.

17.2.1. The cornea

The cornea is an avascular [16], transparent tissue [17] with a major role to play in vision.

Similar to skin, maintenance of the barrier function of the cornea is vital, without which the tissue is prone to infection and disease, causing blurred vision and sometimes blindness. The central cornea does not contain any blood vessels or lymphatic vessels [18] however, the limbal area situated at the periphery of the cornea is highly vascularised [19] supplying nutrients to the residing adult stem cells and highly proliferative epithelial cells. A continuous supply of tear fluid to the front of the eye keeps the cornea from drying out. The tears play a crucial role in washing away debris which can lead to infection but at the same time provide nutrients to cells.

The cornea is $\sim 500 \mu\text{m}$ thick [15] and consists of five different layers: the epithelium, Bowman's membrane, stroma, Descemet's membrane and endothelium (Figure 2).

Figure 2. Schematic of the cross-section of the cornea

The corneal epithelium is the outermost layer of the cornea and is $\sim 50\ \mu\text{m}$ thick. Below the epithelium is the Bowman's membrane, a $10\ \mu\text{m}$ thick layer composed of collagen fibrils below which is the stroma [20]. The stroma is composed of 78 % water, 16 % collagen fibres and quiescent keratocytes [21] and makes up 90 % of the cornea [22]. The collagen fibrils (mainly collagen I) in the stroma are arranged to form lamellae and there are several lamellae that run in parallel to the corneal surface [23]. Due to the arrangement of fibrils the cornea as a whole is transparent [24]. Descemet's membrane, the basement membrane of the endothelium [25] comprises of collagen fibres [26] while the endothelium is a single layer of endothelial cells that do not proliferate once mature, even if injury has taken place [27]. However, *in vitro*, these cells can be cultured, which offers the opportunity to transplant cells back into the eye to restore vision [28]. The corneal endothelium has a very important pump-leak mechanism which maintains hydration of the cornea [29]. Here, solutes and fluid are pumped from the stroma into the aqueous humor while at the same time solutes and fluids leaks into of the stroma from the aqueous humor. This mechanism is driven by osmosis and prevents stromal oedema [30], thus maintaining the transparency of the cornea.

Each of these layers is responsible for the transparency and maintenance of vision. Usually loss of transparency is indicative of disruption to one or more of the corneal layers.

17.2.1.1. The corneal epithelium

The corneal epithelium is comprised of about 5–7 layers of stratified squamous, non-keratinised cells [31] and plays an important role in allowing oxygen [32] entry into the eye but at the same time prevents bacterial entry [33] which may lead to infection. The corneal epithelium consists of three layers of cells: the basal columnar monolayer, 2–3 layers of wing cells and the uppermost two to three layers of superficial cells [34].

The stem cells of the corneal epithelium lie in small pockets / niches about 120 μm in length, popularly known as limbal stem cell niches which are present in structures known as the Palisades of Vogt [35]. The stem cells are reported to lie in the basal part of the niche, well protected from external factors, maintained by several intrinsic factors [36].

As stem cells divide, they produce one cell that remains a stem cell and a daughter cell that eventually differentiates into corneal epithelial cells [37]. The cells in the limbus (located at the periphery of the cornea) are stratified columnar, non-keratinised cells which are about 7–10 layers in thick [37]. As the daughter cells/transient amplifying cells divide they move upwards and inwards from the periphery of the cornea toward the central cornea region where they eventually terminally differentiate and are washed away with the blinking action of the eye. An equilibrium exists in the rate at which the limbal stem cells divide and the cells slough off at the central corneal region. The stem cells under normal functioning are considered to be quiescent even though there is some level of proliferation to maintain the homeostasis of the corneal epithelium [38]. However, upon the onset of injury, the stem cells switch to a highly proliferative state where they divide rapidly to heal corneal wounds, and switch back to the quiescent state once the wound has healed [36].

Apart from housing the stem cell niches, the limbus also serves as a barrier between the conjunctiva and the cornea, preventing the vascularised conjunctiva from moving on to the cornea and vascularising the region [39].

17.2.2. Limbal stem cell deficiency

During injury, the proliferation of the stem cells must be sufficient to prevent the conjunctival cells from encroaching into the corneal region. This may happen if the proliferation of the stem cells is slow or the stem cells are completely absent due to damage [36]. Damage to the limbus is known as limbal stem cell deficiency and may occur due to genetic diseases such as aniridia [40], thermal and chemical burns [41], extensive radiation, surgery, cryotherapy [42], inflammatory diseases such as Stevens-Johnson syndrome [43], as well as contact lens use [44]. Clinical symptoms include pain, blurred vision, tearing, inflammation and in extreme cases blindness [45]. Using impression cytology, limbal stem cell deficiency can be confirmed by identifying goblet cells [46] and cytokeratins characteristic of conjunctival cells [47].

Limbal stem cell deficiency can be divided into partial or total deficiency (Figure 3).

Figure 3. Schematic of an eye showing a normal limbus (red), partial limbal stem cell deficiency (red partly absent) and total limbal stem cell deficiency (the red is absent)

17.2.2.1. Partial limbal stem cell deficiency

When only a part of the limbus is absent, it is known as partial limbal stem cell deficiency. The disease may range from mild, where an abnormal epithelium is present on the corneal surface to severe, where vision is affected and a fibrovascular pannus is present.

The deficiency may affect vision, in which case intervention is necessary or may not affect vision, in which case minimal intervention or medication alone is necessary [45]. Surgical intervention usually involves mechanical debridement of the conjunctival cells that have moved on to the corneal region.

17.2.2.2. Total limbal stem deficiency

When the whole limbal region has been damaged or is absent, this is called total limbal stem cell deficiency. Management in this case would involve debridement of the conjunctival cells along with subsequent transplantation of a new source of limbal epithelium [5]. The source of the new epithelium could be from the patient's undamaged eye if the condition is unilateral damage; however, in the case of bilateral damage, cells are sourced from a donor [48]. Also being explored is the use of other autologous cell types which will be explained later in this chapter.

17.2.3. Clinical treatments of limbal stem cell deficiency

The first successful corneal transplantation was carried out by Zirm in 1905 [49]. Due to the immune privileged nature of the cornea, it is a tissue that can be transplanted without concerns of rejection and thus there is no need for immunosuppression. Attempts have been made to transplant corneas to treat limbal stem cell deficiency; however, they have been unsuccessful as the procedure only includes the central transparent cornea and not the limbal area [48].

Because of this, over the last few decades, researchers have explored the use of several different methods to deliver fresh epithelial tissue or cultured epithelial cells to damaged corneas after mechanical debridement of the conjunctival cells.

17.2.3.1. Conjunctival transplantation

This procedure was first attempted by Thoft in 1977 [50] where he took a few pieces of bulbar conjunctiva from the healthy eye to treat unilateral limbal stem cell deficiency caused by burns. The procedure was based on conjunctival transdifferentiation. It was reported that the conjunctival cells on the cornea underwent a slow transformation into corneal epithelium-like cells, a process known as conjunctival transdifferentiation [50]. Dua studied the concept of conjunctival transdifferentiation on animals and came to the conclusion that it was unlikely that conjunctival transdifferentiation was actually taking place and it was instead squamous metaplasia with the loss of goblet cells. It was also suggested that complete damage of the limbus of the animals had not been carried out in the experiments leading to a partial limbal stem cell deficiency model [51]. This suggests that there is a possibility that even though the diagnosis of the disease was total stem cell deficiency, it may actually have been partial with the corneal epithelial cells trying to cover the corneal region.

17.2.3.2. Keratoepithelioplasty

Keratoepithelioplasty was first carried out by Thoft in 1984 [52]. This procedure was mainly targeted towards patients with bilateral damage, as the tissue transplanted was from cadaveric donors. In this treatment, the whole globe of the cadaver was taken and the partial thickness cornea was excised. The cornea was sectioned into four segments and lenticules comprising the epithelium and stroma from the mid peripheral region were excised and sutured onto the patient's damaged eyes. The vision of three out of four patients improved after the procedure; however, the procedure for the fourth patient failed due to complications [52]. Interestingly, at this time knowledge of limbal stem cells was very incomplete; it is possible that during the keratoepithelioplasty procedure, part of the limbus was excised leading to its transplantation onto the cornea [53].

17.2.3.3. Conjunctival limbal transplantation

This procedure was an extension of the conjunctival transplantation including a part of the limbal region along with the bulbar conjunctiva. A report of the successful use of this technique on 21 patients was published by Kenyon and Tseng [54]. The tissue used in this procedure was autologous tissue.

17.2.3.4. Living related conjunctival limbal allograft

This procedure was similar to conjunctival limbal transplantation except for the fact that the tissue was sourced from a living relative of the patient. This would mean the procedure could be employed for the treatment of bilateral injuries. However, in such cases, tests would need to be carried out in order to determine the human leukocyte antigen (HLA) typing of relatives to avoid rejection of the transplanted tissue [55].

17.2.3.5. Keratolimbal allograft

Keratolimbal allografts involved excising the whole limbus as one tissue from cadaver donors. The ring graft contains a very small amount of sclera, the whole limbus and the peripheral cornea. Clinical outcomes for the use of keratolimbal allografts have shown a success rate of 82–100 % after 1–2 years follow-up but a decrease to 50 % after 3–5 years [56].

17.2.3.6. Ex vivo expansion of limbal epithelial cells

It has become clear over the last few decades that the transplantation of limbal epithelial cells (or limbal tissue explants) is essential to treat limbal stem cell deficiency. Especially in the case of unilateral damage, sacrificing other parts of the eye like the conjunctiva may not be necessary. Pellegrini *et al.* were the first to report the transplantation of a limbal epithelial sheet grown on growth arrested murine 3T3 cells onto the damaged a cornea of an eye. The group reported that this procedure was used on two patients and showed normal epithelia even after a 2 year follow up [57]. This study proved the concept that only the limbal cells were necessary to treat limbal stem cell deficiency.

In summary several treatments have been explored for the treatment of limbal stem cell deficiency using either both conjunctival cells and limbal cells or only limbal cells. Based on the knowledge we have about conjunctival cells being quite different from corneal cells, such as goblet cells, using limbal cells alone has proven to be the best option.

17.3. USING DIFFERENT CELL TYPES AND CARRIERS FOR LIMBAL STEM CELL TRANSPLANTATION

Since the report of Pellegrini *et al.* in 1997, extensive research has been carried out to improve the technique. The use of other cell types to replace limbal stem cells has been explored for transplants involving the eye. This would be either to avoid taking a biopsy from the patient's healthy eye (in the case of unilateral damage) which may cause some damage or because healthy limbal stem cells are not available (in the case of bilateral damage). Even though the concept of transplanting a sheet of limbal epithelial cells seemed promising, handling a sheet of epithelial cells appeared to be a challenge; for this reason, researchers started to explore different carriers to assist with the transfer of limbal epithelial cells.

17.3.1. Transplantation of different cell types

In order to overcome the necessity of using immunosuppressants when tissue is taken from a donor for bilateral damage, the option of using the patient's own cells but from a different source has been explored. Oral mucosal cells have been cultured on either a natural [58] or synthetic [59] substrate and transplanted onto the eye. These cells are similar to limbal epithelial cells, they are stratified squamous non-keratinised cells which express CK3, P63 and ABCG2, as in corneal / limbal epithelial cells but not CK12 [60]. Clinical studies have shown that even though the oral mucosal cells improve visual acuity, there have been reports of neovascularisation at the peripheral region of the cornea [59].

Blazejewska *et al.* explored the use of hair follicle stem cells as an alternative to the limbal epithelial cells by culturing the cells on Laminin 5 in corneal stromal conditioned medium [61]. There was an up-regulation of CK12 and Pax6 and a down-regulation of epidermal keratinocyte marker CK10, indicating that hair follicle stem cells differentiate into corneal like cells [62]. Using an animal model of limbal epithelial stem cell deficiency, hair follicle cells were cultured on a 3T3 feeder layer after which they were placed on a fibrin carrier and transplanted onto the eye. Using this technique, an 80 % success rate was achieved with the hair follicle cells differentiating into a corneal epithelial phenotype [61].

Similarly embryonic stem cells were cultured on collagen IV in limbal fibroblast-conditioned media the embryonic stem cells differentiated into corneal epithelial phenotype expressing p63 and CK3/12. CK10 was also expressed indicating that the cells also had a skin epithelial phenotype [63]. Hanson *et al.* demonstrated the transplantation of differentiated human embryonic cells onto corneal buttons; 6 days after transplantation Pax6 and CK3 were detected [64]. However, there have been no reports of using these techniques in the clinic as yet.

Mesenchymal stem cells have also been studied in rabbits by performing amniotic membrane transplantation followed by injection of mesenchymal stem cells. The study resulted in cells that were positive for CK3/12, but there was no clear difference in the expression of ABCG2 between the transplanted group and the control (limbal stem cell deficient eyes), which is usually positive for limbal epithelial cells [65].

Also, sheets of dental pulp stem cells have been transplanted on to a limbal stem cell deficiency animal model and the amniotic membrane placed over the sheet. The results for mild chemical burn animal models showed less neovascularisation and expression of CK3, ABCG2 and P63, while this was not seen in the severe chemical burn models [66].

The exploration of mesenchymal and dental pulp stem cells for limbal stem cell deficiency treatment has not progressed to clinical evaluation to date.

17.3.1.1. Limbal cell carriers

17.3.1.1.1. Amniotic membrane

The amniotic membrane is the innermost layer of the foetal membrane [67] formed during the course of pregnancy. It consists of an epithelial layer, a thick basement membrane and an avascular stroma which has the ability to promote healing and exclude inflammatory cells [68].

The amniotic membrane is commonly used to deliver limbal epithelial cells to damaged corneas. It is known to preserve the stemness of cells, helps in the growth and proliferation of cells and also lacks immunogenicity [69]. It has been shown that the amniotic epithelial cells do not express HLA-A, -B, or -DR but do express HLA-G [69]; therefore using denuded amniotic membrane is preferred for transplantation [70] as it lowers the chance of rejection. Studies have shown that limbal cells grow much better on amniotic membranes denuded of their epithelium than amniotic membranes with intact epithelium [71]. In addition, using cryopreserved tissue is preferred to fresh as any amniotic epithelial cells remaining on the amniotic membrane would probably be non-viable [72].

Before using the amniotic membrane for limbal stem cell deficiency, it needs to undergo a screening process in order to lower the risk of disease transmission [73]. The process in Western countries must take place by law, with blood from the donor being screened for human immunodeficiency virus (HIV), hepatitis B, and C, cytomegalovirus and syphilis at the time of donation and once more after 6 months giving enough time for the donor to develop antibodies for the disease if actually infected [72]. In developing countries such screening is not yet a legal requirement [67].

Once obtained from the donor, the amniotic membrane is washed with media to remove any blood clots after which it is spread epithelial side up on a 0.22 μ m nitrocellulose membrane. The membrane along with the amniotic

membrane, is placed in a container containing glycerol or dimethyl sulfoxide (DMSO) and media containing antibiotics; this is then stored at -80°C [74] until its use after 6 months. The amniotic membrane may also be cryopreserved, irradiated or lyophilised [75].

The amniotic membrane was first used for therapeutic purposes in 1910 by Davis for skin transplantation and for the eye by de Rotth in 1940 [76] as reported by Rahman *et al.* [67]. Studies have shown that the amniotic membrane can be used to treat partial limbal stem cell deficiency without the need for limbal cells [77]. The amniotic membrane possess both anti-inflammatory and anti-angiogenic properties [70], which assist in the treatment of limbal stem cell deficiency.

In the case of total limbal stem cell deficiency cells are cultured on the amniotic membrane and then transplanted to the damaged eye [77]. In some cases where the cornea is seriously damaged, corneal transplantation is carried out after limbal stem cell transplantation [78]. This method has now become the gold standard for treating total limbal stem cell deficiency [77].

Studies have shown that processing of the amniotic membrane can lead to inter-donor and intra-donor variation, as a result it has been suggested that a standard protocol [79] as well as its use [80] should be followed globally. Also, obtaining the amniotic membrane for transplantation purposes is highly dependent on donors and once obtained needs to go through a tissue bank which could take several months. Sangwan *et al.* reported the clinical outcome of limbal cell transplantation using the amniotic membrane on 200 patients [81]. After a one year period, the success rate was 76 %, dropping to 68 % after 4 years. It is not known why the technique fails after several years for some patients. It could be due to an inherent condition of the patient or to the percentage of limbal stem cells transplanted or to the limbal stem cell niches not providing a long-term secure “home” for the transplanted cells. Variation in the amniotic membrane itself could also be a factor. In practice there are many factors that could affect the long-term outcome and this remains an area for research for the future [82]. Also, despite the success in using the amniotic membrane as a cell carrier, researchers are still exploring alternatives.

17.3.1.1.2. Other natural polymers as a carrier

Using natural polymers as delivery systems has been popular due to their availability.

The potential of using recombinant highly cross linked collagen scaffolds to culture limbal epithelial cells has been explored. The study showed that the cells maintained their stem cell properties and also could differentiate into corneal epithelial cells upon stratification [83]. However, there have been no reports on clinical studies using this approach as yet. Levis *et al.* studied the use of plastic compressed collagen I from rat tail to deliver cells to the cornea and showed that the cells cultured on the compressed collagen resembled

those on native central cornea and could possibly be used as an alternative to the amniotic membrane. However there is no indication on whether, if taken to the clinic, a recombinant form of collagen will be used or not [84].

Rama *et al.* explored the use of autologous fibrin as a cell carrier for limbal stem cell transplantation (clinically known as Holoclar®). A clinical study using this method to transfer the cells showed that the technique was successful in 76.6 % of the patients after a 10 year period and all failures occurred within the first year after surgery [85]. Last year, Holoclar® was given the go ahead by the European Medicines Agency to be used in the clinic, becoming the first stem cell-based medicinal product approved in the Western world. The approval of this product is an assurance to other stem cell researchers that there is some chance that the novel techniques that are being developed for treatment could reach the clinic.

Culture of limbal epithelial cells on *Bombyx mori* silk fibroin films [86] and chitosan-gelatine membranes [87] have also been studied as alternatives to the amniotic membrane. The stem cell properties and the potential to differentiate into corneal epithelial cells were maintained in these studies with the films / membranes being easy to handle, similar to the amniotic membrane.

The anterior lens capsule has also been studied as a cell carrier system. Anterior lens capsules were removed from patients during cataract surgery and were processed to remove the crystalline epithelium. Limbal biopsies from the patients were cultured on the capsule (limbal biopsy and capsule from the same eye – autologous; limbal biopsy and capsule from two different eyes–allogenic) showed that limbal cells which migrated out from limbal explants could be cultured on the capsules under both autologous and allogeneic conditions with a cell viability of > 95 % [88].

17.3.1.1.3. Synthetic polymers as a carrier

The use of synthetic polymers has also been an option to develop cell carrier systems. Natural polymers need to be isolated from human / animal tissue and can be inherently variable; by using synthetic polymers this can be avoided.

A temperature-responsive polymer, poly(*N*-isopropylacrylamide) has been used clinically by Nishida's group. This polymer which changes its hydration with temperature has been used to culture limbal epithelial cells [89], as well as oral mucosal cells [59]. Cells were cultured on the temperature-sensitive polymer at 37 °C for 2 weeks after which the temperature was reduced to 30 °C. This caused the polymer to swell, releasing the sheet of cells without using any proteases, to be transferred onto the damaged eye [59]. These methodologies allow the initial production of sheets of cells but these delicate cell sheets must then be transferred to the cornea.

Another temperature-sensitive polymer has been explored as a carrier for limbal cells, Melbiol, a thermoresponsive polymer block [poly(*N*-isopropylacrylamide-*co*-*n*-butyl methacrylate) (poly(NIPAAm-*co*-BMA))] and

the hydrophilic polymer block [poly(ethylene glycol) (PEG)]. Similar to Nishida's work [59], cells were cultured on the Mebiol gel surface at 37 °C; after 3 weeks, the temperature was dropped to 4 °C releasing the sheet of cells [90]. Cells cultured on the gel showed expression of ABCG2, P63 as well as CK3 [91]. Animal studies using the gel were carried out showing a 58 % total and 17 % partial success rate [90].

Contact lenses have been used as a delivery system for limbal epithelial cells. Di Girolamo *et al.* explored the use of lotrafilcon A contact lenses to culture limbal epithelial cells [92]. Most recent clinical studies using the contact lens to deliver the cells to the cornea showed a 63 % success rate after a 2.5 year median follow-up period [93]. Deshpande *et al.* coated a commercially available contact lens with acrylic acid using plasma polymerisation. This surface supported the attached of limbal epithelial cells but over a period of time the surface degraded releasing the cells on the surface of an *ex vivo* rabbit cornea model [94]. Similar studies using plasma coated lenses have been explored by other groups [95] including animal studies on rabbits [96]. Here, a model of limbal stem cell deficiency was created on the rabbit eyes and the acrylic acid coated lenses along with the limbal epithelial cells were transferred onto the model. Twenty six days after transplantation a stratified epithelium had formed on the surface of the rabbit eye. After staining the cells on the contact lens with BrdU prior to transplantation, results showed that some cells in the basal and suprabasal layers of the stratified epithelium post transfer retained BrdU suggesting the maintenance of highly proliferating cells after such a procedure [96].

Another popular technique in the field of tissue engineering is the use of electrospun scaffolds as these can resemble the structure of the extracellular matrix [97]. Electrospinning is based on electrostatic forces, where a highly charged polymer jet falls towards an oppositely charged or earthed surface (the collector). As the polymer jet falls onto the collector, the solvent evaporates, leaving fibres of the polymer to make up the scaffold. In order to achieve reproducibility, the technique must be carried out using constant parameters in particular, voltage, temperature, humidity, flow rate of polymer jet, distance from needle to the collector and concentration. These parameters can control fibre diameter, whole scaffold thickness, orientation of the fibres and pore size all which affect the degradation rate, if a biodegradable polymer is spun. Electrospun scaffolds can be spun as random [98], aligned [99] or even diagonal mats of fibres [100].

The choice of the polymer and solvent also is highly dependent on the type of scaffold required; for example, dissolving 10 % poly(ϵ -caprolactone) in glacial acetic acid, 90 % acetic acid, methylene chloride (MC) / Dichloroformamide (DMF) (4 / 1), glacial formic acid, and formic acid / acetone (4 / 1) at a constant voltage, distance to collector and flow rate, resulted in nanofibres with spindle like beads when dissolved in both glacial acetic acid and 90 % acetic acid. In MC / DMF, a mixture of micro and nanofibres was observed with

no beads, in glacial formic acid, brittle nanofibres were observed again with no beads, and in formic acid and acetone, branched nanofibres were noted along with many beads [101].

Electrospun scaffolds are being used for ophthalmology [102], skin [103], spinal cord [104] and bone [105]. With respect to limbal stem cell deficiency, electrospun scaffolds are being explored as an alternative to the amniotic membrane. A study by Deshpande *et al.* has used poly(lactide-co-glycolide) (PLGA) electrospun scaffolds (Figure 4) to culture limbal epithelial cells along with an *ex vivo* rabbit cornea model. The study showed the transfer of cells from the biodegradable electrospun scaffold to the rabbit after 4 weeks in culture. The cells cultured on the scaffold and those that transferred from the scaffold onto the cornea model expressed CK3 and P63. Additionally, in this study, outgrowth of limbal epithelial cells from corneal limbal explants was demonstrated, showing that limbal epithelial cells grew out from the explants and also transferred successfully to an *ex vivo* rabbit cornea model. These *in vitro* studies also showed that these scaffolds degraded within weeks and could be terminally sterilized using gamma irradiation and stored for long periods if kept dry and cold [98].

Sharma *et al.* have also used poly(ϵ -caprolactone) electrospun scaffolds to support limbal epithelial cell growth. The cells on the scaffold expressed CK3/12, Integrin β 1 and ABCG2 [106]. On plasma coating the scaffold with helium-oxygen, studies showed that there was an improvement in the cell attachment and proliferation [107]; however, no transfer studies have yet been reported with this membrane.

Figure 4. (A) Photograph and (B) scanning electron microscope (SEM) of the PLGA electrospun scaffold used to transfer cells onto an *ex vivo* rabbit cornea model

Ortega *et al.* [108] went one step further and introduced micro-pockets within PLGA electrospun scaffolds similar to those used by Deshpande *et al.* [98].

Using a combination of microstereolithography and electrospinning to develop a template, an electrospun scaffold was fabricated with small pockets at the periphery (Figure 5) that could potentially house stem cells. The long-term failure of limbal stem cell transplantation could be due to the fact that the stem cells that are transplanted back onto the eye are not well protected. The technique described by Ortega *et al.* opens up the possibility of keeping the limbal tissue explants well protected from the external environment within the micro-pockets, as the pockets act as artificial stem cell niches [109].

Figure 5. (A) Photograph and (B) SEM of PLGA electrospun scaffold with micropockets (indicated by arrows)

The cell carrier used to transplant cells is of utmost importance as it needs to support the initial cell growth as well as release cells onto the cornea in a controlled manner. As explained, several different techniques have been explored for more than a decade and there appear to be several that work clinically. It is not yet clear whether any will prove better than the others in terms of consistency of delivery of cells or long-term outcomes. With the approval of the Holoclar® system by the European Medicines Agency, it is just a matter of time until the outcome of the technique is revealed on a larger number of patients and the way is paved for other techniques to be introduced to the market.

An overview of the cells and substrates used to treat limbal stem cell deficiency is summarized in Figure 6.

Figure 6. Schematic of the different cell sources and different substrates used to treat limbal stem cell deficiency

17.4. NANOMATERIALS FOR OCULAR REPAIR

Nanotechnology is the engineering of material at a submicron scale [110] and a quite common use of nanotechnology is drug delivery, where drugs are incorporated into nanoparticles [111]. Nanotechnology is also broadly used in tissue engineering applications for cell delivery, for example, by using nanofibrous scaffolds for cell delivery purposes.

Studies have shown that neural stem cell differentiation was higher on nanofibrous electrospun scaffolds compared to microfibrous scaffolds [112]. Some groups have fabricated the nanofibrous scaffolds so that cell penetration is not possible due to the presence of nanopores preventing the cells from moving into the scaffold. This encourages cross talk between the cells on the nanofibrous scaffold surface and any other cells that are present in the culture system but not in direct contact.

Over the last few years, researchers have started to use nanotechnology to treat ocular diseases in the form of nanoparticles [113] and nanofibrous

scaffolds [106]. Nanoparticles have mainly been used for drug delivery such as in the case of PLGA loaded with pirfenidone introduced to an alkali burn rat model. In this study, loading the drug into nanoparticles significantly decreased collagen production, haze and increased corneal re-epithelisation [114]. Also the use of silicate nanoparticles to provide an anti-angiogenic effect on corneal vascularisation has been explored [113]. Figure 7 gives an overview of the use of nanotechnology to treat diseases of the cornea.

Figure 7. Schematic showing the use of nanotechnology in the treatment of diseases of the cornea

With respect to delivering cells for treatment of limbal stem cell deficiency, some studies have employed the use of nanofibrous electrospun scaffolds as an alternative to the amniotic membrane under *in vitro* conditions [115]. This may be similar to culturing limbal cells on the amniotic membrane as the cells do not penetrate into the membrane and remain on the surface [116] unlike when microfibrous scaffolds are used [98]. Low *et al.* studied the use of thermally-oxidised, aminosilanised porous silicon membranes with nanopores as another alternative to the amniotic membrane. The study showed that the limbal epithelial cells cultured on these silicon membranes expressed both CK3/12 and P63 and using these nanoporous membrane increases the surface area and improves cellular contact with the physiological environment, while at the same time supporting cell attachment [117]. Another interesting study explored the use of a gel that contains self-assembled nanocapsules of chitosan to support the culture of keratocyte reprogrammed induced pluripotent stem cells (iPSCs). Using a limbal stem cell deficiency rat model, the iPSCs combined with the nanogel showed restoration of the corneal epithelium [118].

17.5. EXPANSION OF CELLS ON THE CARRIER

A problem using the above techniques which has not been given enough importance has been expanding the cells on the carriers before transplantation. Once a limbal biopsy is obtained from the patient's healthy eye (in the case of unilateral damage), the common method is to place the pieces of the limbal biopsy (~ 500 μm in size) on the amniotic membrane (for example) and allow the cells to migrate out onto the membrane over 7–10 days [119]. Cleanrooms are extremely expensive to set up and run and such facilities are usually only available in specialized hospitals in urban areas in developing countries [120]. In order to overcome the inconvenience and costs, Sangwan *et al.* developed the technique of simple limbal epithelial transplantation (SLET) [121]. Here, limbal explants from the healthy eye (in the case of unilateral damage) were finely dissected and fibrin glued onto the amniotic membrane in theatre and placed on the patient's eye in one operation. This obviates the need to expand the cells in the expensive-to-run cleanrooms [122]. The first clinical study of six patients showed a 100 % success rate 6 weeks after surgery which was maintained after 9 months [121]. Longer term studies are in progress (personal communication). A similar approach was proposed in the study of Deshpande *et al.* to use limbal tissue explants with a synthetic biodegradable electrospun PLGA membrane [98]. This technique needs to be evaluated clinically, but if successful, would then allow surgeons to take an “off-the-shelf” sterilised, prepacked membrane and combine it with limbal tissue explants in theatre, obviating the need for access to clean room facilities or to tissue banks to supply amniotic membrane. [123].

17.6. CONCLUSIONS

Where diseases of the cornea result in limbal stem cell deficiency there are now a small number of centres (estimated at 10 at the time of writing) worldwide with expertise in using cell therapy and a range of delivery membranes to treat limbal stem cell deficiency. The challenge is to make this technology, which is very successful at least in the short-term, available to more surgeons and hence more patients worldwide.

The exploration of nanotechnology in treating diseases of the cornea has recently started and has a promising future. Thus, drug delivery *via* nanotechnology in corneal diseases has reached the stage of animal experimentation but has not yet been trialled clinically.

REFERENCES

1. P. Macchiarini, P. Jungebluth, T. Go, M.A. Asnaghi, L.E. Rees, T.A. Cogan, A. Dodson, J. Martorell, S. Bellini, P.P. Parnigotto, S.C. Dickinson, A.P. Hollander, S. Mantero, M.T. Conconi, M.A. Birchall. *Lancet* **372** (2008) 2023–2030.
2. A.M. Raya-Rivera, D. Esquiliano, R. Fierro-Pastrana, E. Lopez-Bayghen, P. Valencia, R. Ordorica-Flores, S. Soker, J.J. Yoo, A. Atala. *Lancet* **384** (2014) 329–336.
3. M. Moustafa, C. Simpson, M. Glover, R.A. Dawson, S. Tesfaye, F.M. Creagh, D. Haddow, R. Short, S. Heller, S. MacNeil. *Diabet. Med.* **21** (2004) 786–789.
4. S. Bhargava, J.M. Patterson, R.D. Inman, S. MacNeil, C.R. Chapple. *Eur. Urol.* **53** (2008) 1263–1269.
5. H.S. Dua, A. Azuara-Blanco. *Br. J. Ophthalmol.* **83** (1999) 414–419.
6. E.A. Ryan, B.W. Paty, P.A. Senior, D. Bigam, E. Alfadhli, N.M. Kneteman, R.T.J. Lakey, A.M.J. Shapiro. *Diabetes* **54** (2005) 2060–2069.
7. P. Deshpande, D.R. Ralston, S. MacNeil. *Burns* **39** (2013) 1170–1177.
8. S.K. Goh, S. Bertera, P. Olsen, J.E. Candiello, W. Halfter, G. Uechi, M. Balasubramani, S. Johnson, B. Sicari, E. Kollar, S.F. Badylak, I. Banerjee. *Biomaterials* **34** (2013) 6760–6772.
9. D.G. Wallace, J. Rosenblatt. *Adv. Drug Deliv. Rev.* **55** (2003) 1631–1649.
10. E.S. Place, J.H. George, C.K. Williams, M.M. Stevens. *Chem. Soc. Rev.* **38** (2009) 1139–1151.
11. P. Gentile, V. Chiono, F. Boccafroschi, F. Baino, C. Vitale-Brovarone, E. Verne, N. Barbani, G. Ciardelli. *J. Biomater. Sci. Polym. Ed.* **21** (2010) 1207–1226.
12. S. Rimmer, C. Johnson, B. Zhao, J. Collier, L. Gilmore, S. Sabnis, P. Wyman, C. Sammon, N.J. Fullwood, S. Macneil. *Biomaterials* **28** (2007) 5319–5331.
13. K.A. Blackwood, R. McKean, I. Canton, C.O. Freeman, K.L. Franklin, D. Cole, I. Brook, P.M. Farthing, S. Rimmer, J.W. Haycock, A. Ryan, S. Macneil. *Biomaterials* **29** (2008) 3091–3104.
14. J. Morales Pedraza, M. Herson. *Cell Tissue Bank.* **13** (2012) 103–117.
15. J.J. Douth, A.J. Quantock, N.C. Joyce, K.M. Meek. *Biophys. J.* **102** (2012) 1258–1264.
16. B.K. Ambati, M. Nozaki, N. Singh, A. Takeda, P.D. Jani, T. Suthar, R.J. Albuquerque, E. Richter, E. Sakurai, M.T. Newcomb, M.E. Kleinman, R.B. Caldwell, Q. Lin, Y. Ogura, A. Orecchia, D.A. Samuelson, D.W. Agnew, J. St Leger, W.R. Green, P.J. Mahasreshti, D.T. Curiel, D. Kwan, H. Marsh, S. Ikeda, L.J. Leiper, J.M. Collinson, S. Bogdanovich, T.S. Khurana, M. Shibuya, M.E. Baldwin, N. Ferrara, H.P. Gerber, S. De Falco, J. Witta, J.Z. Baffi, B.J. Raisler, J. Ambati. *Nature* **443** (2006) 993–997.
17. D.C. Beebe. *Semin. Cell Dev. Biol.* **19** (2008) 125–133.
18. C. Cursiefen, U. Schlötzer-Schrehardt, M. Kuchle, L. Sorokin, S. Breiteneder-Geleff, K. Alitalo, D. Jackson. *Invest. Ophthalmol. Vis. Sci.* **43** (2002) 2127–2135.
19. J. Zhao, V. Mo, T. Nagasaki. *J. Histochem. Cytochem.* **57** (2009) 177–185.
20. N. Pinnamaneni, J.L. Funderburgh. *Stem cells* **30** (2012) 1059–1063.
21. L.A. Pruitt, A.M. Chakravartula, *Mechanics of Biomaterials: Fundamental Principles for Implant Design*, Cambridge University Press, 2011.
22. J.A. West-Mays, D.J. Dwivedi. *Int. J. Biochem. Cell Biol.* **38** (2006) 1625–1631.
23. A. Daxer, P. Fratzl. *Invest. Ophthalmol. Vis. Sci.* **38** (1997) 121–129.

24. D.M. Maurice. *J. Physiol.* **136** (1957) 263–286.
25. M.C. Kenney, P.D. Benya, M.E. Nimni, R.E. Smith. *Invest. Ophthalmol. Vis. Sci.* **18** (1979) 527–532.
26. S.G. Levy, J. Moss, H. Sawada, P.J.C. Dopping-Hepenstal, A.C.E. McCartney. *Curr. Eye Res.* **15** (1996) 45–52.
27. N.C. Joyce. Proliferative capacity of the corneal endothelium. *Prog. Retin. Eye Res.* **22** (2003) 359–389.
28. S.P. Patel, W.M. Bourne. *Invest. Ophthalmol. Vis. Sci.* **50** (2009) 2742–2746.
29. W.M. Bourne. *Trans. Am. Ophthalmol. Soc.* **96** (1998) 229–239.
30. M.R. O'Neal, K.A. Polse. *Invest. Ophthalmol. Vis. Sci.* **26** (1985) 849–856.
31. D.Z. Reinstein, T.J. Archer, M. Gobbe, R.H. Silverman, D.J. Coleman. *J. Refract. Surg.* **24** (2008) 571–581.
32. I. Fatt, M.T. Bieber. *Exp. Eye Res.* **7** (1968) 103–112.
33. S. Kinoshita, W. Adachi, C. Sotozono, K. Nishida, N. Yokoi, A.J. Quantock, K. Okubo. *Prog. Retin. Eye Res.* **20** (2001) 639–673.
34. N. Ehlers, S. Heegaard, J. Hjortdal, A. Ivarsen, K. Nielsen, J.U. Prause. *Acta Ophthalmol.* **88** (2010) 858–861.
35. W. Li, Y. Hayashida, Y.-T. Chen, S.C.G. Tseng. *Cell Res.* **17** (2007) 26–36.
36. M.A. Stepp, J.D. Zieske. *Ocul. Surf.* **3** (2005) 15–26.
37. H.S. Dua, J.S. Saini, A. Azuara-Blanco, P. Gupta. *Indian J. Ophthalmol.* **48** (2000) 83–92.
38. R.A. Thoft, J. Friend. *Invest. Ophthalmol. Vis. Sci.* **24** (1983) 1442–1443.
39. H.S. Dua, A. Azuara-Blanco. *Surv. Ophthalmol.* **44** (2000) 415–425.
40. K. Ramaesh, T. Ramaesh, G.N. Dutton, B. Dhillon. *Int. J. Biochem. Cell Biol.* **37** (2005) 547–557.
41. D. Meller, R.T. Pires, R.J. Mack, F. Figueiredo, A. Heiligenhaus, W.C. Park, P. Prabhasawat, T. John, S.D. McLeod, K.P. Steuhl, S.C. Tseng. *Ophthalmology* **107** (2000) 980–989.
42. S. Ahmad. *Stem Cells Transl. Med.* **1** (2012) 110–115.
43. J.A. Gomes, M.S. Santos, A.S. Ventura, W.B. Donato, M.C. Cunha, A.L. Hofling-Lima. *Arch. Ophthalmol.* **121** (2003) 1369–1374.
44. C.C. Chan, E.J. Holland. *Am. J. Ophthalmol.* **155** (2013) 544–549.
45. H.S. Dua, A. Joseph, V.A. Shanmuganathan, R.E. Jones. *Eye* **17** (2003) 877–885.
46. V. Puangsricharern, S.C. Tseng. *Ophthalmology* **102** (1995) 1476–1485.
47. I. Garcia, J. Etxebarria, A. Boto-de-Los-Bueis, D. Diaz-Valle, L. Rivas, I. Martinez-Soraa, N. Saenz, C. López, A. Del-Hierro-Zarzuelo, R. Méndez, J. Soria, N. González, T. Suárez, A. Acera. *Ophthalmology* **119** (2012) 923–929.
48. S. Burman, V. Sangwan. *Clin. Ophthalmol.* **2** (2008) 489–502.
49. W.J. Armitage, A.B. Tullo, D.F.P. Larkin. *Br. J. Ophthalmol.* **90** (2006) 1222–1223.
50. R.A. Thoft. *Arch. Ophthalmol.* **95** (1977) 1425–1427.
51. H. Dua. *Br. J. Ophthalmol.* **82** (1998) 1407–1411.
52. R.A. Thoft. *Am. J. Ophthalmol.* **97** (1984) 1–6.
53. E. Holland, G. Schwartz. *Ocular Surface Disease Medical and Surgical Management*, Springer, New York, USA, 2002, pp. 149–157.
54. K.R. Kenyon, S.C. Tseng. *Ophthalmology* **96** (1989) 709–722.
55. P. Bakhtiari, A. Djalilian. *Middle East Afr. J. Ophthalmol.* **17** (2010) 9–14.
56. E.M. Espana, M. Di Pascuale, M. Grueterich, A. Solomon, S.C.G. Tseng. *Eye* **18** (2004) 406–417.

57. G. Pellegrini, C.E. Traverso, A.T. Franzi, M. Zingirian, R. Cancedda, M. De Luca. *Lancet* **349** (1997) 990–993.
58. T. Nakamura, T. Inatomi, C. Sotozono, T. Amemiya, N. Kanamura, S. Kinoshita. *Br. J. Ophthalmol.* **88** (2004) 1280–1284.
59. K. Nishida, M. Yamato, Y. Hayashida, K. Watanabe, K. Yamamoto, E. Adachi, S. Nagai, A. Kikuchi, N. Maeda, H. Watanabe, T. Okano, Y. Tano. *N. Engl. J. Med.* **351** (2004) 1187–1196.
60. S. Krishnan, G.K. Iyer, S. Krishnakumar. *Indian J. Med. Res.* **131** (2010) 422–428.
61. EA Meyer-Blazejewska, MK Call, O Yamanaka, H Liu, U Schlötzer-Schrehardt, F.E. Kruse, W.W. Kao. *Stem cells* **29** (2011) 57–66.
62. E.A. Blazejewska, U. Schlötzer-Schrehardt, M. Zenkel, B. Bachmann, E. Chankiewicz, C. Jacobi, F.E. Kruse. *Stem Cells* **27** (2009) 642–652.
63. S. Ahmad, R. Stewart, S. Yung, S. Kolli, L. Armstrong, M. Stojkovic, F. Figueiredo, M. Lako. *Stem Cells* **25** (2007) 1145–1155.
64. C. Hanson, T. Hardarson, C. Ellerstrom, M. Nordberg, G. Caisander, M. Rao, J. Hyllner, U. Stenevi. *Acta Ophthalmol.* **91** (2013) 127–130.
65. H. Reinshagen, C. Auw-Haedrich, R.V. Sorg, D Boehringer, P. Eberwein, J. Schwartzkopff, R. Sundmacher, T. Reinhard. *Acta Ophthalmol.* **89** (2011) 741–748.
66. J.Á.P. Gomes, B. Gerales Monteiro, G.B. Melo, R.L. Smith, M. Cavenaghi Pereira da Silva, N.F. Lizier, A. Kerkis, H. Cerruti, I. Kerkis. *Invest. Ophthalmol. Vis. Sci.* **51** (2010) 1408–1414.
67. I. Rahman, D.G. Said, V.S. Maharajan, H.S. Dua. *Eye* **23** (2009) 1954–1961.
68. M. Dogru, M. Yildiz, M. Baykara, H. Ozcet, H. Erturk. *Eye* **17** (2003) 139–148.
69. M. Kubo, Y. Sonoda, R. Muramatsu, M. Usui. *Invest. Ophthalmol. Vis. Sci.* **42** (2001) 1539–1546.
70. Y. Hao, D.H. Ma, D.G. Hwang, W.S. Kim, F. Zhang. *Cornea* **19** (2000) 348–352.
71. N. Koizumi, N.J. Fullwood, G. Bairaktaris, T. Inatomi, S. Kinoshita, A.J. Quantock. *Invest. Ophthalmol. Vis. Sci.* **41** (2000) 2506–2513.
72. H. Niknejad, H. Peirovi, M. Jorjani, A. Ahmadiani, J. Ghanavi, A.M. Seifalian. *Eur. Cell Mater.* **15** (2008) 88–99.
73. P.J. Addis, C.J. Hunt, J.K.G. Dart. *Br. J. Ophthalmol.* **85** (2001) 905–907.
74. H.S. Dua. *Br. J. Ophthalmol.* **83** (1999) 748–752.
75. H.N. Madhavan, K. Priya, J. Malathi, P.R. Joseph. *Indian J. Ophthalmol.* **50** (2002) 227–231.
76. A. de Roth. *Arch. Ophthalmol.* **23** (1940) 522–525.
77. S.G. Tseng, P. Prabhasawat, K. Barton, T. Gray, D. Meller. *Arch. Ophthalmol.* **116** (1998) 431–441.
78. V.S. Sangwan, M. Fernandes, A.K. Bansal, G.K. Vemuganti, G.N. Rao. *Indian J. Ophthalmol.* **53** (2005) 31–35.
79. A. Hopkinson, R.S. McIntosh, P.J. Tighe, D.K. James, H.S. Dua. *Invest. Ophthalmol. Vis. Sci.* **47** (2006) 4316–4322.
80. V.S. Maharajan, V. Shanmuganathan, A. Currie, A. Hopkinson, A. Powell-Richards, H.S. Dua. *Clin. Exp. Ophthalmol.* **35** (2007) 140–147.
81. V.S. Sangwan, S. Basu, G.K. Vemuganti, K. Sejal, S.V. Subramaniam, S. Bandyopadhyay, S. Krishnaiah, S. Gaddipati, S. Tiwari, D. Balasubramanian. *Br. J. Ophthalmol.* **95** (2011) 1525–1529.

82. V.S. Sangwan, R. Jain, S. Basu, A.B. Bagadi, S. Sureka, I. Mariappan, S. Macneil. *Indian J. Ophthalmol.* **62** (2014) 29–40.
83. S. Dravida, S. Gaddipati, M. Griffith, K. Merrett, S. Lakshmi Madhira, V.S. Sangwan, G.K. Vemuganti. *J. Tissue Eng. Regen. Med.* **2** (2008) 263–271.
84. H.J. Levis, R.A. Brown, J.T. Daniels. *Biomaterials* **31** (2010) 7726–7737.
85. P. Rama, S. Matuska, G. Paganoni, A. Spinelli, M. De Luca, G. Pellegrini. *N. Engl. J. Med.* **363** (2010) 147–155.
86. J. Liu, B.D. Lawrence, A. Liu, I.R. Schwab, L.A. Oliveira, M.I. Rosenblatt. *Invest. Ophthalmol. Vis. Sci.* **53** (2012) 4130–4138.
87. A. de la Mata, T. Nieto-Miguel, M. Lopez-Paniagua, S. Galindo, M.R. Aguilar, L. Garcia-Fernandez, S. Gonzalo, B. Vázquez, J. San Roman, R.M. Corrales, M. Calonge. *J. Mater. Sci. Mater. Med.* **24** (2013) 2819–2829.
88. A. Galal, J.J. Perez-Santonja, J.L. Rodriguez-Prats, M. Abad, J. Alio. *Cornea* **26** (2007) 473–478.
89. K. Nishida, M. Yamato, Y. Hayashida, K. Watanabe, N. Maeda, H. Watanabe, K. Yamamoto, S. Nagai, A. Kikuchi, Y. Tano, T. Okano. *Transplantation* **77** (2004) 379–385.
90. G. Sitalakshmi, B. Sudha, H.N. Madhavan, S. Vinay, S. Krishnakumar, Y. Mori, H. Yoshioka, S. Abraham. *Tissue Eng. Part A* **15** (2009) 407–415.
91. B. Sudha, H.N. Madhavan, G. Sitalakshmi, J. Malathi, S. Krishnakumar, Y. Mori, H. Yoshioka, S. Abraham. *Indian J. Med. Res.* **124** (2006) 655–664.
92. N. Di Girolamo, J. Chui, D. Wakefield, M.T. Coroneo. *Br. J. Ophthalmol.* **91** (2007) 459–464.
93. S. Bobba, S. Chow, S.L. Watson, N. Di Girolamo. *Stem Cell Res. Ther.* **6** (2015).
94. P. Deshpande, M. Notara, N. Bullett, J.T. Daniels, D.B. Haddow, S. MacNeil. *Tissue Eng. Part A* **15** (2009) 2889–2902.
95. H. Zhang, K.D. Brown, S.P. Lowe, G.S. Liu, D. Steele, K. Abberton, D. Mark. *Tissue Eng. Part A* **20** (2014) 1593–1602.
96. K.D. Brown, S. Low, I. Mariappan, K.M. Abberton, R. Short, H. Zhang, S. Maddileti, V. Sangwan, D. Steele, M. Daniell. *Tissue Eng. Part A* **20** (2014) 646–655.
97. Y.Z. Zhang, B. Su, J. Venugopal, S. Ramakrishna, C.T. Lim. *Int. J. Nanomed.* **2** (2007) 623–638.
98. P. Deshpande, C. Ramachandran, F. Sefat, I. Mariappan, C. Johnson, R. McKean, M. Hannah, V.S. Sangwan, F. Claeysens, A.J. Ryan, S. MacNeil. *Biomaterials* **34** (2013) 5088–5106.
99. R.M. Delaine-Smith, N.H. Green, S.J. Matcher, S. MacNeil, G.C. Reilly. *PLoS ONE* **9** (2014) e89761.
100. W.E. Teo, M. Kotaki, X.M. Mo, S. Ramakrishna. *Nanotechnology* **16** (2005) 918.
101. A.G. Kanani, S.H. Bahrami. *J. Nanomat.* **2011** (2011) ID 724153, 10 pages.
102. S. Zhong, W.E. Teo, X. Zhu, R. Beuerman, S. Ramakrishna, L.Y. Yung. *Biomacromolecules* **6** (2005) 2998–3004.
103. K. Sharma, A. Bullock, D. Ralston, S. MacNeil. *Burns* **40** (2014) 957–965.
104. C. Liu, Y. Huang, M. Pang, Y. Yang, S. Li, L. Liu, T. Shu, W. Zhou, X. Wang, L. Rong, B. Liu. *PLoS One* **10** (2015) e0117709.
105. G. Tetteh, A.S. Khan, R.M. Delaine-Smith, G.C. Reilly, I.U. Rehman. *J. Mech. Behav. Biomed. Mater.* **39** (2014) 95–110.
106. S. Sharma, S. Mohanty, D. Gupta, M. Jassal, A.K. Agrawal, R. Tandon. *Mol. Vis.* **17** (2011) 2898–2910.

107. S. Sharma, D. Gupta, S. Mohanty, M. Jassal, A.K. Agrawal, R. Tandon. *Invest. Ophthalmol. Vis. Sci.* **55** (2014) 899–907.
108. I. Ortega, A.J. Ryan, P. Deshpande, S. MacNeil, F. Claeysens. *Acta Biomater.* **9** (2013) 5511–5520.
109. I. Ortega, P. Deshpande, A.A. Gill, S. MacNeil, F. Claeysens. *Biofabrication* **5** (2013) 025008.
110. O.C. Farokhzad, R. Langer. *ACS Nano* **3** (2009) 16–20.
111. F.Q. Hu, Y. Hong, H. Yuan. *Int. J. Pharm.* **273** (2004) 29–35.
112. F. Yang, R. Murugan, S. Wang, S. Ramakrishna. *Biomaterials* **26** (2005) 2603–2610.
113. M. Mohammadpour, M. Jabbarvand, E. Delrish, A. Khoshzaban. *J. Med. Hypotheses Ideas* **8** (2014) 14–20.
114. S. Chowdhury, R. Guha, R. Trivedi, U.B. Kompella, A. Konar, S. Hazra. *PLoS One* **8** (2013) e70528.
115. V. Holan, A. Zajicova, A. Lencova, K. Pokorna, E. Svobodova, M. Krulova. *Acta. Ophthalmol.* **87** (2009) 0.
116. J. Shimazaki, M. Aiba, E. Goto, N. Kato, S. Shimmura, K. Tsubota. *Ophthalmology* **109** (2002) 1285–1290.
117. S.P. Low, N.H. Voelcker, L.T. Canham, K.A. Williams. *Biomaterials* **30** (2009) 2873–2880.
118. Y. Chien, Y.W. Liao, D.M. Liu, H.L. Lin, S.J. Chen, H.L. Chen, C.H. Peng, C.M. Liang, C.Y. Mou, S.H. Chiou. *Biomaterials* **33** (2012) 8003–8016.
119. V.S. Sangwan, S. Burman, S. Tejwani, S.P. Mahesh, R. Murthy. *Indian J. Ophthalmol.* **55** (2007) 251–260.
120. G.N. Rao, R.C. Khanna, S.M. Athota, V. Rajshekar, P.K. Rani. *Indian J. Ophthalmol.* **60** (2012) 396–400.
121. V.S. Sangwan, S. Basu, S. MacNeil, D. Balasubramanian. *Br. J. Ophthalmol.* **96** (2012) 931–934.
122. C. Ramachandran, S. Basu, V.S. Sangwan, D. Balasubramanian. *Stem Cells Transl. Med.* **3** (2014) 1160–1168.
123. F. Sefat, R. McKean, P. Deshpande, C. Ramachandran, C.J. Hill, V.S. Sangwan, A.J. Ryane, S. MacNeila. *Procedia Eng.* **59** (2013) 101–116.

GREEN NANOMATERIALS FOR PSORIATIC LESIONS

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18.1. INTRODUCTION

Many diseases of the skin (*e.g.* psoriasis, dermatitis, eczema) are thought to be caused by infections, diet, and environmental factors, and in particular stress and anxiety. Psoriasis is a chronic inflammatory disease characterized by erythematous lesions with thick silvery scales on the surface, which are sometimes itchy [1]. Clinical medicine describes forms with fewer plaques, located on the elbows, knees or scalp; there are also generalized forms that affect large skin surfaces. Its prevalence in various populations is 1–3 %. The cause remains unknown but it has a strong genetic basis.

Mild forms receive a hygienic dietary regime and topical treatment. This type of treatment is long lasting, and must be adapted to the clinical form regarding the use of different ointments and creams. For the silver-white scaly forms, keratolytic or pickling substances are indicated and are included in ointments, such as salicylic acid, lactic acid (2–10 %) and urea (15–20 %). Also, topical treatment can include substances called tars which are obtained from petroleum distillation such as Ichthyol and Oleum Cadinum.

Other drugs are used that inhibit the proliferation of epidermal cells and regulate cell differentiation, including analogs of vitamin D3, *i.e.* calcipotriol and tacalcitol, found in creams and ointments. Topical corticosteroids provide a rapid and effective way to control localized psoriasis. For this reason, these compounds are frequently used by patients. However, ultra-high potency corticosteroids are not recommended for long-term therapy because there may be a rebound when they are discontinued and adverse effects such as atrophy and telangiectasia may occur. In general, topical treatment is indicated for patients with lesions that occupy less than 20 % of the body.

Systemic treatment is reserved for skin lesions that affect more than 20 % of the body surface and for severe forms of psoriasis (psoriatic erythroderma, psoriatic arthritis and pustular psoriasis). Biologicals are effective modern therapies that are targeted against psoriatic immune pathology and can induce long remission periods. In general, the treatment of psoriasis is prolonged and often combines topical and systemic therapy, based on the doctor's experience and patient compliance.

Although there are numerous therapies, psoriasis therapy has been and remains a challenge for researchers. In order to improve existing therapies or to discover new classes of drugs, there researchers have investigated the use of natural extracts and nanotechnology.

It is well-known that plants (roots, leaves and fruits) containing phenolic compounds have very good anti-inflammatory effects. Anthocyanins, the pigments that provide color to different plant tissues, are organic molecules that prevent the destructive action of free radicals in cells are thus very good

antioxidants. The majority of biologically active components from plant extracts are poorly absorbed, so their action is very slow [2]. Due to their biological activities, these organic compounds are of interest in the food industry and in medicine [3-5]. For use in medicine, to potentiate the therapeutic role of these molecules, they could be attached as ligands to gold or silver nanoparticles. Both gold and silver colloids are non-allergenic. Furthermore, neither gold nor silver induce side effects. These metals have been intensively used in medicine for the treatment of different diseases (arthritis, infections, *etc.*). Nowadays, silver / gold colloid solutions are promoted as dietary supplements against different diseases. It has been found that colloidal metals formed in living organisms are assimilated faster than natural herbal remedies.

To be maintained in colloidal form, noble metals have to be surrounded by ligands with the same electrical charge. These nanomaterials with attractive new properties are synthesized directly or modified with chemical functional groups [6-8]. They are tailored to function in a desired application. In medicine, these particles are often conjugated with antibodies or drugs which permit their use as vehicles for drug delivery, diagnosis imaging or contrast agents [9-12].

To be used in medicine, metallic nanoparticles have to be non-toxic; as such, many studies have been performed on nanomaterials from a toxicological point of view. Most studies using these nanomaterials have been performed on different cell types *in vitro*. Even so, new nanomaterials are prepared and presented in the literature on a frequent basis. It is very important to verify if the new material is toxic or not, especially prior to use in medicine [13].

The metallic nanoparticles are prepared by many methods: chemical, physical and biological (bacteria, fungus, *etc.*) [14-21]. Some researchers have found that high concentrations of metallic nanoparticles are toxic to the human body. Release of these particles into the environment can cause ecological problems, as well. So, their use must have some limitations [22-24].

This is why the green approach is a very important way to prepare nanomaterials based on metallic nanoparticles using natural extracts from different parts of plants. Such methods, called “eco-friendly synthesis”, allow us to obtain nanomaterials appropriate for use in medicine [25-42]. Studies on anthocyanins have been performed investigating their use as sensors for metallic ions [43], in surface-enhanced raman scattering (SERS) preparation [44], in clinical medicine [45-51], and as antioxidants [52,53], when bonded to different non-metallic nanoparticles [54]. Some studies have been performed using nanomaterials on porcine skin [55] or for applications such as clinical ultrasound gels and topical antimicrobial gels [56,57], for dermal fibroblast cells in humans [58], skin diseases [59] and in cosmetics [60]. To our knowledge, metallic nanoparticles bonded to anthocyanins as ligands have not

been prepared and used in medicine, but are used in the energy industry to obtain dye-sensitized solar cells [61,62].

The majority of papers that refer to the green synthesis of metallic nanoparticles use extracts from leaves or fruits at high temperatures, such that the anthocyanins are decomposed. In these cases, metallic nanoparticles are surrounded by organic molecules from plants other than anthocyanins.

Our team is involved in obtaining metallic nanoparticles conjugated with anthocyanins from fruit extracts from the *Adoxaceae* and *Cornaceae* families. Here, we present a part of our study and the use of an eco-friendly synthesis method which allowed us to obtain new nanomaterials based on gold / silver nanoparticles with anthocyanin molecules from fruit extracts of *Viburnum opulus L.* (VO), which contains the anthocyanin kuromanin chloride (K) as the ligand [63]. Comparatively, we have prepared, using the same method, nanomaterials with pure anthocyanin kuromanin chloride.

The prepared nanomaterials were characterized by ultraviolet-visible (UV-Vis) and Fourier transform infrared (FTIR) spectroscopy, transmission electron microscopy (TEM), X-ray diffraction (XRD), energy dispersive X-ray spectroscopy (EDX), thermogravimetric analysis (TGA) and differential scanning calorimetry (DSC) analysis. After the nanomaterials were analyzed from a toxicological point of view *in vitro* and *in vivo* [63-68], they were added to a cream base and used in medicine to treat psoriatic lesions. The results were statistically assessed.

18.2. EXPERIMENTAL

18.2.1. Materials, apparatus and methods

Chemicals (tetrachloroauric acid, silver nitrate, sodium hydroxide) used for the preparation of nanomaterials were of analytical grade, purchased from Merck, Germany. The pure anthocyanin (kuromanin chloride) was obtained from Sigma-Aldrich. Double distilled water was used for the preparation of solutions.

A JEOL-JEM 1010 (JEOL Inc.) instrument was used to assess the morphology and size distribution of the nanoparticles. The absorption spectra of synthesized nanoparticles were measured with a Shimadzu UV-Vis spectrometer (with a wavelength range between 300–850 nm at room temperature). The characterization of the molecular structures that formed between organic molecules and metallic nanoparticles was performed with a JASCO 6100 spectrometer (spectral domain 5000–500 cm^{-1} ; the resolution was 4 cm^{-1} with the sample as KBr pellets). The crystalline nature of nanoparticles was determined with a D8 Advance Diffractometer with $\text{CuK}\alpha_1$ radiation ($\lambda = 15.4056 \text{ \AA}$) and a Ge (111) monochromator. The elemental analysis (EDX)

was performed with an Oxford Instruments with INCA Energy 300 software. TGA was performed using a SDT Q600 analyzer. The DSC experiments were carried out with a Shimadzu DSC-60 differential scanning calorimeter using Shimadzu TA-WS60 and TA60 2.1 version system software for data acquisition and analysis.

A non-invasive, high frequency ultrasound device Dermascan C 20 MHz (Cortex Technology, Denmark) has been used for the assessment of psoriasis lesions.

18.2.2. Plant materials

The extracts from VO fruits (*Adoxaceae* family) (Figure 1) were obtained according to Moldovan *et al.* [69]. The total anthocyanin content from the crude extract was measured using a pH differential method [70,71]. For the reaction with metallic ions, the concentration of the anthocyanin solution was diluted to 25×10^{-3} mM.

Figure 1. Picture of *Viburnum opulus* fruits

Some important factors in obtaining nanoparticles were closely followed [63,72]. The influence of the VO solution pH on the morphology of gold nanoparticles was studied. Anthocyanins in acidic solutions are found in cationic form (flavylium ions) but at neutral and basic solutions are found in the molecular form and anionic form, respectively (Figure 2).

Figure 2. Different pH structures of the major anthocyanin from VO, K

18.2.3. Optimization method

Experiments were performed in acidic, neutral and basic VO solutions. Depending on the pH used, the final solutions had different shades of red or purple for gold and yellow or yellow-brown for silver. In Figure 3, TEM images of the nanomaterials at different pH levels are shown. At acidic pH, the nanoparticles had diameters larger than 100 nm, peaking at 200 nm. At basic pH, smaller round and triangular shaped nanoparticles were obtained. At pH 7.5, the nanoparticle size was spread over a range of 12–90 nm and particles had round shapes. Although a high concentration of nanoparticles with a round shape was obtained at basic pH, at this pH, anthocyanin is present in chalcone form. Figure 4 shows the UV-Vis spectra of the nanomaterials.

In order to obtain the best results in the reduction reaction, the ratio between the reactants, the temperature of the solution and the concentration of the solution were investigated.

When using pure anthocyanin, the concentration of the necessary solution for the reduction of gold ions was three times higher than when using extracts. It is likely that a greater concentration of pure anthocyanin for reduction is necessary in this case due to fact that there are other reductants in the extract (*e.g.* sugars).

18.2.4. Synthesis of AuNPs / AgNPs-VO and AuNPs / AgNPs-K

The same method was used to prepare the nanomaterials with VO and K in view of the possibility of comparing the new products.

The procedure of Crisan *et al.* [63] was applied. The pH of VO and K solutions was adjusted to 7.5 with 1 M NaOH. The concentration of the metallic ion solutions (HAuCl_4 , AgNO_3) was 1 %. In 200 mL of boiling distilled water, 6.6 mL of the silver ion solution and 16.6 mL of the VO / K solution was added rapidly. In the case of gold ions, 6.6 mL of the metallic ion solution was added to 16.6 mL of VO solution or 50 mL of K solution. The solution was continuously stirred for 10 min. Gold ion reduction took place after a few minutes in all cases; a red-purple color was obtained for VO and a red-mauve color for K. The colors of the silver nanoparticle solution were yellow for VO and yellow-brown for K. The colloids were stable for three weeks at 4 °C. The nanomaterials were centrifuged twice at 15 000 rpm and 4 °C for 10 min, then redissolved in double distilled water. For DSC, XRD and FTIR analyses, colloids were dried at room temperature on watch glasses.

18.2.4.1. Calculation of metallic nanoparticle concentrations

Based on the experimental results and according to the solution quantities and their concentrations used, the molar concentrations of metallic nanoparticle solutions were found to be 0.114 nM for AuNPs-VO, 1.34 nM for AgNPs-K, 0.033 nM for AuNPs-K and 4.71 nM for AgNPs-K (considering the mean particle diameter in the case of VO of 63 nm for gold and 35 nm for silver and in the case of K 95 nm for gold and 23 nm for silver). We found that the nanoparticles had a round form and similar dimensions.

18.3. RESULTS AND DISCUSSION

18.3.1. TEM analysis

TEM is one of the most efficient and versatile methods for the characterization of nanomaterials. TEM analysis provides information about the size and size distribution, morphology and dispersion of nanoparticles [73,74].

Figure 5. Representative TEM images of (a) AuNPs-K and (b) AgNPs-K and the associated particle size histograms

Figure 5 presents TEM images and the associated particle size histograms of AuNPs-K (a) and AgNPs-K (b). Examination of the TEM images of AuNPs-K (Figure 5a) revealed the formation of agglomerated nanoparticles (clusters) having a predominantly spherical shape. The size distribution of AuNPs-K was obtained by counting more than 100 nanoparticles; the mean diameter was found to be 95 nm. The morphology of AgNPs-K (Figure 5b) highlights the formation of well-dispersed spherical nanoparticles. The particle size data

were obtained from the associated histogram; the average diameter was found to be 23 nm.

The nanomaterials based on gold / silver nanoparticles and natural extracts from VO were also characterized by TEM (Figure 6).

Figure 6. Representative TEM images of (a) AuNPs-VO and (b) AgNPs-VO and the associated particle size histograms

One can see the differences between the nanomaterials based on natural extracts (AuNPs-VO and AgNPs-VO) and nanomaterials based on pure anthocyanin (AuNPs-K and AgNPs-K) in terms of both size and shape. This can be explained considering the fact that the natural extract contains, besides anthocyanin, other compounds that have a reducing effect.

The TEM images of AuNPs-VO and AgNPs-VO (Figure 6) reveal the formation of well-dispersed nanoparticles with various shapes (spherical, tetrahedral and triangular). The nanoparticle diameters were obtained from the associated

histograms and were found to be in the range of 30–90 nm for AuNPs-VO (Figure 6a) and 20–70 nm for AgNPs-VO (Figure 6b). The mean diameter obtained for AuNPs-VO was 63 nm, which is smaller than that obtained for AuNPs-K. In the case of AgNPs-VO, the average diameter was 35 and was larger than that obtained for AgNPs-K.

18.3.2. UV-Vis spectroscopy

UV-Vis spectroscopy is a valuable tool for nanomaterial characterization and involves the absorption of near-UV or visible light. The optical properties of noble metal nanoparticles have been intensively studied. Metallic nanoparticles, in particular gold and silver nanoparticles, exhibit a strong absorption band in the visible region of the absorption spectra. This absorption band is a result of the coherent oscillation of conduction band free electrons induced by the interaction with the electromagnetic field of the incident light; this is known as surface plasmon resonance (SPR). This band is characteristic for metallic nanoparticles and is absent in the bulk metal absorption spectra. The position, intensity and shape of the SPR band depend on the size and shape of the particle, interparticle interactions, dielectric properties and the surrounding media. By varying the nanoparticle size and shape, the maximum absorbance of SPR can be tuned anywhere between 520–1000 nm [75-77].

Figure 7 presents the UV-Vis spectra of nanomaterials based on gold / silver and pure anthocyanin. A broad absorption peak was observed in the absorption spectra of AuNPs-K (Figure 7a) at 560 nm, while for AgNPs-K (Figure 7b) the SPR band appeared at 410 nm.

Figure 7. UV-Vis spectra of (a) AuNPs-K and (b) AgNPs-K (dilution 1 : 2)

For nanomaterials based on natural extracts and gold / silver nanoparticles, the SPR band appeared at 545 nm for AuNPs-VO (Figure 8a) and 430 for AgNPs-VO (Figure 8b). One can see that, in the case of AuNPs-VO, the SPR band was narrower and shifted to lower wavelengths, indicating a decrease in nanoparticle size, while for AgNPs-VO, the SPR band underwent a small shift to higher wavelengths, indicating an increase in particle size.

Figure 8. UV-Vis spectra of (a) AuNPs-VO and (b) AgNPs-VO (dilution 1 : 2)

The results obtained from UV-Vis measurements were in concordance with the TEM images and confirmed the morphology of the nanomaterials. These results are presented comparatively in Table 1.

Table1. Comparison data between UV-Vis and TEM measurements

	SPR band position [nm]	Nanoparticle mean diameter [nm]	Absorbance [a.u.]
AuNPs-K	560	95	0.12
AuNPs-VO	545	63	0.70
AgNPs-K	410	23	0.85
AgNPs-VO	430	35	0.80

18.3.3. XRD analysis

The experimental XRD patterns of the nanomaterials based on gold / silver nanoparticles and pure anthocyanin (AuNPs-K and AgNPs-K) are presented in Figure 9. In the case of AuNPs-K, characteristic diffraction peaks were observed and assigned to the (111), (200), (220) and (311) planes of the face centered cubic (FCC) lattice of metallic gold. In the XRD pattern of AgNPs-K, only three diffraction peaks were observed, corresponding to the (111), (200) and (311) planes. The most intense peak was assigned to the (111) plane, suggesting that AgNPs-K have a predominantly crystalline structure.

Figure 9. XRD patterns of the synthesized nanomaterials: AuNPs-K and AgNPs-K

The powder diffraction spectra of the nanomaterials synthesized using natural extracts and gold / silver nanoparticles showed five peaks located at 2θ : 38.14°, 44.31°, 64.56°, 77.64° and 81.85° corresponding to the (111), (200), (220), (311) and (222) planes (Figure 10). The dominant peak corresponded to the (111) plane, suggesting that both AuNPs-VO and AgNPs-VO are crystalline in nature.

Figure 10. XRD patterns of synthesized nanomaterials: AuNPs-VO and AgNPs-VO

The size of the synthesized nanomaterials was calculated using the Debye-Scherrer Equation (1):

$$D = \frac{k\lambda}{\beta_s \cos \theta} \quad (1)$$

where D is the nanomaterial's diameter, k is the Scherrer constant, λ is the wavelength of radiation, β_s is the full peak width and θ is the diffraction angle [78,79]. The Scherrer constant is approximately equal to unity (the most cited value in the literature for k is 0.9) and is related to the crystalline shape [80,81]. Therefore, the following diameters were estimated for the crystallites: $D_{\text{AuNPs-K}} = 30.2$ nm, $D_{\text{AgNPs-K}} = 7.7$ nm, $D_{\text{AuNPs-VO}} = 25$ nm and $D_{\text{AgNPs-VO}} = 24$ nm. Due to the fact that one nanoparticle may contain multiple crystallites, the diameters obtained by XRD are smaller than those obtained by TEM analysis.

18.3.4. FTIR spectroscopy

Vibrational spectroscopy is a useful technique for nanomaterial characterization [82,83]. Both FTIR and Raman spectra can provide information on nanomaterial composition, but some disadvantages are encountered. For FTIR experiments, the complexity of the spectrum is regarded as a problem when working with a complex mixture, while for the Raman studies, fluorescence interference poses important challenges. Nevertheless, FTIR and Raman spectroscopy can be considered for chemical nanoparticle surface characterization [84,85].

In the present chapter, we used FTIR spectroscopy for the identification of molecules linked to the surface of gold and silver nanoparticles. The FTIR spectra of kuromanin chloride (cyanidin-3-glucoside) and gold nanoparticles functionalized with kuromanin chloride (AuNPs-K) are shown in Figure 11. Second derivative spectra were also employed for the separation of overlapping signals.

Figure 11. Solid FTIR spectra of K and gold nanoparticles functionalized with K (AuNPs-K) in the region 1800–500 cm^{-1} ; second derivative spectra of K in the region 1470–1432 cm^{-1} and AuNPs-K in the region 1690–1590 cm^{-1}

In K, the peak centered at 1640 cm^{-1} could be related to the stretching vibrations of the aromatic C=C from anthocyanin ($\nu\text{C}=\text{C}$); the bending vibration of water (from the KBr pellet or the water from K) can also appear in this region [86,87]. In AuNPs-K, the band at 1640 cm^{-1} broadens and new vibrations appear at about 1705, 1658, 1647, 1636, 1626 and 1618 cm^{-1} , indicating the formation of C=O bonds and a change in ring in-plane vibrations from the benzopyrylium unit when anthocyanin is linked to the gold surface (Figure 11; AuNPs-K and AuNPs-K second derivative spectrum) [88]. The FTIR bands located at 1588, 1571, 1519, 1489, 1444 and 1414 cm^{-1} in K can be ascribed to aromatic ring vibrations [86,88]; these bands disappear in AuNPs-K, which is consistent with the changes discussed above. The band observed at 1457 cm^{-1} could be from the β -glucopyranose moiety (O–C–H and C–C–H bending vibrations) [89]. This band is clearly seen in AuNPs-K (Figure 11) and it is masked in K (Figure 11; K second derivative spectrum). Its presence in the AuNPs-K spectrum indicates the existence of a sugar moiety in anthocyanin linked to the gold nanoparticle surface.

The 1400–1200 cm^{-1} region in the anthocyanin spectrum was strongly affected by interaction with the gold surface. The band located at 1329 cm^{-1} may be attributed to inter-ring stretching vibrations and could be used as a marker of the change in aromaticity and π -electron delocalization within the rings [86,90]. The other vibrations appearing in this region are mainly due to in-plane O–H bending mode, $\delta\text{O–H}$ coupled with C–OH stretching vibrations from phenols, $\nu\text{C–O}$, and also to asymmetric C–O–C stretching vibrations from ethers, $\nu_{\text{as}}\text{C–O–C}$ [86–88]. These bands disappear in the spectrum of anthocyanin coated gold nanoparticles. However, in AuNPs-K, new vibrations

were seen in the 1411–1360 cm^{-1} domain; some of these vibrations could be associated with the frequencies from 1705–1610 cm^{-1} and could indicate a C=O / gold interaction. This situation is similar to that observed for metallic chelates of conjugated ketones: when the C=O group is weakened by resonance between C–O–M and C=O–M, the frequencies appear in the 1620–1610 cm^{-1} and 1390–1309 cm^{-1} ranges [91].

In the 1200–1000 cm^{-1} region, related to C–O stretching vibrations from pyranose coupled with C–C stretching and symmetric C–O stretching from ethers, some changes were observed. The difference between the two spectra was probably due to the transformation that occurred in the β -pyranose conformation after linking anthocyanin to gold nanoparticles and also to the disappearance of symmetric C–O–C stretching vibrations of aromatic ethers.

The band at 982 cm^{-1} , ascribed to symmetric ring vibrations of β -pyranose, also reveals the existence of a β -pyranose moiety on the surface of gold nanoparticles in the AuNPs-K spectrum [88].

Taken together, the comparison of the two FTIR spectra, kuromanin chloride and AuNPs-K, indicates that the anthocyanin was bound to the surface of the gold nanoparticles. Significant changes related to π -delocalization between the benzopyrylium and phenol moieties were observed after the interaction of anthocyanin with gold nanoparticles.

European cranberry bush (VO) berries contain chlorogenic acid, catechin, epicatechin, cyanidin-3-glucoside, cyanidin-3-rutinoside and different glucosides of quercetin [92]. According to Velioglu, between the two anthocyanin pigments, cyanidin-3-glucoside (kuromanin) is dominant in VO juice. In the FTIR spectrum of VO, the kuromanin seems to adopt the quinonoidal conformation under our experimental conditions, *i.e.* pH 7.5. Thus, the band located at 1732 cm^{-1} could be the result of C=O stretching, and the absence of this band (Figure 12) may be associated with the aromaticity of kuromanin chloride (see the above discussion and Figure 11).

Figure 12. Solid FTIR spectra of VO extract in the 1800–500 cm^{-1} region

In the 1200–1000 cm^{-1} region, the vibrations can be assigned to C–O stretching from pyranose coupled with C–C stretching and symmetric C–O stretching from ethers. The hydroxybenzoic acid, epicatechin and quercetin derivatives also possess strong vibrations in this spectral domain [93]. The band at 984 cm^{-1} , ascribed to symmetric ring vibrations of β -pyranose, reveals the existence of a β -pyranose moiety in the VO spectrum [88].

In Figure 13, the solid FTIR spectra of gold nanoparticles obtained using kuromanin chloride (AuNPs-K) or VO extract solution (AuNPs-VO) are presented. For better comparison, the difference spectrum between AuNPs-VO and AuNPs-K is also shown.

Figure 13. Solid FTIR spectra of gold nanoparticles obtained using kuromanin chloride (AuNPs-K) or VO extract (AuNPs-VO) and their difference spectrum in the 1800–500 cm^{-1} region

As shown in Figure 13, only few differences in the vibrational features of the two types of gold nanoparticles could be detected: an increase in the intensity of the peaks from 1398 and 1144 cm^{-1} and a reduction in the intensity of the frequencies located at 1385, 1161 and 1113 cm^{-1} when VO was used for gold nanoparticle synthesis.

The FTIR spectrum of silver nanoparticles obtained with kuromanin chloride (AgNPs-K) reveals almost the same bands as AuNPs-K; some differences seem to appear in the region 1711–1611 cm^{-1} and 1470–1450 cm^{-1} (Figures 11 and 14).

Figure 14. Solid FTIR spectra of silver nanoparticles functionalized with kuromanin chloride (AgNPs-K) in the 1800–500 cm^{-1} region and second derivative spectrum in the 1690–1600 cm^{-1} region

The presence of anthocyanin on the surface of silver nanoparticles could also be seen when the VO extract was used for the synthesis of silver nanoparticles (Figure 15). The difference spectrum between the two types of silver nanoparticles, $\text{diff}[(\text{AgNPs-VO})-(\text{AgNPs-K})]$, highlights a decrease in band intensities from 1631 and 1465 cm^{-1} and a significant increase in the intensity of the peak at 1385 cm^{-1} when VO was used as the reducing agent.

Figure 15. Solid FTIR spectra of silver nanoparticles obtained using kuromanin chloride (AgNPs-K) or VO extract (AgNPs-VO) and their difference spectrum in the 1800–500 cm^{-1} region

In conclusion, anthocyanin is the major ligand bound to the surface of gold or silver nanoparticles, regardless of the protocol (with kuromanin chloride or VO extract) used for the synthesis of functionalized metallic nanoparticles.

18.3.5. EDX analysis

The analysis confirmed the presence of Au^0/Ag^0 in the samples by the appearance of a large peak for metals. Weak peaks for oxygen and carbon also confirmed the binding of organic molecules to the surface of gold / silver nanoparticles, as can be seen in Figure 16. In each case, three samples were analyzed and the mean for AuNPs-VO was found: approximately 89.52 % gold, 4.76 % oxygen and 7.30 % carbon. In the case of AgNPs, the results were found to be: 80.76 % silver, 3.62 % oxygen and 5.39 % carbon.

Figure 16. EDX spectra and field-emission scanning electron micrograph (FESEM) of AuNPs / AgNPs-VO

Analyses indicated that the ligand involved in the formation of these nanomaterials contained only carbon and oxygen.

18.3.6. TGA / DSC analysis

It is known that the decomposition of anthocyanins is temperature sensitive. The decomposition of kuromanin chloride was assessed by Wang *et al.* [94], and they concluded that it takes place in three stages between 30–390 °C. In the first stage, glucose is cleaved from the anthocyanin to produce cyanidin. In the second stage, the amount of anthocyanin is decreased to 13 %. Between 300–390 °C, anthocyanin is degraded completely, and a large mass loss appears after heating to 390 °C. In the third stage, the mass loss of 37 % corresponds to the decomposition of sugar.

In our case, in the TGA profile (Figure 17), one can see the decomposition temperature of AuNPs / AgNPs-VO at higher values (800 °C). This indicates the stability of organic molecules when they are bound to the gold surface. In the case of AuNPs-VO, the decomposition of organic molecules from the surface of the metal also took place in three stages between 110–450 °C, 450–800 °C and 800–900 °C. For AgNPs-VO, decomposition occurred in two stages between 200–450 °C and 450–810 °C. The shift in the decomposition temperature towards higher values also occurred in the case of amino acid capped gold nanoparticles [95].

The mass percent of organic molecules bound to gold clusters was approximately 9.93 % (we assume that the first step weight loss of 0.6746 % represents the desorption of water). Thus, 89.40 % of the gold remained, which is comparable with the EDX results for AuNPs-VO, *i.e.* 89.52 %. In the case of AgNPs-VO, the percent desorption of water was 1.033 % and the mass percent of organic molecules bound to silver nanoparticles was approximately 23.7 %. The remaining mass percent of silver was 75.27 %, comparable with the results obtained by EDX, *i.e.* 80.76 %.

Figure 17. TGA profile for AuNPs-VO and AgNPs-VO

TGA and DSC analysis of nanomaterials based on metallic nanoparticles are very commonly studied with respect to sintering nanoparticle inks for printed electronics [96-100]. Yu *et al.* [99] presented TGA results and DSC profiles of AgNPs–organic molecules (in their case, the silver nanoparticles were obtained using sodium polyacrylate and ascorbic acid) with sizes of 20–30, 30–40 and 100–120 nm. The DSC analyses showed some exothermic peaks in the temperature range of 200–280 °C. They concluded that these peaks were due to the surface sintering reaction of AgNPs or the recrystallization of nanoparticles by heating. For AgNPs 100 nm in size, there was no exothermic peak in the DSC curve, likely due to the fact that surface sintering is more difficult for larger AgNPs. At low temperatures, smaller AgNPs readily coalesce.

The research of Smith and Hutchinson [100] indicated the dependency of the sintering process on the size of the nanoparticle and ligand shell. TGA revealed that the free ligand decomposed at lower temperatures in comparison with the ligand bound to the nanoparticle. They showed that degradation is dependent on the composition rather than the size of the molecule. When the core size of nanoparticles was different but they bore the same ligand, it was found that larger nanoparticles lose ligands and sinterize more readily than smaller nanoparticles. This behavior suggests a greater stability of the ligand shell on smaller nanoparticles. Based on TGA and DSC analyses, they deduced that sintering is triggered by a very small amount of ligand loss. The sintering process is initiated and then the ligand is rapidly removed from the metallic surface and forms a porous film. This study showed that it is important to take into consideration both the nanoparticle core size and the ligand identity in the process of sinterization.

A DSC thermogram of free anthocyanin was presented by Santos *et al.* [3]. The decomposition of free anthocyanin took place in the range of 150–200 °C.

In our case, thermal effects on nanomaterials were measured by DSC under a nitrogen flow. The endothermic peak showed the decomposition of pure anthocyanin in the range of 210–240 °C [101]. The AuNPs-K presented an endothermic peak at 50 °C, which represents water desorption, and an exothermic peak at 160–210 °C, which represents crystalline rearrangement by the sintering of metallic aggregates. For AgNPs-K, a small endothermic peak was seen at 160 °C, representing some decomposition in organic molecules; after that, a peak at 170–210 °C representing crystalline rearrangement was found. This behavior suggests that, in the case of AuNPs-K, the lack of an organic molecule decomposition signal is due to the large particle diameter (Figure 18).

In the case of AuNPs-VO, there was a large exothermic peak at 300–400 °C and for AgNPs-VO at 280–450 °C, both representing the recrystallization of nanoparticles by heating.

Figure 18. DSC curves for K, AuNPs-K, AgNPs-K, VO, AuNPs-VO and AgNPs-VO

We estimated by computation the number of ligand molecules coordinated onto the metallic nanoparticle as being 525 for gold and 355 in the case of silver (knowing the concentrations and the volumes of the solutions used in the synthesis).

A schematic representation of the formation of colloidal metallic nanoparticles is presented in Figure 19.

Figure 19. Schematic diagram for AuNPs / AgNPs-K formation

18.4. APPLICATION OF NANOMATERIALS ON PSORIATIC LESIONS

The AuNPs / AgNPs-VO and AuNPs / AgNPs-K described above [102,103] were added to an emollient cream (patent in preparation).

The aim of this study was to assess changes to the skin induced by topical therapy based on VO / K conjugated with metal nanoparticles in patients with psoriasis. The research incorporated a clinical study, an ultrasound evaluation and statistical evaluations.

The clinical study was performed at Dermatology Clinic, Cluj-Napoca, Romania. All patients with a diagnosis of *psoriasis vulgaris*, confirmed by pathology, were aged between 35–63. The skin erythematous squamous plaques covered less than 15 % of the body surface. The study was approved by the Ethical Committee of the University of Medicine and Pharmacy Iuliu Hatieganu Cluj-Napoca Romania. The picture in Figure 20 shows one of these cases.

Figure 20. Psoriatic lesion – before treatment

In recent decades, high frequency ultrasonography using probes of at least 20 MHz has been used in dermatology to non-invasively assess different skin lesions and the efficacy of therapy. Different published studies have shown that following different topical treatment for psoriasis plaques, there is a decrease in dermal thickness and a corresponding increase in dermal echo density. Consequently, a ultrasound evaluation was performed with a Dermascan C device (Cortex Technology, Denmark) at 20 MHz, which permits the *in vivo* acquisition of cross-sectional images of the skin up to 2 cm in depth. The device is made up of a transducer, an elaboration system and a data storage system. The ultrasonographic images were processed using specific software (Dermavision). The amplitudes of the echoes of the pixels are given as a value on a numerical scale that ranges from 0–255. The low-echogenicity pixel area corresponds to the interval 0–30, the high echogenicity pixels to the interval

200–255 and the medium echogenicity pixels to 509–150. On a normal cutaneous image, the epidermal echogenicity appears as a white band, and the dermis is a combination of pixels of different amplitudes that may indicate different local processes. An increased number of low echogenicity pixels organized as a band or as diffuse localized process may indicate an inflammatory reaction or edema.

For every subject, we assessed the thickness of the epidermis and dermis in millimeters and the echogenicity variation by comparing the number of pixels with different amplitudes before and after therapy, at the same lesion.

For six weeks, patients underwent only topical treatment. They used creams with 2 % AuNPs-VO or AgNPs-VO every day, and then creams with Au-K and Ag-K, for different lesions. Figure 21 shows the good effect of the creams on the same patient whose knee was shown in Figure 20.

Figure 21. Psoriatic lesions – after treatment with AuNPs-VO cream

In order to determine the significance of the observed results, the ultrasonographic data were mathematically assessed.

Concerning the nanomaterials based on AuNPs-VO / AgNPs-VO, the anti-inflammatory effect was the best (compared with all other creams) in the case of the gold product (the mean value of skin thickness decreased from 3437.75 mm to 938 mm). The good results were statistically significant according to a two-tailed Student's t-test, a good correlation was found in the data. The good correlation is between the thickness of skin and the antiinflammatory effect of the cream based on gold nanoparticles, in the sense that the thickness decreases is perfectly correlated with the using of this cream. The p value was 0.005 (level of significance) and the correlation coefficient $R = 87.4\%$. Figure 22 also shows the good anti-inflammatory effect of AuNPs-VO.

Figure 22. Anti-inflammatory effect of AuNPs-VO / AgNPs-VO after treatment

Concerning the nanomaterials AuNPs-K / AgNPs-K, good results were also found for the gold nanomaterial. The mean value of skin thickness decreased from 1438.1667 at 1164.8333 mm. Unfortunately, the data did not correlate, so we cannot state that the result is statistically significant, and may have occurred due to chance.

18.5. CONCLUSION

Bio-nanomaterials based on gold / silver nanoparticles and anthocyanins from natural extracts, such as pure kuromanin chloride, were prepared using a green synthesis method. These nanomaterials were investigated from the morphological, structural and thermal point of view. The obtained nanomaterials were stable for 3 weeks, if kept at a temperature of 4 °C. Due to their biocompatibility and stability, these nanomaterials were successfully used as a medical treatment. For this reason, gold and silver nanoparticles were added to emollient creams, which were used on psoriatic lesions in several groups of patients; remarkable improvements in disease conditions were observed. The best results were obtained using the AuNPs-VO nanomaterial. The use of creams based on new hybrid materials may be

extended to other types of dermatoses that have topical therapy as the first indication.

18.6. FUTURE WORK

In the near future, we intend to prepare new gold / silver nanoparticles based on semisynthesis compounds with pure anthocyanin and tryptophan. These new nanoproducts will be introduced in creams for psoriatic lesions, taking into consideration that lower levels of epidermal tryptophan are involved in many types of skin disease.

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Aspects of AuNPs / AgNPs-VO optimization procedure were adapted from the paper: *Effect of natural extract pH on morphological characteristics of hybrid materials based on gold nanoparticles*, published in AIP Conference Proceedings [73], by permission of AIP Publishing.

Aspects of AuNPs / AgNPs-K preparation, characterization and application have not been published before.

REFERENCES

1. Arcadia Blog, <http://www.arcadiamedical.ro/blog/2014/09/psoriazisul-o-privire-moderna-asupra-unei-afectiuni-vechi/> (accessed on March 18, 2015).
2. R.V. Filimon, D. Beceanu, M. Niculaua, C. Arion. *Agronom. Res. Mold.* **44** (2011) 81–91.
3. D.T. Santos, J.Q. Albarelli, M.M. Beppu, M.A.A. Meireles. *Food Res. Int.* **50** (2013) 617–624.
4. T. Tsuda, F. Horio, K. Uchida, H. Aoki, T. Osawa. *J. Nutr.* **133** (2003) 2125–2130.
5. I.T. Nizamutdinova, Y.M. Kim, J. Chung, S.C. Shin, Y.K. Jeong, H.G. Seo, J.H. Lee, K.C. Chang, H.J. Kim. *Food Chem. Toxicol.* **47** (2009) 2806–2812.
6. W.L. Barnes, A. Dereux, T.W. Ebbesen. *Nature* **424** (2003) 824–830.
7. G. Raschke, S. Kowarik, T. Franzl, C. Sonnichsen, T.A. Klar, J. Feldmann. *Nano Lett.* **3** (2003) 935–938.
8. C.F. Bohren, D.R. Huffman. *Absorption and scattering of light by small particles*; John Wiley & Sons: New York, USA 1983, 1–544.
9. H.H. Lara, N.V. Ayala-Nunez, L. Ixtepan-Turrent, C. Rodriguez-Padilla. *J. Nanobiotechnol.* **8** (2010) 1–10.
10. J.D. Trono, K. Mizuno, N. Yusa, T. Matsukawa, K. Yokoyama, M. Uesakaj. *Radiat. Res.* **52** (2011) 103–109.
11. R. Sinha, G.J. Kim, S. Nie, D.M. Shin. *Mol. Cancer Ther.* **5** (2006) 1909–1917.
12. Y. Wang, X. Xie, X. Wang, G. Ku, K.L. Gill, D.P. O’Neal, G. Stoica, L.V. Wang. *Nano Lett.* **4** (2004) 1689–1692.
13. M. Auffan, J. Rose, M.R. Wiesner, J.-Y. Bottero. *Environ. Pollut.* **157** (2009) 1127–1133.
14. C.N.R. Rao, G.U. Kulkarni, P.J. Thomas, P.P. Edwards. *Chem. Soc. Rev.* **29** (2000) 27–35.
15. F. Mafune, J.-Y. Kohno, Y. Takeda, T. Kondow. *J. Phys. Chem. B* **106** (2002) 7575–7577.
16. K.N. Thakkar, S.S. Mhatre, R.Y. Parikh. *Nanom. Nanotech. Biol. Med.* **6** (2010) 257–262.
17. N. Kulkarni, U. Muddapur. *J. Nanotechnol.* **2014** (2014) 1–8.
18. S. Prabhu, E.K. Poulouse. *Int. Nano Lett.* **2** (2012) 32–42.
19. A. Chauhan, S. Zubair, S. Tufail, A. Sherwani, M. Sajid, S.C. Raman, A. Azam, M. Owais. *Int. J. Nanomedicine* **6** (2011) 2305–2319.
20. N. Pantidos, L.E. Horsfall. *J. Nanomed. Nanotechnol.* **5** (2014) 1–10.
21. S.S. Shankar, A. Ahmad, R. Pasricha, M. Sastry. *J. Mater. Chem.* **13** (2003) 1822–1826.
22. M. Ahamed, M.S. AlSalhi, M.K.J. Siddiqui. *Clin. Chim. Acta* **411** (2010) 1841–1848.
23. L. Ge, Q. Li, M. Wang, J. Ouyang, X. Li, M.M.Q. Xing. *Int. J. Nanomedicine* **9** (2014) 2399–2407.
24. B. Fadeel, A.E. Garcia-Bennett. *Adv. Drug Deliv. Rev.* **62** (2010) 362–374.
25. J.Y. Song, H.-K. Jang, B.S. Kim. *Process Biochem.* **44** (2009) 1133–1138.
26. B. Baishya, M.C. Kalita. *Int. J. Pharm. Bio. Sci.* **4** (2013) 873–881.
27. D.D. Lekeufack, A. Brioude. *Dalton Trans.* **41** (2012) 1461–1464.
28. N. Ahmad, S. Sharma. *Int. J. Nanopart.* **5** (2012) 185–195.
29. I.T. Muralikrishna, M. Pattanayak, P.L. Nayak. *J. Nano Sci. Technol.* **3** (2014) 45–51.

30. P. Elia, Z. Rach, S. Hazan, S. Kolusheva, Z. Porat, Y. Zeiri. *Int. J. Nanomedicine* **9** (2014) 4007–4021.
31. N. Chanda, R. Shukla, A. Zambre, S. Mekapothula, R.R. Kulkarni, K. Katti, K. Bhattacharyya, G.M. Fent, S.W. Casteel, E.J. Boote, J.A. Viator, A. Upendran, R. Kannan, K.V. Katti. *Pharm. Res.* **28** (2011) 279–291.
32. J. Pulit, M. Banach. *Dig. J. Nanomater. Bios.* **8** (2013) 1295 – 1300.
33. E. Rodriguez-Leon, R. Iniguez-Palomares, R.E. Navarro, R. Herrera-Urbina, J. Tanori, C. Iniguez-Palomares, A. Maldonado. *Nanoscale Res. Lett.* **8** (2013) 1–9.
34. P. Logeswari, S. Silambarasan, J. Abraham. *J. Saudi Chem. Soc.* **19** (2012) 311–317.
35. P. Logeswari, S. Silambarasan, J. Abraham. *Sci. Iran.* **20** (2013) 1049–1054.
36. A.M. Abdelmonema, R.M. Amin. *Int. J. Sci. Basic Appl. Res.* **15** (2014) 57–65.
37. D. Mubarak Ali, N. Thajuddin, K. Jeganathan, M. Gunasekaran. *Colloids Surf. B Biointerfaces* **85** (2011) 360–365.
38. J. Kasthuri, S. Veerapandian, N. Rajendiran. *Colloids Surf. B Biointerfaces* **6** (2009) 55–60.
39. S.K. Sivaraman, I. Elango, S. Kumar, V. Santhanam. *Curr. Sci.* **97** (2009) 1055–1059.
40. N. Ahmad, S. Sharma. *GSC* **2** (2012) 141–147
41. S.S. La, P.L. Nayak. *IJSID* **2** (2012) 325–350.
42. R.S. Varma, B. Baruwati, G.E. Hoag, J.B. Collins, US Patent US 2011/0110723.
43. H. Devnani, S.P. Satsangee. *Int. J. Environ.Sci.Technol.* **12** (2015) 1269–1282.
44. S. Kalmodia, J. Harjwani, R. Rajeswari, W. Yang, C.J. Barrow, S. Ramaprabhu, S. Krishnakumar, S.V. Elchuri. *Int. J. Nanomedicine* **8** (2013) 4327–4338.
45. P.N. Chen, C.S.C. Hu, C.H.L. Hiou, W.H. Kuo, C.L. Chiang. *Cancer Lett.* **235** (2006) 248–259.
46. M.J. Awika, W.L. Rooney, D.R. Waniska. *Food Chem.* **90** (2004) 293–301.
47. F. Mattivi, R. Guzzon, U. Vrhovsek, M. Stefanini, R. Velasco. *J. Agric. Food Chem.* **54** (2006) 7692–7702.
48. B. Jayaprakasam, S.K. Vareed, L.K. Olson, M.G. Nair. *J. Agric. Food Chem.* **53** (2005) 28–31.
49. S.Y. Kang, N.P. Seeram, M.G. Nair, L.D. Bourquin. *Cancer Lett.* **194** (2003) 13–19.
50. H. Wang, M.G. Nair, G.M. Strasburg, Y.C. Chang, A.M. Booren, I.J. Gray, D.L. DeWitt. *J. Nat. Prod.* **62** (1999) 294–296.
51. T. Tsuda, F. Horio, K. Uchida, H. Aoki, T. Osawa. *J. Nutr.* **133** (2003) 2125–2130.
52. R.M. Kershi, N. Ebrahim. *Int. J. Mater. Biomater. Appl.* **4** (2014) 14–18.
53. V.K. Ivanov, A.V. Usatenko, A.B. Shcherbakov. *Russ. J. Inorg. Chem.* **54** (2009) 1522–1527.
54. J. Chen, X. Tao, M. Zhang, A. Sun, L. Zhao. *J. Sci. Food Agr.* **94** (2014) 1781–1786.
55. P.L. Nadworny, J. Wang, E.E. Tredget, R.E. Burrell. *J. Inflamm. (Lond)* **7** (2010) 1–20.
56. Y. He, Z. Du, H. Lv, Q. Jia, Z. Tang, X. Zheng, K. Zhang, F. Zhao. *Int. J. Nanomedicine* **8** (2013) 1809–1815.
57. J. Jain, S. Arora, J.M. Rajwade, P. Omay, S. Khandelwal, K.M. Paknikar. *Mol. Pharm.* **6** (2009) 1388–1401.
58. I.I. Stepniak, M.J. Santos-Martinez, C. Medina, M.W. Radomski. *Int. J. Nanomedicine* **9** (2014) 1677–1687.

59. F.K. Santos, M.H. Oyafuso, C.P. Kiill, G.M.P.D. Remiao, M. Chorilli. *Curr. Nanosci.* **9** (2013) 159–167.
60. C. Polinicencu, H. Popescu, C. Nistor. *Clujul Medical* **53** (1980) 160–163.
61. H. Chang, M.-J. Kao, T.-L. Chen, H.-G. Kuo, K.-C. Choand, X.-P. Lin. *Am. J. Eng. Appl. Sci.* **4** (2011) 214–222.
62. M. Alhamed, A.S. Issa, A. Wael Doubal. *J. Electron Dev.* **16** (2012) 1370–1383.
63. M. Crisan, L. David, B. Moldovan, A. Vulcu, S. Dreve, M. Perde-Schrepler, C. Tatomir, A.G. Filip, P. Bolfa, M. Achim, I. Chiorean, I. Kacso, C. Berghian Grosan, L. Olenic. *J. Mater. Chem. B* **1** (2013) 3152–3158.
64. A. Filip, D. Daicoviciu, P. Bolfa, S. Clichici, A. Muresan, L. David, L. Olenic, *IUPS, Birmingham, UK*, July 21–26, 2013, Abstract No. PCB 149.
65. A. Filip, S. Clichici, P. Bolfa, A. Muresan, I. Baldea, D. Olteanu, L. David, L. Olenic, *XII International Conference on Nanostructured Materials (NANO 2014)* Moscow, Russia July 13–18 2014, p. 802.
66. A. Filip, S. Clichici, L. David, L. Olenic, P. Bolfa, G. Borza, D. Olteanu, I. Baldea, M. Achim, A. Muresan, *XXVIIth National Conference on Physiology of the Romanian Society of Physiology*, Romania 5–7 June 2014, p. 9.
67. M. Perde-Schrepler, E. Fischer-Fodor, P. Virag, O. Soritau, I. Brie, C. Tatomir, F. Imre-Lucaci, A. Florea, L. David, L. Olenic, *The VIth International Conference on oxidativ stress in Dermatology and Biology*, Andros, Greece, 28–31 August 2014, p. 113.
68. M. Perde-Schrepler, E. Fischer-Fodor, P. Virag, O. Soritau, I. Brie, L. David, L. Olenic, *OECI 2014 Oncology Days*, Cluj-Napoca, Romania, 13–14 June 2014, p. 63.
69. B. Moldovan, O. Ghic, L. David, C. Chisbora. *Rev. Chim. Bucharest* **63** (2012) 463–464.
70. M.M. Giusti, L.E. Rodriguez-Saona, R.E. Wrolstad. *J. Agric. Food Chem.* **47** (1999) 4631–4635.
71. M.M. Giusti, R.E. Wrolstad, in *Current Protocols in Food Analytical Chemistry*, New York: John Wiley & Sons, 2001, p. 1.
72. L. Olenic, A. Vulcu, I. Chiorean, M. Crisan, C. Berghian-Grosan, S. Dreve, L. David, L.B. Tudoran, I. Kacso, I. Bratu, C. Neamtu, C. Voica. *AIP Conf. Proc.* **1565** (2013) 238–242.
73. G.H. Woehrle, J.E. Hutchinson, S. Ozkar, R.G. Finke. *Turk. J. Chem.* **30** (2006) 1–13.
74. D.B. Williams, C.B. Carter, *Transmission Electron Microscopy A Textbook for Materials Science*, Springer, New York, USA, 2009, p. 5.
75. S.K. Ghosh, T. Pal. *Chem. Rev.* **107** (2007) 4797–4862.
76. M.M. Alvarez, J.T. Khouri, T.G. Schaaff, M.N. Shafigullin, I. Vezmar, R.L. Whetten. *J. Phys. Chem. B* **101** (1997) 3706–3712.
77. X. Huang, M.A. El-Sayed. *J. Adv. Res.* **1** (2010) 13–28.
78. S.P. Dubey, M. Lahtinen, M. Sillanpaa. *Process. Biochem.* **45** (2010) 1065–1071.
79. D.S. Sheny, J. Mathew, D. Philip. *Spectrochim. Acta A* **79** (2011) 254–262.
80. D.M. Smilgies. *J. Appl. Crystallogr.* **42** (2009) 1030–1034.
81. N. Ahmad, S. Sharma, V.N. Singh, S.F. Shamsi, A. Fatma, B.R. Mehta. *Biotechnol. Res. Int.* **2011** (2011) 1–8.
82. E.J. Espinosa-Ortiz, M. Vaca-Mier, in *Nanotechnology for Water and Wastewater Treatment*, P. Lens, J. Virkutyte, V. Jegatheesan, S.-H. Kim, S.-Al Abed (Eds.), IWA Publishing, London, UK, 2013, p. 113.

83. C.S.S.R. Kumar, *Raman Spectroscopy for Nanomaterials Characterization*, Springer-Verlag, Berlin Heidelberg, Ger, 2012, p. 1.
84. B. Zhang, B. Yan. *Anal. Bioanal. Chem.* **396** (2010) 973–982.
85. J.E. Herrera, N. Sakulchaicharoen, in *Drug Delivery Nanoparticles Formulation and Characterization*, Y. Pathak, D.Thassu (Eds.), Informa Healthcare USA, Inc, New York, USA, 2009, p. 239.
86. J.C. Merlin, J.P. Cornard. *Spectrochim. Acta* **50A** (1994) 703–712.
87. N.B. Colthup, L.H. Daly, S.E. Wiberley, *Introduction to Infrared and Raman Spectroscopy*, Academic Press Inc., San Diego, USA, 1990, p. 1.
88. G. Socrates, *Infrared and Raman Characteristic Group Frequencies Tables and Charts*, John Wiley and Sons Ltd., Chichester, England, 2001, p. 1.
89. M. Hineno. *Carbohydr. Res.* **56** (1977) 219–227.
90. S. Tozzi, N. Zanna, P. Taddei. *Food Chem.* **141** (2013) 3586–3597.
91. A. Piccolo, F.J. Stevenson. *Geoderma* **27** (1982) 195–208.
92. Y.S. Velioglu, L. Ekici, E.S. Poyrazoglu. *Int. J. Food Sci. Technol.* **41** (2006) 1011–1015.
93. Spectral Database for Organic Compounds SDBS, http://sdbs.db.aist.go.jp/sdbs/cgi-bin/direct_frame_top.cgi (accessed on April 2015).
94. D. Wang, Y. Ma, C. Zhang, X. Zhao. *LWT - Food Sci. Technol.* **55** (2014) 645–649.
95. S. Aryal, B.K.C. Remant, N. Dharmaraj, N. Bhattarai, C.H. Kim, H.Y. Kim. *J. Coll. Interf. Sci.* **299** (2006) 191–197.
96. J. Sopousek, J. Bursik, J. Zalesak, Z. Pesina. *J. Min. Metall. Sect. B-Metall.* **48** (2012) 63–71.
97. Z. Yang, *Nanoparticle Inks for Printed Electronics* www.nanomastech.com (accessed on April 2015)
98. C.M.J. Coutts, M.B. Cortie, M.J. Ford, A.M. McDonagh. *J. Phys. Chem. C* **113** (2009) 1325–1328.
99. H. Yu, L. Li, Y. Zhang. *Scripta Materialia* **66** (2012) 931–934.
100. B.L. Smith, J.E. Hutchison. *J. Phys. Chem. C* **117** (2013) 25127–25137.
101. L. Olenic, I. Chiorean, M. Crisan, A. Vulcu, C. Grosan, L.B. Tudoran, I. Kacso, L. Magerusan, I. Bratu, *ATOM N 2014 7th Edition*, 21–24 August 2014, Constanta, Romania, p. 1.
102. L. Olenic, A. Vulcu, A.C. Grosan, S. Dreve, (Romania), Patent no. A/00805 (submitted to OSIM in 2013)
103. L. Olenic, A. Vulcu, A.C. Grosan, S. Dreve, (Romania), Patent no. A/00263 (submitted to OSIM in 2014).

CANCER TARGETING STRATEGIES OF NANOMATERIALS

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19.1. INTRODUCTION

Cancer (a malignant tumor) is the leading cause of disease-related death, and 8.2 million cancer-related deaths were reported by the World Health Organization in 2012 [1]. Among cancer types, the most common causes of cancer death are lung, liver, and stomach cancers [1,2]. Surgery, chemotherapy, immunotherapy, and radiotherapy, either alone or in combination, can be used to treat cancer [3-5]. In recent years, there has been increasing interest in targeted delivery of therapeutic molecules (*e.g.*, genes, drugs, or proteins), which has several advantages over other forms of treatment: (1) enhanced therapeutic efficacy, (2) avoidance of undesired side effects caused by the delivery of therapeutic molecules to normal (healthy) cells; and (3) reduction of the efficacious dose of therapeutic molecules [6,7].

Cancer-targeted delivery systems for therapeutic molecules can be grouped into three categories: viral nanomaterials (*e.g.*, inactivated retroviruses, adenoviruses, adeno-associated viruses, and herpes simplex viruses), non-viral nanomaterials (*e.g.*, synthetic polymers and liposomes), and bacterial carriers (*e.g.*, *Clostridium*, *Salmonella*, and *Bifidobacterium*). Viral nanomaterials show high transfection efficiency, but have clinical safety issues (*e.g.*, immune responses) that must be solved before their use in clinical trials. Furthermore, because viral nanomaterials themselves do not have the ability to target cancer cells, their conjugation to cancer-specific ligands or promoters are required for cancer targeting [8-10]. Non-viral nanomaterials have several advantages such as low pathogenicity and that they easily can be mass-produced, but their disadvantages are low transfection efficiency and low cancer targeting capabilities; thereby, also requiring the use of cancer-specific ligands [11,12]. Anaerobic bacteria are mainly used as bacterial carriers for the delivery of therapeutic molecules to hypoxic cancer cells. They have high transfection efficiency, but also cause clinical safety problems such as inflammation and immune responses [13].

In this chapter, we focus on cancer targeting methods using nanomaterials for cancer cell-targeted therapy.

19.2. CANCER-TARGETING METHODS

Cancer targeting by nanomaterials is accomplished by either passive or active targeting. In the case of active targeting, nanomaterials typically recognize or respond to overexpressed receptors, overexpressed intracellular signals, or hypoxic regions in cancer cells and tissues (Table 1).

Table 1. Advantages and disadvantages of cancer targeting methods for clinical applications [6]

Passive cancer targeting method, the enhanced permeability and retention effect (EPR)		Active cancer targeting method					
		Nanomaterials targeting overexpressed receptors in cancer cells		Nanomaterials targeting overexpressed cellular signals (proteases and protein kinases) in cancer cells		Nanomaterials targeting hypoxic cancer regions	
Advantages	Disadvantages	Advantages	Disadvantages	Advantages	Disadvantages	Advantages	Disadvantages
1. High specificity for solid cancers 2. Applicable to various types of drugs or nanomaterials 3. Reduced side effects	1. Low EPR effect in poorly vascularized regions 2. Low therapeutic value in spreading and metastatic cancers	1. High specificity for cancers 2. Reduced side effects 3. Enhanced therapeutic efficacy	1. Lack of ligands responding to overexpressed receptors 2. Different activity levels of receptors between patients	1. High specificity for cancers 2. Reduced side effects 3. Enhanced therapeutic efficacy	1. Lack of selective peptides or proteins responding to overexpressed cellular signals 2. Different activity levels of cellular signals between patients	1. High specificity for hypoxic cancers 2. Enhanced therapeutic efficacy	1. Lack of useful vectors 2. Clinical safety issues and risk of side effects

19.2.1. Passive cancer targeting method

Cancer cells require oxygen and nutrients to promote their growth and progress. Therefore, the formation of new blood vessels *via* angiogenesis is essential for cancer growth and metastasis. Cancer angiogenesis is triggered by multiple pro-angiogenic factors, including vascular endothelial growth factor (VEGF), angiopoietins, basic fibroblast growth factor (bFGF) and cytokines [interleukin-1 (IL-1), and tumor necrosis factor α (TNF α)]. In addition to chemoattractants, integrins ($\alpha_v\beta_3$ and $\alpha_5\beta_1$), receptors (VEGF receptors), and circulating bone marrow-derived cells (F4/80⁺CD11b⁺ macrophages and Gr1⁺CD11b⁺ neutrophils) directly and/or indirectly participate in the angiogenic process of cancer [14,15]. Blood vessels near cancer cells are often dilated, leaky, tortuous, and heterogeneous.

The passive cancer targeting method is based on the EPR effect that is characteristic of blood vessels near cancer cells. Therefore, when nanomaterials or anti-cancer drugs are injected into the blood stream, they accumulate in cancer cells through these dilated, leaky, tortuous, and heterogeneous blood vessels near cancer tissues when compared with normal tissues that have blood vessels showing a well-organized and functional structure (Figure 1) [16]. However, the vessel structure and pore size of blood vessels within individual cancer types is different [17]. Thus, EPR-based cancer therapy using nanoparticles can exhibit different therapeutic efficacies depending upon the cancer type. The synthesis of optimized nanomaterials for each target cancer type is essential for efficient EPR-based cancer therapy.

Figure 1. Schematic illustration of the EPR effect. Unlike normal blood vessels showing a well-organized and functional structure, cancer blood vessels are leaky, tortuous, and heterogeneous. Nanomaterials injected into the blood stream can pass through these cancer blood vessels and tend to accumulate in cancer tissues, but none or very few pass through normal blood vessels that have a well-organized and functional structure.

Figure 1 has been reproduced from a previous publication [6].

19.2.1.1. Characteristics of nanomaterials that influence EPR

Several nanomaterial characteristics might affect the EPR effect, such as size [17-19], surface charge [17-19], hydrophobicity (high hydrophobicity increases the affinity for the cell membrane) [20], flexibility (high flexibility leads to rapid renal clearance) [18,19], and shape (spheres have a higher uptake by macrophages as compared with rods) [18,19]. Among these factors,

size and surface charge of nanomaterials may be the most important factors influencing the EPR effect.

19.2.1.1.1. Size of nanomaterials

Nanomaterials smaller than 5.5 nm mainly undergo kidney excretion and those larger than 8 nm are rapidly cleared through the reticuloendothelial system organs (liver and spleen) [18,21]. Normal blood vessels can permeate smaller nanomaterials (< 20 nm), but blood vessels near cancer cells are permeable to nanomaterials of < 400 nm in diameter [17,19,22]. Indeed, poly(ethylene glycol) (PEG)-liposomes of 400 nm in diameter can penetrate into the cancer interstitium, but those of 600 nm are excluded from extravascular spaces [22]. However, the most efficient EPR-based therapy might be obtained using nanomaterials between 10–200 nm in diameter [23]. A previous study reported that nanomaterials with a molecular weight (MW) greater than 40 kDa exhibited a high EPR effect [24]. However, other studies suggested that the EPR effect did not depend on the molecular weight (MW) of nanomaterials [22,25]. Therefore, the size of nanomaterials may be more important for efficient EPR than is the MW.

19.2.1.1.2. Charge of nanomaterials

Negatively charged nanomaterials are taken up rapidly by phagocytes (macrophages) and are degraded or discharged from the body. Nanomaterials with high positive charges can bind to blood proteins or to the surface of blood vessels, resulting in rapid kidney excretion and low accumulation in cancer cells [17,19]. The uptake of nanomaterials (150 nm) by macrophages was increased in the order of $-40 > -25 > -15$ mV negative surface charge and $+35 > +25 > +15$ mV positive surface charge [26]. Therefore, neutral or weakly positive-charged nanomaterials are more efficient for EPR compared with strong negatively-charged or positively-charged nanomaterials.

19.2.2. Active cancer targeting method

19.2.2.1. Overexpressed receptors in cancer cells

Cancer cells overexpress numerous receptors. The binding of ligands to overexpressed target receptors plays a key role in the growth, survival, metastasis, angiogenesis, and apoptosis of cancer cells (Figure 2). Examples of this are the binding of EGF and FGF to growth factor receptors (tyrosine kinase receptors) [27,28], and prostaglandin E and lysophosphatidic acid to G-protein-coupled receptors [29]. However, TNF α and TNF-related apoptosis inducing ligand (TRAIL) can stimulate apoptosis of cancer cells by binding to death receptors. However, death receptors and their downstream signals (*e.g.*, caspases) are often suppressed in cancer cells [30–32]. Therefore, nanomaterials recognizing overexpressed receptors in cancer cells might be useful for cancer-targeted therapy.

Figure 2. Signal transduction pathways are different in cancer cells compared with normal cells. Cancer cells overexpress receptors (growth factor receptors and G-protein-coupled receptors) and intracellular signaling molecules (ras/raf/mek/erk and pi3k/akt/mtor) that are related to cell survival and proliferation. However, apoptosis-related receptors (death receptors) and intracellular signals (caspases) are suppressed. Therefore, nanomaterials targeting or responding to overexpressed receptors or cellular signals of cancer cells can be useful for cancer cell-targeted therapy. Figure 2 has been modified from previous publications [59,92].

19.2.2.1.1. Nanomaterials targeting overexpressed receptors in cancer cells

Many receptor-specific ligands (aptamers, proteins, peptides, and antibodies) have been reported to be useful materials for targeting overexpressed receptors on cancer cells. Although transferrin and folate, which recognize transferrin receptor (TfR) and folate receptor, respectively, are extensively used to target various cancer cells, most nanomaterials containing receptor-specific ligands were identified by using limited numbers of cancer cells that overexpressed target receptors (Table 2). For instance, although targeting and therapeutic efficacy of folate-conjugated nanomaterials have been investigated using 9L / LacZ rat gliosarcoma cells [33], IGROV-1 human ovarian cancer cells [34], SKOV-3 human ovarian cancer cells [35], and B16 mouse melanoma cells

[36], the most commonly used cells are KB human epidermoid carcinoma and HeLa human epidermoid carcinoma cells, because they have an especially high overexpression of folate receptors [37-43]. It is important to note that receptor expression may vary according to the cancer cell type. For example, the antisense delivery efficiency of TfR-targeted, protamine-containing lipid nanomaterials was in the order of K562 > MV4-11 > Raji human leukemia cells, which was directly related to the TfR expression level. Thus, the higher the TfR expression, the greater the down-regulation of the target gene by nanomaterials [44].

Several receptor-specific peptides in Table 2 have been investigated, such as homing peptides identified from various library methods (the phage display peptide library method) (for review, see [45-47]). Nanomaterials containing these homing peptides can be used for cancer cell-targeted therapy. For example, the small heat shock protein 16.5-derived nanocage conjugated to a human hepatocellular carcinoma cell-specific peptide SP94 (SFSIIHTPILPL) was identified by an *in vivo* phase display method, and achieved selective targeting to HepG2 and HuH-7 human hepatocellular carcinoma cells, but not to HeLa cells or RLN-8 rat hepatocytes [48].

Double-targeted nanomaterials conjugated to dual-ligands, such as transferrin and folate [49], cyclic RGD (cRGD) and transferrin [50], glucose and folate [51], and RGD and IL-13 peptide (CGEMGWVRC) [52], have been developed to increase therapeutic efficacy against target cancer cells or to target specific cancer cells. Gold nanoparticles conjugated to folate and glucose showed a higher uptake by human epidermoid carcinoma (KB) cells than did gold nanomaterials conjugated to either folate or glucose alone [51]. Similarly, when transferrin and folate were linked to PEG-phosphatidylethanolamine, the transfection efficiency in HepG2 human hepatocellular carcinoma cells was higher for the conjugate than for the single ligand-modified nanomaterials [49]. Furthermore, nanomaterials linked with RGD and IL-3 peptide [52], and cRGD and transferrin [50] successfully targeted human umbilical vein endothelial and C6 rat glioma cells, and human umbilical vein endothelial and HeLa human epidermoid carcinoma cells, respectively.

In contrast, several studies have suggested that cancer-specific ligand-conjugated nanomaterials did not preferentially localize to cancer tissues in comparison with non-conjugated nanomaterials [53-55]. In these studies, despite the lack of change in cancer localization, ligand-conjugated nanomaterials showed enhanced uptake by cancer cells compared with non-conjugated nanomaterials, indicating that the increased therapeutic efficacy against cancer cells by ligand-conjugated nanomaterials was caused by increased uptake by cancer cells rather than by their localization to cancer cells [53-55].

19.2.2.2. Overexpressed cellular signals in cancer cells

Living cells contain numerous intracellular signal transduction pathways that play a key role in cell growth, differentiation, proliferation, and apoptosis. These signal pathways are tightly regulated and function normally in healthy cells. However, in cancer cells, many signal pathways related to cellular survival and proliferation are overexpressed, such as the RAS/RAF/MEK/ERK and PI3K/AKT/mTOR pathway. In some cases, apoptosis-related signal pathways are suppressed, such as the death receptor-mediated pathways (Figure 2). Therefore, nanomaterials that could respond to these overexpressed intracellular signals could also be used for cancer-targeted therapy.

19.2.2.2.1. Nanomaterials targeting overexpressed cellular signals (proteases and protein kinases) in cancer cells

Cellular signal transduction pathways often are stimulated or suppressed through phosphorylation by protein kinases, or by dephosphorylation or cleavage by proteases. Phosphorylation reactions mediated by protein kinases add a phosphate group from adenosine triphosphate to the phosphorylation sites (serine, threonine, and tyrosine) [56,57] located on proteins. Nanomaterials that recognize overexpressed protein kinases are related to the phosphorylation reaction. Phosphorylation of nanomaterials containing target peptides can add two anionic charges of the phosphate to target peptide substrates, thereby weakening electrostatic interactions in the nanomaterials. For example, protein kinase C (PKC) α and protein kinase A (PKA) are overexpressed in several cancer cells, such as breast cancer, lung cancer, melanoma, ovarian cancer, and prostate cancer, but are expressed at very low levels in normal cells or tissues [58-60]. Nanomaterials conjugated to PKC α (FKKQGSFAKKK)- or PKA (ALRRSLG)-specific peptides showed a higher targeting efficacy to cancer cells or tissues than to normal cells or tissues [61-66].

As mentioned in 19.2.2.1., caspase activation in cancer cells is very low, but can be stimulated by the activation of death receptors through stimulators such as TNF α and TRAIL. Caspases induce cell apoptosis by cleavage of their target peptides [30-32]. In a recent study, doxorubicin conjugated to a caspase-3-specific peptide (DEVD) was efficiently cleaved in apoptotic regions of cancer cells induced by radiation exposure, leading to efficient inhibition of cancer growth, with low toxicity in normal tissues [67].

Several protease cleavable nanoparticles have been developed for cancer cell-targeted therapy. Matrix metalloproteinases (MMPs) (MMP-2, -9, and -12) are known as cancer-associated proteases, and participate in the progression of several cancer mechanisms, including migration, invasion, metastasis, and angiogenesis [68,69]. MMP-specific peptides (PVGLIG and GPLGIAGQ for MMP-2) can be selectively cleaved by MMPs that are overexpressed in cancer

cells. Exploiting these functions of MMPs, conjugates of MMP-cleavage peptides and anticancer drugs can be useful for cancer cell-targeted therapies [70-73]. For example, a self-assembled nanoparticle containing PEG 2000-paclitaxel-MMP-2 peptide (GPLGIAGQ) exhibited a higher anticancer efficacy *in vitro* and *in vivo* than did free paclitaxel [70]. Furthermore, nanocages conjugated with a MMP-2-binding peptide (CTTHWGF²TL²C) showed selective uptake into MMP-2 overexpressing cancer cells [74].

19.2.2.3. Nanomaterials targeting hypoxic cancer regions

EPR-mediated or ligand-mediated targeting methods generally have a low targeting efficacy for hypoxic tumor regions. Their therapeutic efficacy against hypoxic tumor cells is also dramatically reduced because of the limited delivery of therapeutic agents to cancer cells and the induction of drug resistance [75,76].

Hypoxia-inducible factor-1 α (HIF-1 α) is an attractive therapeutic target because it is a key regulator of the hypoxic environment and is related to drug resistance and cancer metastasis [76]. Therefore, suppression of HIF-1 α expression leads to an increase in therapeutic efficacy against hypoxic cancer cells. Several nanomaterials that bind to or contain HIF-1 α siRNA or antisense oligonucleotides have been developed and have exhibited a high therapeutic efficacy for hypoxic cancer cells and increased inhibitory efficacy against cancer metastasis [77-80].

For hypoxic cancer cell-targeted therapy, several nanomaterials (mainly viral nanomaterials) incorporating hypoxia-responsive promoters that are specifically expressed in hypoxic cancers, have a high specificity and gene expression for hypoxic cancer cells [81,82]. Furthermore, nanomaterials containing hypoxia-responsive agents (2-nitroimidazole), which can be highly sensitive to hypoxia, are suitable for hypoxic tumor cell-targeted therapy [83,84].

Another strategy for hypoxic cancer cell-targeted therapy is the use of anaerobic bacteria (*e.g.*, *Salmonella* and *Clostridium spp.*). Anaerobic bacteria themselves have the capacity to inhibit the growth of cancer cells and also can increase therapeutic efficacy in combination with anticancer molecules or radiation therapy [13,85-88]. *Salmonella* and *Clostridium* are pathogenic bacteria and therefore cause clinical safety problems such as inflammation and immune responses. To overcome these problems, non-pathogenic anaerobic bacteria (*e.g.* *Bifidobacterium sp.*) [89] and nonpathogenic or attenuated bacteria [90,91] are being developed as bacterial vectors to target hypoxic cancers.

19.3. CLINICAL APPLICATIONS OF NANOMATERIALS IN CANCER TREATMENT

Nanomaterial-anticancer drug complexes are most commonly used in cancer clinical trials. For example, liposomes loaded with anticancer drugs, such as doxorubicin and daunorubicin, have been approved by the U.S. Food and Drug Administration for the treatment of several cancers, such as ovarian and metastatic breast and human immunodeficiency virus-related Kaposi sarcoma (see [93–95] for review). In cancer clinical trials, the use of PEG-conjugated liposomes (rather than PEG-free liposomes) may be a more efficient way to increase therapeutic effects of anticancer drugs, because of their prolonged blood circulation time and enhanced drug accumulation in cancer cells [94,95]. In addition to liposome-drug complexes, several nanomaterial-drug complexes are currently undergoing clinical trials in cancer, such as the polymer-drug conjugate of paclitaxel and poly(L-glutamic acid) (also known as paclitaxel poliglumex, Xyotax, and CT-2103) for the treatment of non-small cell lung cancer [96,97], cyclodextrin-PEG micelle with camptothecin (also known as CRLX-101) for advanced (metastatic and / or unresectable) solid malignancies [98] and PEG-irinotecan conjugate (also known as etirinotecan pegol and NKTR-102) for advanced breast cancer [99]. Targeting delivery of these nanomaterial-drug complexes to cancer cells is based on the EPR effect.

On the other hand, there are very few reports on cancer clinical trials using nanomaterial-gene complexes. Recently, results of a phase I clinical trial using a complex of the human tumor suppressor gene p53 with a liposomal nanomaterial (SGT-53) employing an anti-transferrin receptor single-chain antibody fragment (scFv) as the targeting molecule were reported. The trial supplied evidence of targeted cancer delivery of systemically dosed SGT-53 to metastatic lesions. Furthermore, SGT-53 was well-tolerated and exhibited anticancer activity [100]. Similarly, in a phase I clinical trial using a small interfering RNA targeting the M2 subunit of ribonucleotide reductase (RRM2) complexed with nanomaterials containing a cyclodextrin-containing polymer, a PEG, and human transferrin, the complex efficiently reduced the specific RRM2 messenger RNA and the RRM2 protein levels in cancer tissues [101]. Targeting of nanomaterial-gene complexes containing scFv [100] or transferrin [101] to cancers is based on the active cancer targeting method, and takes advantage of the overexpression of transferrin receptor in cancer cells.

19.4. SUMMARY AND CONCLUSIONS

For cancer cell-targeted delivery of therapeutic molecules, several delivery systems, including viral or non-viral nanomaterials, and anaerobic bacterial carriers, have been developed and utilized for *in vivo* or *ex vivo* / *in vitro* applications. Cancer targeting by nanomaterials is accomplished by both passive and active cancer targeting. Passive targeting methods based on the EPR effect exhibit low therapeutic efficacy in poorly vascularized regions and in spreading and metastatic cancers. Active cancer targeting is based on the recognition or response to overexpressed receptors, overexpressed intracellular signals, and hypoxic regions in cancer cells and tissues. However, a lack of cancer targeting materials (ligands and peptide substrates responding to activated receptors and intracellular signals, respectively) and different activity levels of receptors and intracellular signals between patients may be a serious obstacle to be overcome for the use of nanomaterials based on the active cancer targeting method.

Table 2. Receptor-specific ligands used for the development of cancer cell-targeted nanomaterials

Ligands	Receptors	Target cancer cells	Refs.
<i>Antibodies (Abs)</i>			
Anti-CD20 Ab (Mabthera)	CD20 receptor	Daudi lymphoma cells	[102]
Anti-CD71 Ab	TfR	PC-3 human prostate cancer cells	[103]
Anti-DR5 Ab	Death receptor 5 (DR5; also known as TRAIL-receptor 2)	HCT116 human colon cancer cells	[104]
		A375 human melanoma cells	[105]
Anti-HER2 Ab (Herceptin)	Human epidermal growth factor receptor 2 (HER2)	SKOV-3 human ovarian cancer cells	[102]
		SK-BR-3 human breast cancer cells	[106]
Anti-Met Ab	Hepatocyte growth factor receptor (HGFR; also known as c-Met or Met)	A549 human lung cancer and MKN45 human gastric cancer cells	[107]
Single chain variable fragment anti-EGFR Ab (ScFvEGFR)	EGFR	H292 human lung cancer cells	[108]
Transferrin receptor Ab (OKT9)	TfR	Ramos human Burkitt's lymphoma cells	[109]

Ligands	Receptors	Target cancer cells	Refs.
<i>Aptamers</i>			
Sgc8 DNA aptamer	PTK7 (an orphan tyrosine kinase receptor)	CEM lymphoblastic leukemia cells	[110]
GL21.T aptamer	Axl receptor (a tyrosine kinase receptor)	A549 human lung cancer and U87 human glioma cells	[111]
GS24 aptamer	TfR	B16 mouse melanoma cells	[112]
<i>Proteins (glycoproteins)</i>			
Asialofetuin	Asialoglycoprotein receptor	HepG2 human hepatocellular carcinoma cells	[113]
EGF	EGFR	H22 mouse hepatocellular carcinoma cells	[114]
		MDA-MB-468 human breast cancer cells	[115]
		A431 human epidermoid carcinoma cells	[116]
EphrinA I	EphA2 receptor	PC-3 human prostate cancers	[117]
Transferrin (apotransferrin)	TfR	K562 human leukemia cells	[118]
		K562 > MV4-11 > Raji human leukemia cells	[44]
		LLC1 mouse lung cancer cells	[119]
		ZH5 rat hepatocellular carcinoma cells	[120]
		Ramos human Burkitt's lymphoma cells	[109]
		HepG2 human hepatocellular carcinoma cells	[121]
		SCC-7 murine squamous cell carcinoma cells	[122]
		MIA PaCa-2 human pancreatic cancer cells	[123]
Urokinase plasminogen activator (uPA)	uPA receptor (uPAR)		
VEGF121	VEGF receptor	U87 human glioma cells	[124]

Ligands	Receptors	Target cancer cells	Refs.
<i>Peptides</i>			
Analogue peptide of neuropeptide Y (Arg6, Pro34)	Y1-receptor	MCF-7 human breast cancer cells	[125]
Angiopep-2 (TFFYGGSRGKRNNFKTEEY)	Low-density lipoprotein receptor-related protein-1	C6 rat glioma / U87 human glioma cells	[126]/ [127]
α -conotoxin ImI	$\alpha 7$ nicotinic acetylcholine receptor ($\alpha 7$ nAChR)	MCF-7 human breast cancer cells	[128]
α -melanocyte-stimulating hormone peptide (SYSMEHFRWGKPV)	Melanocortin type 1 receptor	B16F0 mouse melanoma cells	[129]
apoA-1 mimetic peptide (AP) (FAEKFKAEVKDYFAKFWD)	EGFR	KB human epidermoid carcinoma cells	[130]
ATF peptide (amino acids 1 to 135 of mouse uPA)	uPAR	MIA PaCa-2 human pancreatic cancer cells	[131]
Bombesin (BN) peptide (QWAVGHL)	Gastrin releasing peptide receptor	HeLa human epidermoid carcinoma cells	[132]
Chlorotoxin [a 36-amino acid peptide derived from <i>Leiurus quinquestriatus</i> (scorpion) venom]	MMP-2 and receptor-associated protein ClC-3	U89 human glioma/C6 rat glioma cells	[133]/ [134]
Cyclic RGD	Integrin $\alpha_v\beta_3$	4T1 mouse metastatic breast cancer cells	[135]
		M21 human melanoma cells	[136, 137]
		LNCaP human prostate cancer cells	[137]
		SW620 human colon cancer cells	[138]
C16Y peptide (DFKLFAVYIKYR) (D-Lys6)-LHRH	Integrin $\alpha_v\beta_3$	U87 human glioma cells	[139]
		B16 mouse melanoma cells	[140]
	Luteinizing hormone-releasing hormone (LHRH) receptor	A2780 human ovarian cancer cells	[141]
Follicle-stimulating hormone (FSH) β 81–95 peptide	FSH receptor	Caov-3 human ovarian cancer cells	[142]
F3 peptide (KDEPQRRSARLSAKPAPPK)	Nucleolin receptor	MDA-MB-435S, MDA-MB-231, and Hs578T	[143]

Ligands	Receptors	Target cancer cells	Refs.
PEPKPKKAPAKK)		human breast cancer cells	
Gastrin (modified peptide; CKSSEAYGW-Nle-DF)	Cholecystokinin-2 receptor	InR1G9 hamster glucagonoma cells	[144]
GE11 peptide (CYHWYGYTPQNVI)	EGFR	HuH7 human hepatocellular carcinoma cells	[145]
HAIYPRH (knor-specific peptide)	TfR	Bel-7402 human hepatocellular carcinoma/U87 human glioma cells	[146]/[147]
Human ATF (hATF) peptide of uPA	uPAR	MIA PaCa-2 human pancreatic cancer cells	[148]
Interleukin 13 (IL-13) peptide (isolated by phage display; CGEMGWVRC)	IL-13 α 2	U87 human glioma/C6 rat glioma cells	[149]/[150]
iRGD (neuropilin 1-binding peptide; CRGDKGPDC)	Neuropilin 1	AsPC-1 human pancreatic cancer cells	[151]
MC1R agonist peptide (SYS-Nle-EH-d-FRWGKPV)	Melanocortin type-1 receptor (MC1R)	B16F0 / M3 mouse melanoma cells	[152]/[153]
NR7 peptide (NSVRGSR)	EGFR	SKOV-3 human ovarian cancer cells	[154]
PreS1-derived peptide	Asialoglycoprotein receptor	HepaRG human hepatocellular carcinoma cells	[155]
RGD	Vascular endothelial growth factor receptor-2 (VEGF R2)	N2A mouse neuroblastoma cells	[156]
RGDGSSV	Integrin $\alpha_{2b}\beta_3$	GL261 mouse glioma cell / B16F0 mouse melanoma cells	[157]
YCDGFYACYMDV	HER2	SK-BR-3 human breast cancer cells	[158]
YCDPC	Integrin $\alpha_4\beta_1$	KCl-H929 and MM.1S human multiple myeloma cells	[158]
YIGSR (laminin-derived peptide)	Laminin receptor	B16 mouse melanoma cells	[159]

Ligands	Receptors	Target cancer cells	Refs.
YSA peptide (YSAYPDSVPMMMS; mimics the ligand ephrin-A1)	EphA2 receptor	HEY human ovarian cancer cells	[160]
<i>Others (small molecules)</i>			
Anisamide	Sigma receptor	NCI-H460 human lung cancer cells	[161]
		B16F0 mouse melanoma cells	[162]
		H460 human non-small lung cancer cells	[163]
Chondroitin sulfate	CD44 receptor	B16F0 mouse melanoma cells	[164]
Folate	Folate receptor	KB human epidermoid carcinoma cells	[37- 39]
		HeLa human epidermoid carcinoma cells	[39]
		9L / LacZ rat gliosarcoma cells	[33]
		IGROV-1 /SKOV-3 human ovarian cancer cells	[34]/ [35]
		B16 mouse melanoma cells	[36]
Galactose / Galactosamine	Asialoglycoprotein receptor	HepG2 human hepatocellular carcinoma cells	[165]/ [166]
Hyaluronic acid	CD44 receptor	SKOV-3TR human ovarian cancer cells	[167]
Lactose	Asialoglycoprotein receptor	HepG2 human hepatocellular carcinoma cells	[168]
NeuAc α 2-6Gal (residue of glycoprotein glycan)	CD22	Daudi human B-cell lymphoma cells	[169]
Pectin	Asialoglycoprotein receptor	HepG2 human hepatocellular carcinoma cells	[170]

Ligands	Receptors	Target cancer cells	Refs.
Tamoxifen derivative	Estrogen receptor	MCF-7 human breast cancer cells	[171]
Tetraiodothyroacetic acid ($\alpha_v\beta_3$ -antagonist)	Integrin $\alpha_v\beta_3$	A375 human melanoma cells	[172]

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REFERENCES

1. B.W. Stewart, C.P. Wild, *World Cancer Report 2014*, IARC Publisher, Lyon, France, 2014, p. 16.
2. J. Ferlay, I. Soerjomataram, R. Dikshit, S. Eser, C. Mathers, M. Rebelo, D.M. Parkin, D. Forman, F. Bray. *Int. J. Cancer* **136** (2015) E359–E386.
3. M. Vanneman, G. Dranoff. *Nat. Rev. Cancer* **12** (2012) 237–251.
4. R. Baskar, K.A. Lee, R. Yeo, K.W. Yeoh. *Int. J. Med. Sci.* **9** (2012) 193–199.
5. V.T. DeVita. *Cancer Res.* **68** (2008) 8643–8653.
6. J.H. Kang, R. Toita, Y. Katayama. *Biotechno. Adv.* **28** (2012) 757–763.
7. S. Biswas, V.P. Torchilin. *Adv. Drug Deliv. Rev.* **66** (2014) 26–41.
8. S.A. Collines, B.A. Guinn, P.T. Harrison, M.F. Scallan, G.C. O’Sullivan, M. Tangney. *Curr. Gene Ther.* **8** (2008) 866–878.
9. M.Z.I. Pranjol, A. Hajitou. *Viruses* **7** (2015) 268–284.
10. R. Waehler, S.J. Russell, D.T. Curiel. *Nat. Rev. Genetics* **8** (2007) 573–587.
11. Y. Zhang, A. Satterlee, L. Huang. *Mol. Ther.* **20** (2012) 1298–1304.
12. X. Guo, L. Huang. *Acc. Chem. Res.* **45** (2012) 971–979.
13. N. Nair, T. Kasai, M. Seno. *Anticancer Res.* **34** (2014) 6289–6296.
14. R.S. Kerbel. *N. Engl. J. Med.* **358** (2008) 2039–2049.
15. S.M. Weis, D.A. Cheresh. *Nat. Med.* **17** (2011) 1359–1370.
16. Y. Matsumura, H. Maeda. *Cancer Res.* **46** (1986) 6387–6392.
17. U. Prabhakar, H. Maeda, R.K. Jain, E.M. Sevick-Muraca, W. Zamboni, O.C. Farokhzad, S.T. Barry, A. Gabizon, P. Grodzinski, D.C. Blakey. *Cancer Res.* **73** (2013) 1–6.
18. M. Longmire, P.L. Choyke, H. Kobayashi. *Nanomedicine* **3** (2008) 703–717.
19. H. Kobayashi, R. Watanabe, P.L. Choyke. *Theranostics* **4** (2014) 81–89.

20. H. Maeda H. Nakamura, J. Fang. *Adv. Drug Deliv. Rev.* **65** (2013) 71–79.
21. H.S. Choi, W. Liu, P. Misra, E. Tanaka, J.P. Zimmer, B. Itty Ipe, M.G. Bawendi, J.V. Frangioni. *Nat. Biotechnol.* **25** (2007) 1165–1170.
22. F. Yuan, M. Dellian, D. Fukumura, M. Leunig, D.A. Berk, V.P. Torchilin, R.K. Jain. *Cancer Res.* **55** (1995) 3752–3756.
23. T.M. Allen, P.R. Cullis. *Science* **303** (2004) 1818–1822.
24. Y. Noguchi, J. Wu, R. Duncan, J. Strohalm, K. Ulbrich, T. Akaike, H. Maeda. *Jpn. J. Cancer Res.* **89** (1998) 307–314.
25. H. Imoto, Y. Sakamura, K. Ohkouchi, R. Atsumi, Y. Takakura, H. Sezaki, M. Hashida. *Cancer Res.* **52** (1992) 4396–4401.
26. C. He, Y. Hu, L. Yin, C. Tang, C. Yin. *Biomaterials* **31** (2010) 3657–3666.
27. E. Zwick, J. Bange, A. Ullrich. *Endocr. Relat. Cancer* **8** (2001) 161–173.
28. A. Tomas, C.E. Futter, E.R. Eden. *Trends Cell Biol.* **24** (2014) 26–34.
29. R.T. Dorsam, J.S. Gutkind. *Nat. Rev. Cancer* **7** (2007) 79–94.
30. S. Wang, W.S. Ei-Deiry. *Oncogene* **22** (2003) 8628–8633.
31. K.M. Debatin, P.H. Krammer. *Oncogene* **23** (2004) 2950–2966.
32. M.E. Guicciardi, G.J. Gores. *FASEB J.* **23** (2009) 1625–1637.
33. K.M. McNeeley, E. Karathanasis, A.V. Annapragada, R.V. Bellamkonda. *Biomaterials* **30** (2009) 3986–3995.
34. N. Kamaly, T. Kalber, M. Thanou, J.D. Bell, A.D. Miller. *Bioconjug. Chem.* **20** (2009) 648–655.
35. J.C. Fernandes, X. Qiu, F.M. Winnik, M. Benderdour, X. Zhang, K. Dai, Q. Shi. *Int. J. Nanomedicine* **7** (2012) 5833–5845.
36. L. Wang, M. Li, N. Zhang. *Int. J. Nanomedicine* **7** (2012) 3281–3294.
37. P.J. Stevens, M. Sekido, R.J. Lee. *Pharm. Res.* **21** (2004) 2153–2157.
38. R. Rossin, D. Pan, K. Qi, J.L. Turner, X. Sun, K.L. Wooley, M.J. Welch. *J. Nucl. Med.* **46** (2005) 1210–1218.
39. G. Destito, R. Yeh, C.S. Rae, M.G. Finn, M. Manchester. *Chem Biol.* **14** (2007) 1152–1162.
40. A.J. Ditto, K.N. Shah, N.K. Robishaw, M.J. Panzner, W.J. Youngs, Y.H. Yun. *Mol. Pharm.* **9** (2012) 3089–3098.
41. F. Gao, L. Li, T. Liu, N. Hao, H. Liu, L. Tan, H. Li, X. Huang, B. Peng, C. Yan, K. Yang, X. Wu, D. Chen, F. Tang. *Nanoscale* **4** (2012) 3365–3372.
42. K.D. Lee, S.H. Choi, D.H. Kim, H.Y. Lee, K.C. Choi. *Arch. Pharm. Res.* **37** (2014) 1546–1553.
43. Y.Q. Guan, Z. Zheng, Z. Huang, Z. Li, S. Niu, J.M. Liu. *Sci. Rep.* **4** (2014) 4990.
44. X. Yang, C.G. Koh, S. Liu, X. Pan, R. Santhanam, B. Yu, Y. Peng, J. Pang, S. Golan, Y. Talmon, Y. Jin, N. Muthusamy, J.C. Byrd, K.K. Chan, L.J. Lee, G. Marcucci, R.J. Lee. *Mol. Pharm.* **6** (2009) 221–230.
45. A. Gautam, P. Kapoor, K Chaudhary, R. Kumar; Open Source Drug Discovery Consortium, G. P. Raghava. *Curr. Med. Chem.* **21** (2014) 2367–2391.
46. J. Bábíčková, L. Tóthová, P. Boor, P. Celec. *Biotechnol. Adv.* **31** (2013) 1247–1259.
47. A. Rivinoja, P. Laakkonen. *Methods Mol. Biol.* **683** (2011) 401–415.
48. R. Toita, M. Murata, S. Tabata, K. Abe, S. Narahara, J.S. Piao, J.H. Kang, M. Hashizume. *Bioconjug. Chem.* **23** (2012) 1494–1501.
49. F. Jing, D. Li, W. Xu, Y. Liu, K. Wang, Z. Sui. *Pharm. Biol.* **52** (2014) 570–574.
50. Q. Xu, Y. Liu, S. Su, W. Li, C. Chen, Y. Wu. *Biomaterials* **33** (2012) 1627–1639.

51. X. Li, H. Zhou, L. Yang, G. Du, A.S. Pai-Panandiker, X. Huang, B. Yan. *Biomaterials* **32** (2011) 2540–2545.
52. H. Gao, Y. Xiong, S. Zhang, Z. Yang, S. Cao, X. Jiang. *Mol. Pharm.* **11** (2014) 1042–1052.
53. D.B. Kirpotin, D.C. Drummond, Y. Shao, M.R. Shalaby, K. Hong, U.B. Nielsen, J.D. Marks, C.C. Benz, J.W. Park. *Cancer Res.* **66** (2006) 6732–6740.
54. D.W. Bartlett, H. Su, I.J. Hildebrandt, W.A. Weber, M.E. Davis. *Proc. Natl. Acad. Sci. USA* **104** (2007) 15549–15554.
55. C.H. Choi, C.A. Alabi, P. Webster, M.E. Davis. *Proc. Natl. Acad. Sci. USA* **107** (2010) 1235–1240.
56. C. López-Otín, T. Hunter. *Nat. Rev. Cancer* **10** (2010) 278–292.
57. P.J. Utz, P. Anderson. *Cell Death Differ.* **7** (2000) 589–602.
58. O. Konopatskaya, A.W. Poole. *Trends Pharmacol. Sci.* **31** (2010) 8–14.
59. J.H. Kang. *New J. Sci.* **2014** (2014) 231418.
60. S. Naviglio, M. Caraglia, A. Abbruzzese, E. Chiosi, D. Di Gesto, M. Marra, M. Romano, A. Sorrentino, L. Sorvillo, A. Spina, G. Illiano. *Expert. Opin. Ther. Targets* **13** (2009) 83–92.
61. J.H. Kang, D. Asai, J.H. Kim, T. Mori, R. Toita, T. Tomiyama, Y. Asami, J. Oishi, Y.T. Sato, T. Niidome, B. Jun, H. Nakashima, Y. Katayama. *J. Am. Chem. Soc.* **130** (2008) 14906–14907.
62. J.H. Kang, J. Oishi, J.H. Kim, M. Ijuin, R. Toita, B. Jun, D. Asai, T. Mori, T. Niidome, K. Tanizawa, S. Kuroda, Y. Katayama. *Nanomedicine* **6** (2010) 583–589.
63. R. Toita, J.H. Kang, J.H. Kim, T. Tomiyama, T. Mori, T. Niidome, B. Jun, Y. Katayama. *J. Control. Release* **139** (2009) 133–139.
64. R. Toita, J.H. Kang, T. Tomiyama, C.W. Kim, S. Shiosaki, T. Niidome, T. Mori, Y. Katayama. *J. Am. Chem. Soc.* **134** (2012) 15410–15417.
65. T. Tomiyama, R. Toita, J.H. Kang, D. Asai, S. Shiosaki, T. Mori, T. Niidome, Y. Katayama. *J. Control. Release* **148** (2010) 101–105.
66. J. Oishi, K. Kawamura, J.H. Kang, K. Kodama, T. Sonoda, M. Murata, T. Niidome, Y. Katayama. *J. Control. Release* **110** (2006) 431–436.
67. B.S. Lee, Y.W. Cho, G.C. Kim, D.H. Lee, C.J. Kim, H.S. Kil, D.Y. Chi, Y. Byun, S.H. Yuk, K. Kim, I.S. Kim, I.C. Kwon, S.Y. Kim. *J. Natl. Cancer Inst.* In press. doi: 10.1093/jnci/dju403.
68. K. Klessenbrock, V. Plaks, Z. Werb. *Cell* **141** (2010) 52–67.
69. C. Gialeli, A.D. Theocharis, N. Karamanos. *FEBS J.* **278** (2011) 16–27.
70. L. Zhu, T. Wang, F. Perche, A. Taigind, V.P. Torchilin. *Proc. Natl. Acad. Sci. USA* **110** (2013) 17047–17052.
71. D. Bacinello, E. Garanger, D. Taton, K.C. Tam, S. Lecommandoux. *Biomacromolecules* **15** (2014) 1882–1888.
72. C. Nazli, G.S. Demirer, Y. Yar, H.Y. Acar, S. Kizilel. *Colloids Surf. B Biointerfaces* **122** (2014) 674–683.
73. F.B. Cui, R.T. Li, Q. Liu, P.Y. Wu, W.J. Hu, G.F. Yue, H. Ding, L.X. Yu, X.P. Qian, B.R. Liu. *Cancer Lett.* **346** (2014) 53–62.
74. T. Kawano, M. Murata, J.S. Piao, S. Narahara, N. Hamano, J.H. Kang, M. Hashizume. *Int. J. Mol. Sci.* **16** (2015) 148–158.
75. W.R. Wilson, M.P. Hay. *Nat. Rev. Cancer* **11**, (2011) 393–410.
76. J.M. Brown, W.R. Wilson. *Nat. Rev. Cancer* **4** (2004) 437–447.
77. X. Zhao, F. Li, Y. Li, H. Wang, H. Ren, J. Chen, G. Nie, J. Hao. *Biomaterials* **46** (2015) 13–25.

78. X.Q. Liu, M.H. Xiong, X.T. Shu, R.Z. Tang, J. Wang. *Mol. Pharm.* **9** (2012) 2863–2874.
79. W.H. Chen, R.L. Lecaros, Y.C. Tseng, L. Huang, Y.C. Hsu. *Cancer Lett.* **359** (2015) 65–74.
80. Y. Wang, M. Saad, R.I. Pakunlu, J.J. Khandare, O.B. Garbuzenko, A.A. Vetcher, V.A. Soldatenkov, V.P. Pozharov, T. Minko. *Clin. Cancer Res.* **14** (2008) 607–616.
81. R.L. Cowen, E.J. Garside, B. Fitzpatrick, M.V. Papadopoulou, K.J. Williams. *Br. J. Radiol.* **81** (2008) S45–S56.
82. P.B. Card, R.T. Hogg, C.R. Gil Del Alcazar, R.D. Gerard. *Cancer Gene Ther.* **19** (2012) 451–459.
83. T. Thambi, V.G. Deepagan, H.Y. Yoon, H.S. Han, S.H. Kim, S. Son, D.G. Jo, C.H. Ahn, Y.D. Suh, K. Kim, I.C. Kwon, D.S. Lee, J.H. Park. *Biomaterials* **35** (2014) 1735–1743.
84. O. Mazuryk, M. Maciuszek, G. Stochel, F. Suzenet, M. Brindell. *J. Inorg. Biochem.* **134** (2014) 83–91.
85. C. Bettegowda, L.H. Dang, R. Abrams, D.L. Huso, L. Dillehay, I. Cheong, N. Agrawal, S. Borzillary, K.M. McCaffery, E.L. Watson, K.S. Lin, F. Bunz, K. Baidoo, M.G. Pomper, K.W. Kinzler, B. Vogelstein, S. Zhou. *Proc. Natl. Acad. Sci. USA* **9** (2003) 15083–15088.
86. C.H. Lee, C.L. Wu, Y.S. Tai, A.L. Shiau. *Mol. Ther.* **11** (2005) 707–716.
87. M.Q. Wei, R. Ren, D. Good, J. Anné. *Genet. Vaccines Ther.* **6** (2008) 8.
88. B. Yu, M. Yang, L. Shi, Y. Yao, Q. Jiang, X. Li, L. H. Tang, B.J. Zheng, K.Y. Yuen, D.K. Smith, E. Song, J.D. Huang. *Sci. Rep.* **2** (2012) 436.
89. S. Taniguchi, M. Fujimori, T. Sasaki, H. Tsutsui, Y. Shimatani, K. Seki, J. Amano. *Cancer Sci.* **101** (2010) 1925–1932.
90. K.B. Low, M. Ittensohn, T. Le, J. Platt, S. Sodi, M. Amoss, O. Ash, E. Carmichael, A. Chakraborty, J. Fischer, S.L. Lin, X. Luo, S.I. Miller, L. Zheng, I. King, J.M. Pawelek, D. Bermudes. *Nat. Biotechnol.* **17** (1999) 37–41.
91. V. Punj, D. Saint-Dic, S. Daghfal, J.R. Kanwar. *Cancer Biol. Ther.* **3** (2004) 708–714.
92. T. Mori, J.H. Kang, in *Breakthroughs in melanoma research*, Y. Tanaka (Ed.), InTech Publisher, Rijeka, Croatia, 2012, p. 389.
93. A.S. Thakor, S.S. Gambhir. *CA Cancer J. Clin.* **63** (2013) 395–418.
94. R.K. Jain, T. Stylianopoulos. *Nat. Rev. Clin. Oncol.* **7** (2010) 653–664.
95. Y. Barenholz. *J. Control. Release* **160** (2012) 117–134.
96. L. Paz-Ares, H. Ross, M. O'Brien, A. Riviere, U. Gatzemeier, J. Von Pawel, E. Kaukel, L. Freitag, W. Digel, H. Bischoff, R. García-Campelo, N. Iannotti, P. Reiterer, I. Bover, J. Prendiville, A.J. Eisenfeld, F.B. Oldham, B. Bandstra, J.W. Singer, P. Bonomi. *Br. J. Cancer* **98** (2008) 1608–1613.
97. M.E. O'Brien, M.A. Socinski, A.Y. Popovich, I.N. Bondarenko, A. Tomova, B.T. Bilynsky, Y.S. Hotko, V.L. Ganul, I.Y. Kostinsky, A.J. Eisenfeld, L. Sandalic, F.B. Oldham, B. Bandstra, A.B. Sandler, J.W. Singer. *J. Thorac. Oncol.* **3** (2008) 728–734.
98. G.J. Weiss, J. Chao, J.D. Neidhart, R.K. Ramanathan, D. Bassett, J.A. Neidhart, C.H. Choi, W. Chow, V. Chung, S.J. Forman, E. Garmey, J. Hwang, D.L. Kalinoski, M. Koczywas, J. Longmate, R.J. Melton, R. Morgan, J. Oliver, J.J. Peterkin, J.L. Ryan, T. Schluep, T.W. Synold, P. Twardowski, M.E. Davis, Y. Yen. *Invest. New Drugs* **31** (2013) 986–1000.

99. A. Awada, A.A. Garcia, S. Chan, G.H. Jerusalem, R.E. Coleman, M.T. Huizing, A. Mehdi, S.M. O'Reilly, J.T. Hamm, P.J. Barrett-Lee, V. Cocquyt, K. Sideras, D.E. Young, C. Zhao, Y.L. Chia, U. Hoch, A.L. Hannah, E.A. Perez. *Lancet Oncol.* **14** (2013) 1216–1225.
100. N. Senzer, J. Nemunaitis, D. Nemunaitis, C. Bedell, G. Edelman, M. Barve, R. Nunan, K.F. Pirollo, A. Rait, E.H. Chang. *Mol. Ther.* **21** (2013) 1096–1103.
101. M.E. Davis, J.E. Zuckerman, C.H. Choi, D. Seligson, A. Tolcher, C.A. Alabi, Y. Yen, J.D. Heidel, A. Ribas. *Nature* **464** (2010) 1067–1070.
102. A. Cirstoiu-Hapca, L. Bossy-Nobs, F. Buchegger, R. Gurny, F. Delie. *Int. J. Pharm.* **331** (2007) 190–196.
103. A.E. Felber, B. Castagner, M. Elsabahy, G.F. Deleavey, M.J. Damha, J.C. Leroux. *J. Control. Release* **152** (2011) 159–167.
104. S.M. Abdelghany, D. Schmid, J. Deacon, J. Jaworski, F. Fay, K.M. McLaughlin, J.A. Gormley, J.F. Burrows, D.B. Longley, R.F. Donnelly, C.J. Scott. *Biomacromolecules* **14** (2013) 302–310.
105. B. Ding, X. Wu, W. Fan, Z. Wu, J. Gao, W. Zhang, L. Ma, W. Xiang, Q. Zhu, J. Liu, X. Ding, S. Gao. *Int. J. Nanomedicine* **6** (2011) 1991–2005.
106. Y. Liu, K. Li, B. Liu, S.S. Feng. *Biomaterials* **31** (2010) 9145–9155.
107. R. Heukers, I. Altintas, S. Raghoenath, E. De Zan, R. Pepermans, R.C. Roovers, R. Haselberg, W.E. Hennink, R.M. Schiffelers, R.J. Kok, P.M. van Bergen en Henegouwen. *Biomaterials* **35** (2014) 601–610.
108. X.H. Peng, Y. Wang, D. Huang, Y. Wang, H.J. Shin, Z. Chen, M.B. Spewak, H. Mao, X. Wang, Y. Wang, Z.G. Chen, S. Nie, D.M. Shin. *ACS Nano* **5** (2011) 9480–9493.
109. J. Wang, S. Tian, R.A. Petros, M.E. Napier, J.M. Desimone. *J. Am. Chem. Soc.* **132** (2010) 11306–11313.
110. T. Chen, M.I. Shukoor, R. Wang, Z. Zhao, Q. Yuan, S. Bamrungsap, X. Xiong, W. Tan. *ACS Nano* **5** (2011) 7866–7873.
111. L. Cerchia, C.L. Esposito, S. Camorani, A. Rienzo, L. Stasio, L. Insabato, A. Affuso, V. de Franciscis. *Mol. Ther.* **20** (2012) 2291–303.
112. M.Z. Zhang, R.N. Yu, J. Chen, Z.Y. Ma, Y.D. Zhao. *Nanotechnology* **23** (2012) 485104.
113. S. Díez, G. Navarro, C.T. de Ilarduya. *J. Gene Med.* **11** (2009) 38–45.
114. Y.C. Yao, X.Y. Zhan, J. Zhang, X.H. Zou, Z.H. Wang, Y.C. Xiong, J. Chen, G.Q. Chen. *Biomaterials* **29** (2008) 4823–4830.
115. M.A. Sandoval, B.R. Sloat, D.S. Lansakara, A. Kumar, B.L. Rodriguez, K. Kiguchi, J. Digiovanni, Z. Cui. *J. Control. Release* **157** (2012) 287–296.
116. C. Fortier, G. De Crescenzo, Y. Durocher. *Biomaterials* **34** (2013) 1344–1353.
117. A.M. Gobin, J.J. Moon, J.L. West. *Int. J. Nanomedicine* **3** (2008) 351–358.
118. N.C. Bellocq, S.H. Pun, G.S. Jensen, M.E. Davis. *Bioconjug. Chem.* **14** (2003) 1122–1132.
119. R.A. Abela, J. Qian, L. Xu, T.S. Lawrence, M. Zhang. *Cancer Gene Ther.* **15** (2008) 496–507.
120. A.D. Krishna, R.K. Mandraju, G. Kishore, A.K. Kondapi. *PLoS One* **4** (2009) e7240.
121. W. Wang, F. Zhou, L. Ge, X. Liu, F. Kong. *Int. J. Nanomedicine* **7** (2012) 2513–2522.
122. J.Y. Yhee, S.J. Lee, S. Lee, S. Song, H.S. Min, S.W. Kang, S. Son, S.Y. Jeong, I.C. Kwon, S.H. Kim, K. Kim. *Bioconjug. Chem.* **24** (2013) 1850–1860.

123. G.Y. Lee, W.P. Qian, L. Wang, Y.A. Wang, C.A. Staley, M. Satpathy, S. Nie, H. Mao, L. Yang. *ACS Nano* **7** (2013) 2078–2089.
124. S. Goel, F. Chen, H. Hong, H.F. Valdovinos, R. Hernandez, S. Shi, T.E. Barnhart, W. Cai. *ACS Appl. Mater. Interfaces* **6** (2014) 21677–21685.
125. W. Hild, K. Pollinger, A. Caporale, C. Cabrele, M. Keller, N. Pluym, A. Buschauer, R. Rachel, J. Tessmar, M. Breunig, A. Goepferich. *Proc. Natl. Acad. Sci. USA* **107** (2010) 10667–10672.
126. R. Huang, W. Ke, L. Han, J. Li, S. Liu, C. Jiang. *Biomaterials* **32** (2011) 2399–2406.
127. L. Mei, Q. Zhang, Y. Yang, Q. He, H. Gao. *Int. J. Pharm.* **474** (2014) 95–102.
128. D. Mei, Z. Lin, J. Fu, B. He, W. Gao, L. Ma, W. Dai, H. Zhang, X. Wang, J. Wang, X. Zhang, W. Lu, D. Zhou, Q. Zhang. *Biomaterials* **42** (2015) 52–65.
129. L. Vannucci, E. Falvo, M. Fornara, P. Di Micco, O. Benada, J. Krizan, J. Svoboda, K. Hulikova-Capkova, V. Morea, A. Boffi, P. Ceci. *Int. J. Nanomedicine* **7** (2012) 1489–1509.
130. Z. Zhang, J. Chen, L. Ding, H. Jin, J.F. Lovell, I.R. Corbin, W. Cao, P.C. Lo, M. Yang, M.S. Tsao, Q. Luo, G. Zheng. *Small* **6** (2010) 430–437.
131. L. Yang, H. Mao, Z. Cao, Y.A. Wang, X. Peng, X. Wang, H.K. Sajja, L. Wang, H. Duan, C. Ni, C.A. Staley, W.C. Wood, X. Gao, S. Nie. *Gastroenterology* **136** (2009) 1514–1525.
132. L. Hosta-Rigau, I. Olmedo, J. Arbiol, L.J. Cruz, M.J. Kogan, F. Albericio. *Bioconjug. Chem.* **21** (2012) 1070–1078.
133. C. Qin, B. He, W. Dai, Z. Lin, H. Zhang, X. Wang, J. Wang, X. Zhang, G. Wang, L. Yin, Q. Zhang. *Biomaterials* **35** (2014) 5908–5920.
134. S. Huang, J. Li, L. Han, S. Liu, H. Ma, R. Huang, C. Jiang. *Biomaterials* **32** (2011) 6832–6838.
135. C. Qin, B. He, W. Dai, H. Zhang, X. Wang, J. Wang, X. Zhang, G. Wang, L. Yin, Q. Zhang. *Mol. Pharm.* **11** (2014) 3233–3241.
136. C.A. Boswell, P.K. Eck, C.A. Regino, M. Bernardo, K.J. Wong, D.E. Milenic, P.L. Choyke, M.W. Brechbiel. *Mol. Pharm.* **5** (2008) 527–539.
137. H.S. Choi, W. Liu, F. Liu, K. Nasr, P. Misra, M.G. Bawendi, J.V. Frangioni. *Nat. Nanotechnol.* **5** (2010) 42–47.
138. L. Li, J. Yang, W.W. Wang, Y.C. Yao, S.H. Fang, Z.Y. Dai, H.H. Hong, X. Yang, X.T. Shuai, G.Q. Gao. *Int. J. Pharm.* **438** (2012) 1–10.
139. Y. Zhong, C. Wang, R. Cheng, L. Cheng, F. Meng, Z. Liu, Z. Zhong. *J. Control. Release* **195** (2014) 63–71.
140. N. Hamano, Y. Negishi, A. Fujisawa, M. Manandhar, H. Sato, F. Katagiri, M. Nomizu, Y. Aramaki. *Int. J. Pharm.* **428** (2012) 114–117.
141. N.V. Nukolova, H.S. Oberoi, Y. Zhao, V.P. Chekhonin, A.V. Kabanov, T.K. Bronich. *Mol. Pharm.* **10** (2013) 3913–321.
142. X. Zhang, J. Chen, Y. Kang, S. Hong, Y. Zheng, H. Sun, C. Xu. *Int. J. Pharm.* **453** (2013) 498–505.
143. V. Moura, M. Lacerda, P. Figueiredo, M.L. Corvo, M.E. Cruz, R. Soares, M.C. de Lima, S. Simões, J.N. Moreira. *Breast Cancer Res. Treat.* **133** (2012) 61–73.
144. C. Sanchez, D. El Hajj Diab, V. Connord, P. Clerc, E. Meunier, B. Pipy, B. Payré, R.P. Tan, M. Gougeon, J. Carrey, V. Gigoux, D. Fourmy. *ACS Nano* **8** (2014) 1350–1363.

145. F.M. Mickler, L. Möckl, N. Ruthardt, M. Ogris, E. Wagner, C. Bräuchle. *Nano Lett.* **12** (2012) 3417–3423.
146. L. Han, R. Huang, J. Li, S. Liu, S. Huang, C. Jiang. *Biomaterials* **32** (2011) 1242–1252.
147. Y. Kuang, S. An, Y. Guo, S. Huang, K. Shao, Y. Liu, J. Li, H. Ma, C. Jiang. *Int. J. Pharm.* **454** (2013) 11–20.
148. Y.S. Cho, G.Y. Lee, H.K. Sajja, W. Qian, Z. Cao, W. He, P. Karna, X. Chen, H. Mao, Y.A. Wang, L. Yang. *Small* **9** (2013) 1964–1973.
149. H. Gao, Z. Yang, S. Zhang, S. Cao, S. Shen, Z. Pang, X. Jiang. *Sci. Rep.* **3** (2013) 2534.
150. B. Wang, L. Lv, Z. Wang, Y. Zhao, L. Wu, X. Fang, Q. Xu, H. Xin. *Biomaterials* **35** (2014) 5897–5907.
151. M. Murata, S. Narahara, T. Kawano, N. Hamano, J.S. Piao, J.H. Kang, K. Ohuchida, T. Murakami, M. Hashizume. *Mol. Pharm.* **12** (2015) 1422–1430.
152. W. Lu, C. Xiong, R. Zhang, L. Shi, M. Huang, G. Zhang, S. Song, Q. Huang, G.Y. Liu, C. Li. *J. Control. Release* **161** (2012) 959–966.
153. M.O. Durymanov, E.A. Beletkaia, A.V. Ulasov, Y.V. Khramtsov, G.A. Trusov, N.S. Rodichenko, T.A. Slastnikova, T.V. Vinogradova, N.Y. Uspenskaya, E.P. Kopantsev, A.A. Rosenkranz, E.D. Sverdlov, A.S. Sobolev. *J. Control. Release* **163** (2012) 211–219.
154. C.W. Liu, W.J. Lin. *Int. J. Nanomedicine* **7** (2012) 4749–4767.
155. M. Murata, J.S. Piao, S. Narahara, T. Kawano, N. Hamano, J.H. Kang, D. Asai, R. Ugawa, M. Hashizume. *Protein Expr. Purif.* **110** (2015) 52–56.
156. R.M. Schiffflers, A. Ansari, J. Xu, Q. Zhou, Q. Tang, G. Storm, G. Molema, P.Y. Lu P.V. Scaria, M.C. Woodle. *Nucleic Acids Res.* **32** (2004) e149.
157. D. Hallahan, L. Geng, S. Qu, C. Scarfone, T. Giorgio, E. Donnelly, X. Gao, J. Clanton. *Cancer Cell* **3** (2003) 63–74.
158. J.F. Stefanick, J.D. Ashley, B. Bilgicer. *ACS Nano* **7** (2013) 8115–8127.
159. G. Sarfati, T. Dvir, M. Elkabets, R.N. Apte, S. Cohen. *Biomaterials* **32** (2011) 152–161.
160. W.H. Blackburn, E.B. Dickerson, M.H. Smith, J.F. McDonald, L.A. Lyon. *Bioconjug. Chem.* **20** (2009) 960–968.
161. S.D. Li, Y.C. Chen, M.J. Hackett, L. Huang. *Mol. Ther.* **16** (2008) 163–169.
162. Y. Yang, J. Li, F. Liu, L. Huang. *Mol. Ther.* **20** (2012) 609–615.
163. S.K. Kim, M.B. Foote, L. Huang. *Cancer Lett.* **334** (2013) 311–318.
164. T. Kurosaki, T. Kitahara, S. Kawakami, K. Nishida, J. Nakamura, M. Teshima, H. Nakagawa, Y. Kodama, H. Sasaki. *Biomaterials* **30** (2009) 4427–4434.
165. F.L. Mi, Y.Y. Wu, Y.L. Chiu, M.C. Chen, H.W. Sung, S.H. Yu, S.S. Shyu, M.F. Huang. *Biomacromolecules* **8** (2007) 892–898.
166. H.F. Liang, C.T. Chen, S.C. Chen, A.R. Kulkarni, Y.L. Chiu, M.C. Chen, H.W. Sung. *Biomaterials* **27** (2006) 2051–2059.
167. X. Yang, A.K. Lyer, A. Singh, E. Choy, F.J. Hornicek, M.M. Amiji, Z. Duan. *Sci. Rep.* **5** (2015) 8509.
168. X. Zhou, M. Zhang, B. Yung, H. Li, C. Zhou, L.J. Lee, R.J. Lee. *Int. J. Nanomedicine* **7** (2012) 5465–5474.
169. W.C. Chen, D.S. Sigal, A. Saven, J.C. Paulson. *Leuk. Lymphoma.* **53** (2012) 208–210.
170. C.Y. Yu, Y.M. Wang, N.M. Li, G.S. Liu, S. Yang, G.T. Tang, D.X. He, X.W. Tan, H. Wei. *Mol. Pharm.* **11** (2014) 638–644.

171. E.C. Dreaden, S.C. Mwakwari, Q.H. Sodji, A.K. Oyelere, M.A. El-Sayed. *Bioconjug. Chem.* **20** (2009) 2247–2253.
172. S. Lee, J. Kim, G. Shim, S. Kim, S.E. Han, K. Kim, I.C. Kwon, Y. Choi, Y.B. Kim, C.W. Kim, Y.K. Oh. *J. Control. Release* **164** (2012) 213–220.

MATERIALS FOR CARDIAC TISSUE ENGINEERING

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20.1. INTRODUCTION

Stem cell research is poised to revolutionise many aspects of biomedical research [1,2]. In consequence, in this field of study, research output and the number of active researchers have rapidly grown in a broad range of areas, including stem cell-based clinical applications, and basic research on fundamental cell biology and development. Thus, stem cell research has expanded in emerging topics such as embryonic stem (ES) and induced pluripotent stem (iPS) cells, bioethics and social science [3].

This is, for instance, the case of cardiovascular diseases, which are at present the leading cause of death worldwide, according to the World Health Organisation [4]. Once damaged, cardiac muscle has little intrinsic repair capability due to the poor regeneration potential exhibited by remaining cardiac muscle cells (cardiomyocytes). For decades, heart damage in patients has been typically managed using medical (aspirin, β -blockers, or angiotensin-converting enzyme inhibitors) or mechanical means, such as percutaneous coronary intervention and coronary artery bypass graft surgery [5]. Heart function, however, recovers completely only after cardiac transplantation, which is restricted by heart donor availability and deleterious immunological responses. One alternative is promoting repair by the delivery of functional cells to the injured myocardium. Thus, over the last 10 years, there has been tremendous effort in developing therapies based on stem cells and, more recently, tissue engineering [6-8].

In this context, nanostructure materials (within the overall size range of 1–1,000 nm) offer appealing strategies to allow greater means of regenerating cardiac cells and to strengthen weakened scarred heart tissue. For example, researchers recognise carbon nanotubes as reactive to electrical stimulation, and they then use these nanoparticles to create therapeutic cells with the characteristics of genuine cardiac progenitors [9,10]. Moreover, novel engineered cardiac patches are developed by seeding cells with regenerative potential onto porous scaffolds that give the appropriate shape and organisation to the tissue. Nevertheless, while the heart is an electrically conductive organ, the majority of materials used for scaffold assembly are non-conductive; thus, the resultant engineered tissue does not contract as normal heart tissue does. To solve this problem, some researchers have generated gold-infused cardiac patches, whose cells all beat synchronously [11-13]. Taken together, preliminary data from these studies could have implications for millions of people around the world, and show the undoubted potential held by this emerging therapy.

Herein, we examine and discuss the growing demonstrations of damaged heart repair involving approaches to cell- and tissue engineering-based therapy and nanotechnology. In the following text, we thus envision that we are moving

from traditional medicine (that addresses the symptoms of heart diseases) to being rightfully able to heal these debilitating diseases.

20.2. SCAFFOLD-FREE CARDIAC TISSUE ENGINEERING

20.2.1. Cell sheets

The scaffold-free cell sheets consist of a number of monolayers of therapeutic cells cultured on temperature-responsive polymer surfaces that allow non-enzymatic detachment [14]. Remarkably, monolayers or sheets of distinct cell types (*i.e.* mesenchymal stem cell (MSC)), through the establishment of tight and functional cellular connections, are able to propagate electrochemical signals, improving cardiac function when implanted to cover the infarcted myocardium in rodents [14-16]. Moreover, specific layers comprising endothelial cells can be placed between therapeutic cell sheets to enhance vascularisation of the implant in various animal models [17]. A similar strategy of scaffold-free tissue engineering is based on aggregations of embryonic stem cell (ESC)-derived cardiomyocytes, which will generate synchronously contracting cardiac tissue [18]. Despite the beneficial effects of scaffold-free constructs, the translation of this approach to the clinics is challenging, due to the difficulties of adaptation to human heart proportions.

20.2.2. Injectable nanomaterials

One of the most widely used injectable nanomaterials for cardiac regeneration are those referred to as hydrogels. In particular, hydrogels, which can be natural or synthetic, are three-dimensional (3D) cross-linked polymer networks that reproduce the extracellular matrix (ECM) and the natural microenvironment [19]. Hydrogels are used as a vehicle to inject therapeutic cells, proteins and genes into injured myocardium, conferring efficient cell retention into the place of injection. Commonly, hydrogels are injected transendocardially, epicardially or intracoronary for minimally invasive delivery. For this purpose, they are liquid and jellyfy upon injection *via* physical or chemical factors such as temperature and pH. Injectable hydrogels have been developed using numerous natural and synthetic biomaterials.

- Natural hydrogels: The most common natural hydrogels are collagen, gelatin, laminin, matrigel, hyaluronic acid (HA), ECM, alginate and chitosan. Importantly, the structure of these compounds is highly similar to the molecules found in biological organisms, thus reducing possible harmful immunoreactions after *in vivo* implantation.
- Synthetic hydrogels include poly(ethylene glycol) (PEG), polylactide (PLA), poly(lactic-co-glycolic acid) (PLGA), poly(caprolactone) (PCL), poly(acrylamide), polyurethane, poly(*N*-isopropylacrylamide), and self-assembling peptides hydrogels, just as examples. In terms of

advantages, these polymers can be easily modulated to have physical and chemical properties suitable for cardiac tissue engineering. However, they can induce cytotoxicity. Alternatively, a mixture of natural and synthetic hydrogels can be used, combining the advantages of both types of polymers [20-22].

Pre-clinically, application of hydrogels increases cell survival in cellular cardiomyoplasty [23-32], and contributes to restoring damaged myocardium in both small and large animal models of myocardial infarction (MI) [33-38]. Interestingly, injectable hydrogels also preserve cardiac function when administrated without cells, demonstrating that the cells may not be responsible [39-54]. For instance, promising preliminary results obtained in rodents and pigs using alginate [45,46] led to the first clinical trials in MI patients (NCT00557531 and NCT01226563) [55].

Injectable hydrogels can also be used to deliver proteins, genes and nanoparticles as therapeutics themselves or to improve cell survival and retention further, prolonging the release into the target tissue and ultimately improving cardiac function after MI. To date, a number of growth factors has been delivered using this strategy, including hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1), stem cell derived factor-1 (SDF-1) and transforming growth factor beta (TGF- β 1) [56-63]. Similarly, plasmids containing VEGF and pleiotrophin have been administered as injectable hydrogels, in order to improve transfection efficiency [40].

More recently, the use of other nanomaterials in combination with therapeutic cells has been explored. As examples:

- Superparamagnetic oxide, iron oxide and ferumoxytol nanoparticles: have been developed to deliver therapeutic cells into infarcted animals. Magnetic targeting was used to direct cells with regenerative potential to damaged areas, and to enhance cell retention and engraftment [64-66]. The incorporation of iron oxide nanoparticles into microcapsules comprising MSCs, agarose, ECM proteins, collagen, and fibrin highly increases cell survival and retention [67]. Also, the use of antibodies to target exogenous therapeutic cells and endogenous injured cells has been combined with iron oxide to enhance cell targeting by magnetic attraction [68].
- Magnetic nanobeads: have been employed to deliver adenoviral vectors encoding hVEGF to enhance transduction efficiency [69].
- Heparin-presenting self-assembling peptides nanofibers: used to deliver paracrine factors derived from hypoxic conditioned stem cell media into the ischemia-reperfusion model of MI. As a result, a significant preservation of hemodynamic function is obtained [70].

20.2.3. Exosomes

Exosomes are specialised lipid membranous nano-sized vesicles released by many cell types, containing a variety of RNA species (including mRNAs, and miRNAs), soluble (cytosolic) and transmembrane proteins presented in the appropriate and functional orientation. Their intrinsic properties make exosomes an ideal therapeutic candidate in regenerative medicine, such as for MI patients [71]. Exosomes have unique tissue / cell-specific proteins that reveal their cellular source. Their functions are still unknown, but they are believed to be also important for intercellular communication. Exosomes of a particular type of cells can have a therapeutic use on a specific tissue due to their paracrine effect. As an example, delivery of MSC-derived exosomes to the myocardial ischemia / reperfusion mouse model enhanced myocardial viability and prevented adverse remodelling [72]. Moreover, extracellular vesicles derived from human bone marrow MSC promoted angiogenesis in a rat MI model [73].

20.3. SCAFFOLDS FOR CARDIAC TISSUE ENGINEERING

20.3.1. Artificial cardiac tissue

The microenvironment in which cells live is crucial for the maintenance of their basic properties and functions. In particular, the microarray signalling and response of the cells rely on their interactions with the components of the ECM where they reside [74]. This is also the case for regenerative cells. In consequence, cardiac tissue engineering hopes to profit from the formation of new 3D functional artificial heart tissue that mimics the physicochemical and physiological properties of cardiac ECM. In this context, different natural and synthetic biocompatible and biodegradable materials (Figure 1) have appeared in the experimental set.

Figure 1

Figure 1. Schematic illustration of different scaffolds used in cardiac tissue engineering. On the left, the most commonly used matrices from natural (top) and synthetic (bottom) materials are listed. Several organs from which ECM scaffolds can be obtained are shown on the right. The figure was designed and hand-drawn by C.G-M.

- Natural materials such as fibrin [75], chitosan [76], alginate [10] or collagen mixtures [77], are highly flexible, allowing different sizes and shapes according to the needs of the individual recipient and the implanted cells. For instance, fibrin patches embedded with cardiac adipose tissue-derived progenitor cells demonstrated significant improvement in cardiac function in MI murine model after 30 days of follow up (Figure 2 A-D) [75].

Figure 2. *In vivo* study results. (A) Picture of an excised mouse heart showing fibrin patch (dotted line) covering infarcted area. (B) Masson's trichrome staining cross section of cardiac ATDPCs fibrin (dotted line) treated and control animals. (C) Image exhibiting green fluorescent protein (GFP)-positive cardiac ATDPCs (green) and the presence of Isolectin B4 (white) positive vessels in the patch. (D) Histogram representing percentage of ejection fraction values of control-MI, fibrin-MI and fibrin with cardiac ATDPCs-MI animals at post-MI and pre-sacrifice time points [75]. (E) Image of an excised porcine heart, showing the pericardial descellularised scaffold (green dotted line) adhesion to myocardium after 30 days of follow up. (F) Images of two heart sections from treated and control animals exhibiting the infarct area. Zoomed-in photographs show transmurality degree differences between groups. (G) Histogram representing the mean $\pm$ scanning electron microscope (SEM) of infarcted area in control and treated animals after 1 month of follow up. (H) Green positive immunostaining against isolectin B4 (green) and elastin (white) indicates the presence of newly formed vessels (arrow) [110]. (I) Immunofluorescence against β III tubulin (green), cTnI (white), and elastin (red) labelled nerve fibres (arrows), M, myocardium and P, patch, respectively [111]. Nuclei are counterstained with DAPI (blue). Scale bars = 1 mm (A, B), 75 μ m (C), 50 μ m (H, I).

Synthetic materials: PLA [78], poly(glicolic acid) [79], PEG [80], PLGA [81], or PCL are the most commonly used for cardiac regeneration purposes [82]. They have been extensively analysed with bioreactors [83] and in animal studies and clinical trials [7,84]. But the aforementioned examples are only a short list of many existing synthetic biomaterials continuously emerging [85]. However, to date,

the U.S. Food and Drug Administration (FDA) has only approved the clinical use of PEG, PLA and PLGA [86].

Collectively, these new generated scaffolds should be porous to ensure the oxygen and nutrients' diffusion, and also should warrant the cell-cell interaction avoiding anoikis process. However, the similarity of the artificial cardiac matrix to cardiac tissue is not perfect, and the implanted cells only colonise the surface or not more than a few microns of thickness [87]. Latterly, in order to unravel this critical point, two different alternatives have appeared, as follows:

- Carbon nanotubes (CNT): have emerged as a novel alternative to improve biochemical and mechanical properties of the aforementioned materials. CNTs are composed of sheets of graphite rolled into cylindrical tubes and could be single- or multi-walled. In addition to their optimal conductive, mechanical and thermal features, CNTs are porous structures (1 nm in single-walled and 4–30 nm in multi-walled) mimicking natural collagen fibers of the ECM, favouring cell adhesion, proliferation and differentiation [88]. Regarding cardiac tissue repair, several studies have included the use of CNTs into their original scaffolds. For instance, Pok and colleagues have developed a chitosan scaffold with biocompatible CNTs acting as electrical nanobridges between cardiomyocytes. An improved electrical coupling, synchronous beating, and cardiomyocyte function were described [89]. Most recently, a CNTs scaffold has been generated to show its cytocompatibility, cell viability, attachment, proliferation and, also, cell infiltration [90].
- Electrospinning technology: has emerged to create new nanostructured scaffolds [91]. This method is based on immobilising different cell types with a wide range of molecules simultaneously within a fibre during the generation of the scaffold [92]. Interestingly, electrospinning technology can process both a small and large number of progenitor cells creating different 3D architectures. In this field of search, several studies have demonstrated the notable advantages of 3D synthetic electrospun scaffolds. As an example, Fleischer and colleagues have developed an electrospun scaffold made of PCL, dichloromethane and dimethylformamide with gold nanoparticles able to favour cell-cell coupling at the electrical level [11]. However, despite the electrospinning of synthetic polymers warranting a porous network of fibers allowing the diffusion of oxygen and nutrients, their mechanical properties do not match those of ECM, lacking the interaction between cells. In contrast, natural materials offer functional bioactive properties supporting tissue assembly, but cannot be electrospun because of their lack of viscoelasticity. Consequently, both materials are used together in search for the ideal electrospun scaffold for cardiac repair [93].

20.3.2. Extracellular matrix derived from natural tissues

ECM is a cell-secreted heterogeneous mixture composed of water, saccharides and numerous functional and structural proteins combined and spatially organised by tissue type [94,95]. Because of their importance in many processes, including cell proliferation and differentiation, guiding cell migration and modulating cellular responses, ECM is recognised as an attractive biomaterial for tissue engineering-based regenerative medicine [96]. In this context, scaffolds derived from ECM can be generated by decellularisation of biological tissue samples. This procedure removes cellular and nuclear content by physical (*i.e.* agitation, sonication, direct pressure, and freeze / thaw cycles), chemical (detergent and salts) and enzymatic treatments (*i.e.* trypsin) [97], producing 3D acellular matrices with identically retained anatomical geometry and vascular architecture [98,99]. In order to select the optimal tissue-specific decellularisation protocol, cell removal efficiency and the adequacy of ECM retention are crucial parameters to be considered [100]. In line, decellularisation affects not only ECM composition but also scaffold structure and mechanics, depending on a variety of factors such as detergent, concentration and, exposure time [101]. Importantly, collagen and laminin retention after the decellularisation process could facilitate subsequent scaffold recellularisation with regenerative cells by providing spatial orientation [100], while glycosaminoglycans and adhesive proteins removal might slow cell migration onto the scaffold and its bioactivity [101]. Before cell reseeding and surgical application, ECM-derived scaffolds need further processing, *i.e.* disinfection, lyophilisation and sterilisation, which adjust matrix integrity and architecture [98]. Preliminary results in both pre-clinical and clinical contexts show that management of allogeneic and xenogeneic ECM is safe and beneficial for cardiac tissue engineering [102].

In this context, different decellularised scaffolds from animal and human origins have been used (Figure 1) [103].

- Small intestinal submucosa: In brief, a commercial ECM patch derived from porcine small intestinal submucosa (SIS) (CorMatrix-ECM®) attenuates myocardial remodelling and improves cardiac performance in rats after MI [104]. Currently, a clinical trial is enrolling participants to evaluate its safety and functional benefit in subjects with ischemic myocardium (NCT02139189).
- Urinary bladder matrix: On the other hand, application of cell-free urinary bladder matrix (UBM) in the MI dog model increases myofibroblast recruitment, reduces inflammatory response and limits thrombus expansion, although no improvements in cardiac function, left ventricular dilation or contractility were observed [105]. Moreover, a similar study has shown increased contraction and myocyte recruitment and proliferation 8 weeks after treatment [106].

- Pericardium: Pericardial tissue is routinely resected or even removed during cardiothoracic surgery without adverse consequences [107] and shows similar properties within human individuals, presenting a porous ECM, which facilitates cell retention and vascularisation [108]. *In vitro* studies demonstrate that pericardial-derived ECM allows cell survival, growth and attachment induced pluripotent stem cells (iPSCs)-derived cardiomyocytes and MSCs [109], and also facilitates differentiation of progenitor cells derived from adipose [110] and cardiac [108] tissue towards endothelial or cardiac cell lineage, respectively. Furthermore, decellularised human pericardium embedded with self-assembling peptide RAD16-I and adipose tissue-derived progenitor cells (ATDPCs) increases scaffold vascularisation and reduces infarct size one month after implantation in the swine MI model (Figure 2 E-G) [110]. Hence, although the pericardial matrix is not an exact match to myocardial ECM, it favours cell retention and cardiac differentiation, offering an additional autologous / allogeneic therapeutic option for cardiac repair. Most recently, it has been demonstrated that an acellular decellularised pericardium implanted over infarction in swine is neovascularised and neoinnervated by host cells and improves ventricular function (Figure 2 H,I) [111].
- Myocardium: Decellularised myocardial ECM shows better preservation of the original composition, 3D-architecture and ECM microenvironment [99] than natural matrices and conserves similar native myocardium stiffness [112]. *In vitro* studies using myocardial ECM as a scaffold demonstrate cell engraftment and differentiation towards cardiac [109,112-115] and endothelial lineage [113-115], beside spontaneous recellularised scaffold contraction [112,114]. These promising findings may promote *in vivo* testing of cell reseeded decellularised myocardial ECM scaffolds for MI repair. The first challenge is combining decellularised myocardial ECM with fibrin embedded with MSCs, already released to encourage pre-clinical outcomes in the near future [116].

20.4. BIOARTIFICIAL HEARTS

In 2008, a decellularisation protocol was adapted to generate a 3D whole-heart scaffold, using sodium dodecyl sulfate (SDS) by antegrade coronary perfusion in cadaveric rat hearts [117]. Acellular generated heart preserved main matrix proteins, vascular architecture, valves and chambers and, importantly, the recellularised heart exhibited contractile activity when reseeded with cardiomyocytes and endothelial cells [117]. In recent times, further studies have reported decellularisation of porcine hearts, which is a model scalable to human proportions [118-120]. The perfusion-decellularisation procedure is particularly efficient for whole-organ decellularisation, since it reduces the diffusion distance required for decellularising agents to reach cells [121], and makes use of convective forces facilitating tissue removal of cellular components [122]. In order to improve decellularisation of whole hearts, attempts integrating a software-controlled automatic coronary perfusion system have been developed, obtaining reproducible scaffolds [123,124].

Recellularisation is a final mandatory step that involves seeding cells onto the decellularised scaffold, which could be placed in a bioreactor to support tissue growth [7]. The heart has a high cellular density, with around 10^8 cardiomyocytes/cm³, and its 3D scaffold has to be fully recellularised at the time of implantation in order to be completely functional [125]. Therefore, seeding such large numbers of cells would require several weeks of organ culturing in the bioreactor before implantation [117].

Despite whole-organ providing a promising platform for cardiac tissue engineering techniques, many unresolved issues and challenges need to be addressed before engineered hearts can be a clinical reality.

20.5. PITFALLS, CONCLUSIONS AND PERSPECTIVES

Researchers and clinicians agree in the commitment to supply new and better treatments for patients, including those suffering heart diseases. In line, the most immediate benefit of the converging research areas (*i.e.* those dedicated to stem cells, tissue engineering and nanotechnology) is essentially in regenerative medicine.

However, there are hurdles on the road ahead, regulatory as well as technical, and those related to deleterious side-effects for treated patients. For instance, although the engineering of nanomaterials holds great promise for development of innovative immunomodulatory approaches [126,127], the potential risk of their accelerating use has become relevant. Respiratory exposure to manufactured magnetic iron oxide nanoparticles generates a great number of extracellularly secreted membrane vesicles (exosomes) capable of transferring activation signals to the immune system [128]. In particular, in those individuals who already have pre-existing allergic conditions (known as

sensitised individuals), the generated exosomes may result in a delayed type of hypersensitivity reaction and subsequent severe allergic responses; whereas in unsensitised individuals, the resulting immune activation response may be much lower. Furthermore, a key role of nanoparticle-induced exosomes as signalling mediators in the induction of immune activation *via* T helper cell type 1 has been reported [129]. Taken together, these studies suggest that the generated exosomes aggravate the immune activation and inflammatory responses induced by exposure to nanoparticles. Alternatively, in order to regulate potential harmful immune response *in vivo* further, nanoparticles could be co-administered with immunosuppressive cells or cell-free immunomodulatory agents [130,131].

In sum, cell- and tissue engineering-based therapies involving nanostructure materials are exciting exploration areas, and have shown both intriguing and instructive preliminary results in the treatment of cardiac diseases. However, as clinical trials proceed, our incomplete understanding of the behaviour and functions of regenerative cells and associated nanoparticles is made evident by numerous unresolved concerns, including the optimal therapeutic strategy that can be readily translated to patients.

REFERENCES

1. M. Rao. *Stem Cell. Res. Ther.* **3** (2012) 27.
2. M. Rao, C. Mason, S. Solomon. *Regen. Med.* **10** (2015) 181–191.
3. Stem cell report: Trends and Perspectives on the Evolving International Landscape. EuroStemCell, Kyoto University's Institute for Integrated Cell-Material Sciences (WPI-iCeMS) and Elsevier, Dec 2013.
4. World Health Organization: The top 10 causes of death.
<http://www.who.int/mediacentre/factsheets/fs310/en/> (15/04/2015).
5. C. Gálvez-Montón, J. Ordoñez-Llanos, A.B. de Luna, A. Bayes-Genis. *Eur. Heart J.* **33** (2012) 2888–2891.
6. C. Soler-Botija, J.R. Bagó, A. Bayes-Genis. *Ann. N. Y. Acad. Sci.* **1254** (2012) 57–65.
7. J.C. Garbern, R.T. Lee. *Cell. Stem Cell* **12** (2013) 689–698.
8. C. Gálvez-Montón, C. Prat-Vidal, S. Roura, C. Soler-Botija, A. Bayes-Genis. *Rev. Esp. Cardiol.* **66** (2013) 391–399.
9. J.N. Mackle, D.J. Blond, E. Mooney, C. McDonnell, W.J. Blau, G. Shaw, F.P. Barry, J.M. Murphy, V. Barron. *Macromol. Biosci.* **11** (2011) 1272–1282.
10. E. Mooney, J.N. Mackle, D.J. Blond, E. O'Cearbhaill, G. Shaw, W.J. Blau, F.P. Barry, V. Barron, J.M. Murphy. *Biomaterials* **33** (2012) 6132–6139.
11. T. Dvir, B.P. Timko, M.D. Brigham, S.R. Naik, S.S. Karajanagi, O. Levy, H. Jin, K.K. Parker, R. Langer, D.S. Kohane. *Nat. Nanotechnol.* **6** (2011) 720–725.
12. S. Fleischer, M. Shevach, R. Feiner, T. Dvir. *Nanoscale* **6** (2014) 9410–9414.
13. M. Shevach, S. Fleischer, A. Shapira, T. Dvir. *Nano Lett.* **14** (2014) 5792–5796.
14. T. Shimizu, M. Yamato, Y. Isoi, T. Akutsu, T. Setomaru, K. Abe, A. Kikuchi, M. Umezue, T. Okano. *Circ. Res.* **90** (2002) e40–e48.

15. Y. Miyahara, N. Nagaya, M. Kataoka, B. Yanagawa, K. Tanaka, H. Hao, K. Ishino, H. Ishida, T. Shimizu, K. Kangawa, S. Sano, T. Okano, S. Kitamura, H. Mori. *Nat. Med.* **12** (2006) 459–466.
16. S. Masuda, T. Shimizu, M. Yamato, T. Okano. *Adv. Drug. Deliv. Rev.* **60** (2008) 277–285.
17. W.S. Turner, N. Sandhu, K.E. McCloskey. *J. Vis. Exp.* **3** (2014) e51044.
18. K.R. Stevens, L. Pabon, V. Muskheli, C.E. Murray. *Tissue Eng. Part A* **15** (2009) 1211–1222.
19. L.W. Xia, R. Xie, X.J. Ju, W. Wang, Q. Chen, L.Y. Chu. *Nat. Commun.* **4** (2013) 2226.
20. M. Radisic, K.L. Christman. *Mayo Clin. Proc.* **88** (2013) 884–898.
21. G. Vunjak-Novakovic, K.O. Lui, N. Tandon, K.R. Chien. *Annu. Rev. Biomed. Eng.* **13** (2011) 245–267.
22. J. Radhakrishnan, U.M. Krishnan, S. Sethuraman. *Biotechnol. Adv.* **32** (2014) 449–461.
23. K.L. Christman, A.J. Vardanian, Q. Fang, R.E. Sievers, H.H. Fok, R.J. Lee. *J. Am. Coll. Cardiol.* **44** (2004) 654–660.
24. J.S. Nakamuta, M.E. Danoviz, F.L.N. Marques, L. dos Santos, C. Becker, G.A. Goncalves, P.F. Vassallo, I.T. Schetterert, P.J.F. Tucci, J.E. Krieger. *PLoS One* **4** (2009) e6005.
25. H.D. Guo, H.J. Wang, Y.Z. Tan, J.H. Wu. *Tissue Eng. Part A* **17** (2011) 45–58.
26. M.E. Danoviz, J.S. Nakamuta, F.L.N. Marques, L. dos Santos, E.C. Alvarenga, A.A. dos Santos, E.L. Antonio, I.T. Schetterert, P.J. Tucci, J.E. Krieger. *PLoS One* **5** (2010) e12077.
27. X. Zhang, H. Wang, X. Ma, A. Adila, B. Wang, F. Liu, B. Chen, C. Wang, Y. Ma. *Exp. Biol. Med.* **235** (2010) 1505–1515.
28. T. Kofidis, D.R. Lebl, E.C. Martinez, G. Hoyt, M. Tanaka, R.C. Robbins. *Circulation* **112** (2005) 1173–1177.
29. T. Wang, X.J. Jiang, Q.Z. Tang, X.Y. Li, T. Lin, D.Q. Wu, X.Z. Zhang, E. Okello. *Acta Biomater.* **5** (2009) 2939–2944.
30. S.T. Wall, C.C. Yeh, R.Y. Tu, M.J. Mann, K.E. Healy. *J. Biomed. Mater. Res. A* (2010) **95** 1055–1066.
31. Y.D. Lin, M.L. Yeh, Y.J. Yang, D.C. Tsai, T.Y. Chu, Y.Y. Shih, M.Y. Chang, Y.W. Liu, A.C.L. Tang, T.Y. Chen, C-Y. Luo, K.C. Chang, J.H. Chen, H.L. Wu, T.K. Hung, P.C.H. Hsieh. *Circulation* (2010) S132–S141.
32. W.N. Lu, S.H. Lu, H.B. Wang, D.X. Li, C.M. Duan, Z.Q. Liu, T. Whao, W.J. He, B. Xu, Q. Fu, Y.C. Song, X.H. Xie, C.Y. Wang. *Tissue Eng. Part A* **15** (2009) 1437–1447.
33. E.T. Roche, C.L. Hastings, S.A. Lewin, D.E. Shvartsman, Y. Brudno, N.V. Vasilyev, F.J. O'Brien, C.J. Walsh, G.P. Duffy, D.J. Mooney. *Biomaterials* **35** (2014) 6850–6858.
34. J. Chen, R. Guo, Q. Zhou, T. Wang. *J. Med. Sci.* **30** (2014) 173–180.
35. C.H. Chen, M.Y. Chang, S.S. Wang, P.C. Hsieh. *Am. J. Physiol. Heart Circ. Physiol.* **306** (2014) H1078–H1086.
36. C.C. Huang, Z.X. Liao, D.Y. Chen, C.W. Hsiao, Y. Chang, H.W. Sung. *Adv. Healthc. Mater.* **3** (2014) 1133–1148.
37. H. Wang, J. Shi, Y. Wang, Y. Yin, L. Wang, J. Liu, Z. Liu, C. Duan, P. Zhu, C. Whang. *Biomaterials* **35** (2014) 3986–3998.
38. T. Wang, X.J. Jiang, Q.Z. Tang, X.Y. Li, T. Lin, D.Q. Wu, X.Z. Zhang, E. Okello. *Acta Biomater.* **5** (2009) 2939–2944.

39. K.L. Christman, H.H. Fok, R.E. Sievers, Q. Fang, R.J. Lee. *Tissue Eng.* **10** (2004) 403–409.
40. A.A. Rane, K.L. Christman. *J. Am. Coll. Cardiol.* **58** (2011) 2615–2629.
41. H. Wang, J. Zhou, Z. Liu, C. Wang. *J. Cell Mol. Med.* **14** (2010) 1044–1055.
42. D.M. Nelson, Z. Ma, K.L. Fujimoto, R. Hashizume, W.R. Wagner. *Acta Biomater* **7** (2011) 1–15.
43. E. Tous, B. Purcell, J.L. Ifkovits, J.A. Burdick. *J. Cardiovasc. Trans. Res.* **4** (2011) 528–542.
44. T.D. Johnson, K.L. Christman. *Expert Opin. Drug Deliv.* **10** (2012) 59–72.
45. N. Landa, L. Miller, M.S. Feinberg, R. Holbova, M. Shachar, I. Freeman, S. Cohen, J. Leor. *Circulation* **117** (2008) 1388–1396.
46. J. Leor, S. Tuvia, V. Guetta, F. Manczur, D. Castel, U. Willenz, O. Petnehazy, N. Landa, M.S. Feinberg, E. Konen, O. Goitein, O. Tsur-Gang, M. Shaul, L. Klapper, S. Cohen. *J. Am. Coll. Cardiol.* **54** 2009 1014–1023.
47. J.M. Singelyn, P. Sundaramurthy, T.D. Johnson, P.J. Schup-Magoffin, D.P. Hu, D.M. Faulk, J. Wang, K.M. Mayle, K. Bartels, M. Salvatore, A.M. Kinsey, A.N. Demaria, N. Dib, K.L. Christman. *J. Am. Coll. Cardiol.* **59** (2012) 751–763.
48. R.J. Laham, M. Rezaee, M. Post, F.W. Sellke, R.A. Braeckman, D. Hung, M. Simons. *Drug Metab. Dispos.* **27** (1999) 821–826.
49. R.J. Laham, N.A. Chronos, M. Pike, M.E. Leimbach, J.E. Udelson, J.D. Pearlman, R.I. Pettigrew, M.J. Whitehouse, C. Yoshizawa, M. Simons. *J. Am. Coll. Cardiol.* **36** (2000) 2132–2139.
50. K. Krause, K. Jaquet, C. Schneider, S. Haupt, M.V. Lioznov, K.M. Otte, K.H. Kuck. *Heart* **95** (2009) 1145–1152.
51. J.M. Singelyn, J.A. DeQuach, S.B. Seif-Naraghi, R.B. Littlefield, P.J. Schup-Magoffin, K.L. Christman. *Biomaterials* **30** (2009) 5409–5416.
52. H.D. White, R.M. Norris, M.A. Brown, P.W. Brandt, R.M. Whitlock, C.J. Wild. *Circulation* **76** (1987) 44–51.
53. D.D. McManus, S.J. Shah, M.R. Fabi, A. Rosen, M.A. Whooley, N.B. Schiller. *J. Am. Soc. Echocardiogr.* **22** (2009) 190–197.
54. J.L. Ifkovits, E. Tous, M. Minakawa, M. Morita, J.D. Robb, K.J. Koomalsingh, J.H. Gorman 3rd, R.C. Gorman, J.A. Burdick. *Proc. Natl. Acad. Sci. USA* **107** (2010) 11507–11512.
55. Clinical Trials, <https://clinicaltrials.gov> (04/04/2015).
56. S. Koudstaal, M.M. Bastings, D.A. Feyen, C.D. Waring, F.J. van Slochteren, P.Y. Dankers, D. Torella, J.P. Sluijter, B. Nadal-Ginard, P.A. Doevendans, G.M. Ellison, S.A. Chamuleau. *J. Cardiovasc. Transl. Res.* **7** (2014) 232–241.
57. M. Song, H. Jang, J. Lee, J.H. Kim, S.H. Kim, K. Sun, Y. Park. *Biomaterials* **35** (2014) 2436–2445.
58. A.J. Rufaihah, S.R. Vaibavi, M. Plotkin, J. Shen, V. Nithya, J. Wang, D. Seliktar, T. Kofidis. *Biomaterials* **34** (2013) 8195–8202.
59. J.C. Garbern, E. Minami, P.S. Stayton, C.E. Murry. *Biomaterials* **32** (2011) 2407–2416.
60. X. Hao, E.A. Silva, A. Månsson-Broberg, K.H. Grinnemo, A.J. Siddiqui, G. Dellgren, E. Wårdell, L.A. Brodin, D.J. Mooney, C. Sylvén. *Cardiovasc. Res.* **75** (2007) 178–185.
61. M.A. Laflamme, K.Y. Chen, A.V. Naumova, V. Muskheli, J.A. Fugate, S.K. Dupras, H. Reinecke, C. Xu, M. Hassanipour, S. Police, C. O'Sullivan, L. Collins, Y. Chen,

- E. Minami, E.A. Gill, S. Ueno, C. Yuan, J. Gold, C.E. Murry. *Nat. Biotechnol.* **25** (2007) 1015–1024.
62. Z.X. Lu, L.L. Mao, F. Lian, J. He, W.T. Zhang, C.Y. Dai, S. Xue, W.G. Lu, H.S. Zhu. *BMC Cardiovasc. Disord.* **14** (2014) 53.
63. M.Y. Chang, Y.J. Yang, C.H. Chang, A.C. Tang, W.Y. Liao, F.Y. Cheng, C.S. Yeh, J.J. Lai, P.S. Stayton, P.C. Hsieh. *J. Control. Release* **170** (2013) 287–294.
64. Z. Huang, Y. Shen, A. Sun, G. Huang, H. Zhu, B. Huang, J. Xu, Y. Song, N. Pei, J. Ma, X. Yang, Y. Zou, J. Qian, J. Ge. *Stem Cell Res. Ther.* **4** (2013) 149.
65. J. Han, B. Kim, J.Y. Shin, S. Ryu, M. Noh, J. Woo, J.S. Park, Y. Lee, N. Lee, T. Hyeon, D. Choi, B.S. Kim. *ACS Nano* **9** (2015) 2805–2819.
66. A.C. Vandergriff, T.M. Hensley, E.T. Henry, D. Shen, S. Anthony, J. Zhang, K. Cheng. *Biomaterials* **35** (2014) 8528–8539.
67. A. Blocki, S. Beyer, J.Y. Dewavrin, A. Goralczyk, Y. Wang, P. Peh, M. Ng, S.S. Moonshi, S. Vuddagiri, M. Raghunath, E.C. Martinez, K.K. Bhakoo. *Biomaterials* **53** (2015) 12–24.
68. K. Cheng, D. Shen, M.T. Hensley, R. Middleton, B. Sun, W. Liu, G. De Couto, E. Marbán. *Nat. Commun.* **5** (2014) 4880.
69. Y. Zhang, W. Li, L. Ou, W. Wang, E. Delyagina, C. Lux, H. Sorg, K. Riehemann, G. Steinhoff, N. Ma. *PLoS One* **7** (2012) e39490.
70. M.J. Webber, X. Han, S.N. Murthy, K. Rajangam, S.I. Stupp, J.W. Lomasney. *J. Tissue Eng. Regen. Med.* **4** (2010) 600–610.
71. O.G. De Jong, B.W. Van Balkom, R.M. Schiffelers, C.V. Bouten, M.C. Verhaar. *Front. Immunol.* **5** (2014) 608.
72. F. Arslan, R.C. Lai, M.B. Smeets, L. Akeroyd, A. Choo, E.N. Aguior, L. Timmers, H.V. van Rijen, P.A. Doevendans, G. Pasterkamp, S.K. Lim, D.P. de Kleijn. *Stem Cell Res.* **10** (2013) 301–312.
73. S. Bian, L. Zhang, L. Duan, X. Wang, Y. Min, H. Yu. *J. Mol. Med.* **92** (2014) 387–397.
74. S. Pernagallo, J.J. Diaz-Mochon, M.A. Bradley. *Lab. Chip.* **9** (2009) 397–403.
75. J.R. Bagó, C. Soler-Botija, L. Casaní, E. Aguilar, M. Alieva, N. Rubio, A. Bayes-Genis, J. Blanco. *Int. J. Cardiol.* **169** (2013) 288–295.
76. L. Altomare, E. Guglielmo, E.M. Varoni, S. Bertoldi, A. Cochis, L. Rimondini, L. De Nardo. *Biomater.* **4** (2014) e29506.
77. A. Gishto, K. Farrell, C.R. Kothapalli. *J. Biomed. Mater. Res. A* **103** (2015) 693–708.
78. A. Behfar, S. Yamada, R. Crespo-Diaz, J.J. Nesbitt, L.A. Rowe, C. Perez-Terzic, V. Gaussin, C. Homsy, J. Bartunek, A. Terzic. *J. Am. Coll. Cardiol.* **56** (2010) 721–734.
79. D.A. Stout, B. Basu, T.J. Webster. *Acta. Biomater.* **7** (2011) 3101–3112.
80. S. Dhingra, R.D. Weisel, R.K. Li. *Methods Mol. Biol.* **1181** (2014) 51–59.
81. J. Yu, A.R. Lee, W.H. Lin, C.W. Lin, Y.K. Wu, W.B. Tsai. *Tissue Eng. Part A* **20** (2014) 1896–1907.
82. C. Soler-Botija, J.R. Bagó, A. Llucià-Valldeperas, A. Vallés-Lluch, C. Castells-Sala, C. Martínez-Ramos, T. Fernández-Muiños, J.C. Chachques, M.M. Pradas, C.E. Semino, A. Bayes-Genis. *Am. J. Transl. Res.* **6** (2014) 291–301.
83. M. Radisic, A. Marsano, R. Maidhof, Y. Wang, G. Vunjak-Novakovic. *Nat. Protoc.* **3** (2008) 719–738.
84. L.A. Reis, L.L. Chiu, N. Feric, L. Fu, M. Radisic. *J. Tissue Eng. Regen. Med.* (2014) [Epub ahead of print].

85. Q.Z. Chen, S.E. Harding, N.N. Ali, A.R. Lyon, A.R. Boccaccini. *Mater. Sci. Eng. R* **59** (2008) 1–37.
86. Z. Li, J. Guan. *Polymers* **32** (2011) 740–761.
87. T. Eschenhagen, A. Eder, I. Vollert, A. Hansen. *Am. J. Physiol. Heart Circ. Physiol.* **303** (2012) H133–H143.
88. E.L. Hopley, S. Salmasi, D.M. Kalaskar, A.M. Seifalian. *Biotechnol. Adv.* **32** (2014) 1000–1014.
89. S. Pok, F. Vitale, S.L. Eichmann, O.M. Benavides, M. Pasquali, J.G. Jacot. *ACS Nano* **8** (2014) 9822–9832.
90. G. Lalwani, A. Gopalan, M. D'Agati, J. Srinivas Sankaran, S. Judex, Y.X. Qin, B. Sitharaman. *J. Biomed. Mater. Res. A* (2015) [Epub ahead of print].
91. V. Beachley, V. Kasyanov, A. Nagy-Mehesz, R. Norris, I. Ozolanta, M. Kalejs, P. Stradins, L. Baptista, K. da Silva, J. Grainjero, X. Wen, V. Mironov. *J. Tissue Eng.* **5** (2014) 2041731414556561.
92. E. Ehler, S.N. Jayasinghe. *Analyst* **139** (2014) 4449–4452.
93. R. Lakshmanan, U.M. Krishnan, S. Sethuraman. *Expert Opin. Biol. Ther.* **12** (2012) 1623–1640.
94. G.W. Laurie, S. Horikoshi, P.D. Killen, B. Segui-Real, Y. Yamada. *J. Cell. Biol.* **109** (1989) 1351–1362.
95. H.S. Baldwin. *Cardiovasc. Res.* **31** (1996) E34–E45.
96. K.S. Midwood, L.V. Williams, J.E. Schwarzbauer. *Int. J. Biochem. Cell Biol.* **36** (2004) 1031–1037.
97. T.W. Gilbert. *J. Cell. Biochem.* **113** (2012) 2217–2222.
98. S.F. Badylak, D.O. Freytes, T.W. Gilbert. *Acta. Biomater.* **5** (2009) 1–13.
99. P.M. Crapo, T.W. Gilbert, S.F. Badylak. *Biomaterials* **32** (2011) 3233–3243.
100. M.E. Scarritt, N.C. Pashos, B.A. Bunnell. *Front. Bioeng. Biotechnol.* **3** (2015) 43.
101. T.W. Gilbert, T.L. Sellar, S.F. Badylak. *Biomaterials* **27** (2006) 3675–3683.
102. T.J. Keane, S.F. Badylak. *J. Tissue Eng. Regen. Med.* **9** (2015) 504–511.
103. I. Perea-Gil, C. Prat-Vidal, A. Bayes-Genis. *Stem Cell Res. Ther.* (2015) In Press.
104. H.E. Mewhort, J.D. Turnbull, H.C. Meijndert, J.M. Ngu, P.W. Fedak. *J. Thorac. Cardiovasc. Surg.* **147** (2014) 1650–1659.
105. K.A. Robinson, J. Li, M. Mathison, A. Redkar, J. Cui, N.A. Chronos, R.G. Matheny, S.F. Badylak. *Circulation* **112** (2005) I135–I143.
106. D.J. Kelly, A.B. Rosen, A.J. Schuldt, P.V. Kochupura, S.V. Doronin, I.A. Potapova, E.U. Azeloglu, S.F. Badylak, R.R. Brink, I.S. Cohen, G.R. Gaudette. *Tissue Eng. Part A* **15** (2009) 2189–2201.
107. V. Fuster, R.W. Alexander, R.A. O'Rourke (Eds.), *Hurst's The Heart, 10th Ed.*, NY: McGraw-Hill Medical Publishing Division, New York, 2001.
108. S.B. Seif-Naraghi, M.A. Salvatore, P.J. Schup-Magoffin, D.P. Hu, K.L. Christman. *Tissue Eng. Part A* **16** (2010) 2017–2027.
109. B. Oberwallner, A. Brodarac, P. Anić, T. Šarić, K. Wassilew, K. Neef, Y.H. Choi, C. Stamm. *Eur. J. Cardiothorac. Surg.* **47** (2015) 416–425.
110. C. Prat-Vidal, C. Gálvez-Montón, V. Puig-Sanvicens, B. Sanchez, I. Díaz-Güemes, P. Bogónez-Franco, I. Perea-Gil, A. Casas-Solà, S. Roura, A. Lluçia-Valldeperas, C. Soler-Botija, F.M. Sánchez-Margallo, C.E. Semino, R. Bragos, A. Bayes-Genis. *Int. J. Cardiol.* **174** (2014) 654–661.
111. C. Gálvez-Montón, M.T. Fernandez-Figueras, M. Martí, C. Soler-Botija, S. Roura, I. Perea-Gil, C. Prat-Vidal, A. Lluçia-Valldeperas, Á. Raya, A. Bayes-Genis. *Stem Cell Res. Ther.* **6** (2015) 108.

112. Y. Eitan, U. Sarig, N. Dahan, M. Machluf. *Tissue Eng. Part C Methods* **16** (2010) 671–683.
113. I. Perea-Gil, J. Uriarte, C. Prat-Vidal, C. Gálvez-Montón, S. Roura, A. Lluçà-Valldeperas, C. Soler-Botija, R. Farré, D. Navajas, A. Bayes-Genis. *Am. J. Transl. Res.* **7** (2015) 558–573.
114. T.Y. Lu, B. Lin, J. Kim, M. Sullivan, K. Tobita, G. Salama, L. Yang. *Nat. Commun.* **4** (2013) 2307.
115. B. Wang, A. Borazjani, M. Tahai, A.L. Curry, D.T. Simionescu, J. Guan, F. To, S.H. Elder, J. Liao. *J. Biomed. Mater. Res. A* **94** (2010) 1100–1110.
116. A.F. Godier-Furnémont, T.P. Martens, M.S. Koeckert, L. Wan, J. Parks, K. Arai, G. Zhang, B. Hudson, S. Homma, G. Vunjak-Novakovic. *Proc. Natl. Acad. Sci. USA* **108** (2011) 7974–7979.
117. H.C. Ott, T.S. Matthiesen, S.K. Goh, L.D. Black, S.M. Kren, T.I. Netoff, D.A. Taylor. *Nat. Med.* **14** (2008) 213–221.
118. J.M. Wainwright, C.A. Czajka, U.B. Patel, D.O. Freytes, K. Tobita, T.W. Gilbert, S.F. Badylak. *Tissue Eng. Part C Methods* **16** (2010) 525–532.
119. A. Weymann, S. Loganathan, H. Takahashi, C. Schies, B. Claus, K. Hirschberg, P. Soós, S. Korkmaz, B. Schmack, M. Karck, G. Szabó. *Circ. J.* **75** (2011) 852–860.
120. A. Weymann, N.P. Patil, A. Sabashnikov, P. Jungebluth, S. Korkmaz, S. Li, G. Veres, P. Soos, R. Ishtok, N. Chaimow, I. Pätzold, N. Czerny, C. Schies, B. Schmack, A.F. Popov, A.R. Simon, M. Karck, G. Szabo. *PLoS One* **9** (2014) e111591.
121. F. Moroni, T. Mirabella. *Am. J. Stem Cells* **3** (2014) 1–20.
122. A. Soto-Gutierrez, J.A. Wertheim, H.C. Ott, T.W. Gilbert. *J. Clin. Invest.* **122** (2012) 3817–3823.
123. P. Akhyari, H. Aubin, P. Gwanmesia, M. Barth, S. Hoffmann, J. Huelsmann, K. Preuss, A. Lichtenberg. *Tissue Eng. Part C Methods* **17** (2011) 915–926.
124. H. Aubi, A. Kranz, J. Hülsmann, A. Lichtenberg, P. Akhyari. *Methods Mol. Biol.* **1036** (2013) 163–178.
125. G. Vunjak-Novakovic, N. Tandon, A. Godier, R. Maidhof, A. Marsano, T.P. Martens, M. Radisic. *Tissue Eng. Part B Rev* **16** (2010) 169–187.
126. D.M. Smith, J.K. Simon, J.R. Baker Jr. *Nat. Rev. Immunol.* **13** (2013) 592–605.
127. B.S. Zolnik, A. González-Fernández, N. Sadrieh, M.A. Dobrovolskaia. *Endocrinology* **151** (2010) 458–465.
128. M. Zhu, Y. Li, J. Shi, W. Feng, G. Nie, Y. Zhao. *Small* **6** (2012) 404–412.
129. M. Zhu, X. Tian, X. Song, Y. Li, Y. Tian, Y. Zhao, G. Nie. *Small* **8** (2012) 2841–2848.
130. I. Perea-Gil, M. Monguió-Tortajada, C. Gálvez-Montón, A. Bayes-Genis, F.E. Borràs, S. Roura. *Biomed. Res. Int.* **2015** (2015) 439808.
131. S. Rani, A.E. Ryan, M.D. Griffin, T. Ritter. *Mol. Ther.* **23** (2015) 812–823.

LIVER TISSUE ENGINEERING

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21.1. INTRODUCTION

The only solution for chronic, final-stage liver diseases and acute liver failure is liver transplantation, which is long-term and radical. The causes of these diseases are genetic, toxic, congenital, parasitic, iatrogenic, *etc.* However, the demand far exceeds the number of donors for liver transplantation, as can be seen by analysing waiting lists. In addition, there are religious limitations in some situations that reduce the options in the case of deceased donors.

Various ways to save organs available for donation have been sought: split-liver, domino transplant, living-related transplant, marginal transplant, *etc.* In addition to the lack of organs, there are complications in the post-transplant period that can compromise the graft and are primarily due to the body's immune reaction or immunosuppressive treatment administered to limit the immune response of the body against the graft.

Long waiting lists for organ transplants (liver, kidney, heart and lung) and reduced availability of organs from donors has led to further research in order to obtain organs from other sources.

There were initial hopes regarding transplants from animals to humans (xenotransplantation), but most recent research has focused on obtaining artificial organs.

A number of solutions have been developed utilising extracorporeal liver filtration membranes or absorption systems to remove toxins from the blood. Of these, the most common are molecular adsorbents recirculating system (MARS), liver dialysis devices and Prometheus. BioArtificial Liver (BAL) systems are also extracorporeal systems that perform detoxification, metabolic and synthesis functions. These systems are composed of primary hepatocyte or hepatoma cell lines and membranes that separate functional hepatocytes from the patient's plasma. The best known are HepatAssist and extracorporeal liver assist device (ELAD).

To obtain quality synthetic organs, it is necessary to have a scaffold structure compatible with the host organism and the cells to repopulate it. Stem cells have a promising potential in terms of developing *in vitro* organs due to the proliferative activity and the potential of differentiation into almost any type of mature cell (Figure 1). Stem cells have shown that they have the ability to differentiate into mature liver cells [1-3].

Figure 1. Classic approach to tissue engineering
(from Tiruvannamalai-Annamalai *et al.* 2014) [4]

There are also new developments in tissue engineering such as the modular approach (Figure 2).

Figure 2. Modular tissue engineering (from Tiruvannamalai-Annamalai *et al.* 2014) [4]

Current problems researchers are facing are a lack of a compatible and functional scaffold structures for *in vitro* liver development, a lack of selected stem cells, and immunological barriers, which also constitute an impediment for the construction of synthetic organs.

However, research conducted so far in this area constitutes an important step in obtaining synthetic organs.

Essential characteristics of engineered liver tissue are to perform the essential functions of the liver: plasma protein synthesis; ketogenesis; synthesis of urea; detoxification; and immune function.

The liver is composed mostly of hepatocytes (approx. 70 % of the cell population), Kupffer cells, progenitor cells (Ito cells), stellate cells, biliary epithelial cells, sinusoidal epithelial cells and fibroblasts [5].

21.2. METHODS TO OBTAIN A CELL POPULATION SUITABLE FOR RECELLULARISING THE MATRIX

The source of the cells is particularly important because they must have the potential to differentiate into mature hepatocytes. Different cell types have been tested for liver fibrous scaffold repopulation: hematopoietic cells, stem cells, Ito cells and hepatocyte mature cells [6-8].

Initial experiments were performed with mature liver cells. Mature hepatocytes in laboratory conditions did not sufficiently multiply, nor did they retain their differentiation characteristics [9].

Subsequently, researchers sought other sources for cells to repopulate matrices, testing hematopoietic stem cells from bone marrow, known for their superior plasticity to differentiate into various types of mature cells. Adult stem cells from bone marrow mesenchymal stem cells (BMSCs) can easily be harvested *via* bone marrow puncture, multiplied *in vitro*, and oriented towards subsequent differentiation into mature hepatocytes for liver scaffold repopulation. Adult stem cells are preferred to embryonic stem cells because there are a series of limitations relating to the use of the latter in therapy in humans, including ethical issues.

A number of inductive environmental factors dictate the stem cell differentiation. The cells in the immediate vicinity or scaffold characteristics can also guide the subsequent differentiation that the cell will follow.

Paracrine factors influencing hepatocyte differentiation and cell growth are hepatocyte growth factor (HGF), fibroblastic growth factor (FGF), activin, bone morphogenesis protein (BMP), oncostatin M (OSM), epidermal growth factor (EGF), interleukin-6 (IL-6), transforming growth factor alpha (TGF- α), and insulin growth factor (IGF). There are also drugs that affect hepatocyte differentiation: dimethyl sulfoxide (DMSO), steroids, amino acids, and nicotinamide. Intracellular regulators of gene expression involved in hepatocyte differentiation are known as the following hepatocyte nuclear factors (HNFs): 3 α , β ; 4 α ; 1 α , β ; 6 [10-14].

Controlling all these factors can lead to the better targeting of stem cell differentiation.

Li *et al.* argued that a three-dimensional structure of collagen-modified poly(lactic-co-glycolic acid) (PLGA) favoured growth and the differentiation of BMSCs towards hepatocyte [15].

21.3. METHODS TO OBTAIN AN ORGAN MATRIX

The organ scaffold has a number of roles; it provides a supporting structure, configures the organ in space, guides cell proliferation and differentiation, maintains cells phenotype and apoptosis by facilitating, sensing and responding to the external environment, and maintains optimal distances between cells to allow the diffusion of nutrients and oxygen [16].

Both biological and synthetic matrices have been tested for tissue regeneration.

The support matrix is intended to provide organ-specific elastic-mechanical characteristics, develop vasculature necessary to sustain tissue energy, develop the biliary tract, and provide the specific configuration of the tissue and organ.

The matrix used should be biocompatible and, for synthetic matrices, should be biodegradable with no release of toxic elements into the body.

If a resorbable (biodegradable) polymer matrix is used, it will be degraded over time and replaced with autologous tissue, reducing the foreign body inflammatory reaction [17].

A number of features of the matrix influence the characteristics of the organ: the macroscopic structure – including its appearance and surface coating, method of attachment, inflow and outflow regions of blood and bile, and vascular branching inside the organ; and the microscopic structure – where intercellular distances are evaluated, cell growth support, and the diffusion possibility of nutrients and oxygen.

Natural matrices may be formed from collagen, gelatine, matrigel, and fibronectin, and are suitable for cell growth characteristics, but have poor mechanical properties and are difficult to handle. In addition, a change of the properties of these matrices is difficult and depends on the tissue of origin [18-21].

Natural matrixes were obtained by decellularisation of the organs, maintaining functional molecules and their original, three-dimensional structure, thus being ideal as a scaffold for reconstructing new organs (Figure 3) [22,23]. Decellularisation may be accomplished by physical (agitation, freezing, thawing, pressure, mechanical massage, sonication) or chemical methods (ionic detergent, nonionic, zwitterionic, acidic or alkaline treatments, chelating agents, and hypertonic and hypotonic treatments). For this purpose, an enzyme treatment can also be used (trypsin, endonuclease or exonuclease) [23]. Often, these methods are combined in successive sequences.

Figure 3. Decellulariaation and recellularisation of the explanted liver (from Struecker *et al.* 2014) [24]

Figure 4. Ferret decellularised liver (from Booth *et al.* 2012) [25]

Some authors use scaffold obtained from animal organs (Figure 4); however, with this method, care must be taken to eliminate all immunological stimuli from the scaffolds [26-31].

Synthetic matrices may have varying physical and mechanical properties according to the initial design, including the microscopic and macroscopic structure, which can be configured as needed. In addition, the rate of degradation of synthetic supporting structures can be adjusted as required by changing the polymer composition used [5]. However, at present, it is difficult to simulate perfectly the Nature, with blood vessels, bile ducts, extracellular spaces, *etc.*

There are also fluid matrixes that encapsulate cells and suspend them in the liquid medium (living scaffolds).

Various kinds of materials have been used, such as polyesters, hydrogels, synthetic polypeptides, *etc.*: poly(lactic acid) (PLA); poly(glicolic acid) (PGA); PLGA; polycaprolactone (PCL).

The disadvantages of using these polymers include a lack of stimulation of cell proliferation due to their hydrophobic nature and a lack of recognition of cellular signals [5]. In order to overcome these drawbacks, bioactive molecules are attached to the surface of inert polymers [32,33].

To create a similar structure of the ECM, nanostructured fibres created by electrospinning were used [34]. Kazemnejad *et al.* (2007) created a matrix of poly(ϵ -caprolactone), collagen and poly(ether sulphone) *via* electrospinning [34].

A synthetic scaffold can be used either to be seeded with cells or to provide support for tissue regeneration. The first attempts to generate a scaffold has a limited ability to control the microscopic structure. Various physico-chemical techniques can be used: photolithography, two-photon polymerisation, chemical vapour deposition, foaming, membrane lamination, electrospinning, *etc.* These techniques do not allow to control of porosity and intercommunication between networks of ducts and have no reproducible structure. These drawbacks can be overcome by rapid prototyping 3D printing techniques or fused deposition modelling (FDM).

Singare *et al.* described a method of achieving a basic three-dimensional structure of the liver by computer design in computer-aided design (CAD) and generation by 3D printing using a PolyJet 3D printer. The material used for the mould was poly(dimethyl-siloxane) (PDMS), which was later used for structure creation from biodegradable material [35].

Feng *et al.* developed a galactosylated chitosan (GC) nanofibers scaffold for enhanced properties of 3D hepatocytes growing [36].

21.4. METHODS FOR RECELLULARISING THE SCAFFOLDS

In order to populate the scaffold with cultured cells, various techniques are used. In most cases, the simple immersion in cell suspension is not enough. Populating the scaffold is done predominantly on the surface, which creates uneven results. A colloidal solution containing the cells is injected into the scaffold to populate it. Some authors use an intermittent vacuum in order to aspirate the suspension into the scaffold pores [15].

To further grow and differentiate these cells in a three-dimensional environment, specific growth factors should be administered. Hydrogels or slow release microspheres containing these growth factors can be used. The delivery systems can be filled with genes or peptides that accelerate cell proliferation. Using a protein-composed scaffold is useful because of cell recognition sites localised on protein surfaces that form the matrix [37-40].

It has been shown that the interaction between cells and the scaffold plays an important role in further differentiating the cells. In this interaction, the matrix

topography and surface radicals (or recognition sites) are essential. To modulate this interaction, one can include growth factors and chemical radicals in the matrix, which may favour cell immobilisation (*e.g.*, arginine-glycine-aspartic acid) [41].

Okano *et al.* described another way to obtain an artificial liver, which involved overlapping sheets impregnated with hepatocytes [42].

21.5. APPLICATIONS OF RECONSTRUCTED ORGANS

Multiple techniques for scaffold synthesis and cell culture possibilities from various sources show that this research is still in the initial phase. However, to achieve a functional organ, multiple tissues with complex structures and functionalities must be combined, which will require additional research from experts in bioengineering.

A functional liver must include, besides liver tissue, blood vessels and bile ducts, all integrated into a complex matrix that allows the diffusion of oxygen and nutrients, and the degradation of products to cells that populate the matrix, all which must be biocompatible with the patient immunologic self.

Production of engineered organs will allow the treatment of a number of additional patients with acute or chronic liver failure and, thus, will reduce waiting lists, which presently span 2–3 years. In addition, immunological compatibility will avoid immunosuppression, which has a number of side effects on the body, including infections and malignancies.

21.6. CONCLUSIONS

Regenerative medicine has enormous potential in the creation of artificial organs, which will have a particularly important impact in reducing morbidity and mortality from acute or chronic diseases.

Particularly, the mass production of liver-engineered tissues will allow a large number of patients in the end-stage of diseases or with acute liver failure to be treated, thus reducing mortality and morbidity while on the waiting list for liver transplantation.

The impediments described in this chapter are increasingly overcome by further research, allowing for a prediction that, in 10 years, such a therapy will be the standard.

REFERENCES

1. L.V. di Bonzo, I. Ferrero, C. Cravanzola, K. Mareschi, D. Rustichell, E. Novo, F. Sanavio, S. Cannito, E. Zamara, M. Bertero, A. Davit, S. Francica, F. Novelli, S. Colombatto, F. Fagioli, M. Parola. *Gut*. **57**(2) (2008) 223–231.
2. K.D. Lee, T.K. Kuo, J. Whang-Peng, Y.F. Chung, C.T. Lin, S.H. Chou, J.R. Chen, Y.P. Chen, O.K. Lee. *Hepatology* **40**(6) (2004) 1275–1284.
3. S. Snykers, T. Vanhaecke, A. De Becker, P. Papeleu, M. Vinken, I. Van Riet, V. Rogiers. *BMC Dev. Biol.* **7** (2007) 24.
4. R. Tiruvannamalai-Annamalai, D.R. Armant, H.W. Matthew. *PLoS One* **9**(1) (2014) e84287.
5. S. Kazemnejad. *Avicenna J. Med. Biotechnol.* **1**(3) (2009) 135–145.
6. T. Mitaka, T. Mizuguchi, F. Sato, C. Mochizuki, Y. Mochizuki. *J. Gastroenterol. Hepatol.* **13** Suppl (1998) S70–S77.
7. J.W. Allen, S.N. Bhatia. *Tissue Eng.* **8**(5) (2002) 725–737.
8. S.L. Nyberg, R.P. Remmel, H.J. Mann, M.V. Peshwa, W.S. Hu, F.B. Cerra. *Ann. Surg.* **220**(1) (1994) 59–67.
9. E. Török, J.M. Pollok, P.X. Ma, P.M. Kaufmann, M. Dandri, J. Petersen, M.R. Burda, D. Kluth, F. Perner, X. Rogiers. *Cells Tissues Organs* **169**(1) (2001) 34–41.
10. S. Snykers, J. De Kock, V. Rogiers, T. Vanhaecke. *Stem Cells* **27**(3) (2009) 577–605.
11. D.A. Shafritz, M. Oertel, A. Menthena, D. Nierhoff, M.D. Dabeva. *Hepatology* **43**(2 Suppl 1) (2006) S89–S98.
12. T. Kinoshita, A. Miyajima. *Biochim. Biophys. Acta* **1592**(3) (2002) 303–312.
13. F. Clotman, F.P. Lemaigre. *Cell Cycle* **5**(2) (2006) 168–171.
14. F. Lemaigre, K.S. Zaret. *Curr. Opin. Genet. Dev.* **14**(5) (2004) 582–590.
15. J. Li, R. Tao, W. Wu, H. Cao, J. Xin, J. Li, J. Guo, L. Jiang, C. Gao, A.A. Demetriou, D.L. Farkas, L. Li. *Stem Cells Dev.* **19**(9) (2010) 1427–1436.
16. H. Tabesh, G. Amoabediny, N.S. Nik, M. Heydari, M. Yosefifard, S.O. Siadat, K. Mottaghy. *Neurochem. Int.* **54**(2) (2009) 73–83.
17. J.P. Vacanti, R. Langer, J. Upton, J.J. Marler. *Adv. Drug Deliv. Rev.* **33**(1-2) (1998) 165–182.
18. S.Y. Ong, H. Dai, K.W. Leong. *Biomaterials* **27**(22) (2006) 4087–4097.
19. R.E. Schwartz, M. Reyes, L. Koodie, Y. Jiang, M. Blackstad, T. Lund, T. Lenvik, S. Johnson, W.S. Hu, C.M. Verfaillie. *J. Clin. Invest.* **109**(10) (2002) 1291–1302.
20. H.C. Fiegel M.D., M.V. Lioznov, L. Cortes-Dericks, C. Lange, D. Kluth, B. Fehse, A.R. Zander. *Stem Cells* **21**(1) (2003) 98–104.
21. H. Baharvand, S.M. Hashemi, S. Kazemi Ashtiani, A. Farrokhi. *Int. J. Dev. Biol.* **50**(7) (2006) 645–652.
22. S.F. Badylak. *Transpl. Immunol.* **12**(3-4) (2004) 367–377.
23. T.W. Gilbert, T.L. Sellaro, S.F. Badylak. *Biomaterials* **27**(19) (2006) 3675–3683.
24. B. Struecker, N. Raschzok, I.M. Sauer. *Nat. Rev. Gastroenterol. Hepatol.* **11**(3) (2014) 166–176.
25. C. Booth, T. Soker, P. Baptista, C.L. Ross, S. Soker, U. Farooq, R.J. Stratta, G. Orlando. *World J. Gastroenterol.* **18**(47) (2012) 6926–6934.
26. O. Barakat, S. Abbasi, G. Rodriguez, J. Rios, R.P. Wood, C. Ozaki, L.S. Holley, P.K. Gauthier. *J. Surg. Res.* **173**(1) (2012) e11–e25.

27. B.E. Uygun, A. Soto-Gutierrez, H. Yagi, M.-L. Izamis, M.A. Guzzardi, C. Shulman. *Nat. Med.* **16**(7) (2010) 814–820.
28. P.M. Baptista, M.M. Siddiqui, G. Lozier, S.R. Rodriguez, A. Atala, S. Soker. *Hepatology* **53**(2) (2011) 604–617.
29. K. Schenke-Layland, R.M. Nerem. *Adv. Drug Deliv. Rev.* **63**(4-5) (2011) 195–196.
30. T. Shupe, M. Williams, A. Brown, B. Willenberg, B.E. Petersen. *Organogenesis* **6**(2) (2010) 134–136.
31. A. Soto-Gutierrez, L. Zhang, C. Medberry, K. Fukumitsu, D. Faulk, H. Jiang, J. Reing, R. Gramignoli, J. Komori, M. Ross, M. Nagaya, E. Lagasse, D. Stolz, S.C. Strom, I.J. Fox, S.F. Badylak. *Tissue Eng. Part C Methods* **17**(6) (2011) 677–686.
32. Y.Z. Zhang, J. Venugopal, Z.M. Huang, C.T. Lim, S. Ramakrishna. *Biomacromolecules* **6**(5) (2005) 2583–2589.
33. J. Venugopal, Y.Z. Zhang, S. Ramakrishna. *Nanotechnology* **16**(10) (2005) 2138–2142.
34. S. Kazemnejad, A. Allameh, M. Soleimani, A. Gharehbaghian, Y. Mohammadi, N. Amirizadeh, N. Kaviani. *Iran. J. Biotechnol.* **5**(4) (2007) 201–211.
35. S. Singare, S.Y. Zhong, Z.Z. Sun. *J. Biomim. Biomat. Tissue Eng.* **11** (2011) 73–80.
36. Z.Q. Feng, X. Chu, N.P. Huang, T. Wang, Y. Wang, X. Shi, Y. Ding, Z.Z. Gu. *Biomaterials* **30**(14) (2009) 2753–2763.
37. M.E. Davis, P.C. Hsieh, T. Takahashi, Q. Song, S. Zhang, R.D. Kamm, A.J. Grodzinsky, P. Anversa, R.T. Lee. *Proc. Natl. Acad. Sci. U.S.A.* **103**(21) (2006) 8155–8160.
38. O. Oliviero, M. Ventre, P.A. Netti. *Acta Biomater.* **8**(9) (2012) 3294–3301.
39. S. Zhang, G. Wang, X. Lin, M. Chatzinikolaidou, H.P. Jennissen, M. Laub, H. Uludağ. *Biotechnol. Prog.* **24**(4) (2008) 945–956.
40. M. Nune, P. Kumaraswamy, U.M. Krishnan, S. Sethuraman. *Curr. Protein Pept. Sci.* **14**(1) (2013) 70–84.
41. M. Lutolf, J. Hubbell. *Nat. Biotechnol.* **23**(1) (2005) 47–55.
42. J. Yang, M. Yamato, T. Shimizu, H. Sekine, K. Ohashi, M. Kanzaki, T. Ohki, K. Nishida, T. Okano. *Biomaterials* **28**(34) (2007) 5033–5043.

MECHANICAL GUIDANCE OF CELL MIGRATION

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22.1. INTRODUCTION

Cell development and morphogenesis, wound healing or tumour invasion are all biological processes which require cells to be able to migrate following directional cues. In recent years, much attention has been paid on cell signalling and movement as regulated by many kinds of stimuli such as chemotaxis [1,2], galvanotaxis [3] and extracellular matrix (ECM) compliance/stiffness and topology [4-8] as shown in Figure 1.

Figure 1. Directional control of cell migration can be induced by means of various taxis (moves to/away from stimulus) as schematized

It is widely renowned that cells perceive and explore the chemical and physical properties of a surrounding environment, gathering information at sites of ECM attachment and using them to activate specific signalling pathways within the cells [9,10]. The control of the cell environment by multiple physicochemical cues has therefore emerged as a key tenet to enable functionality, modulate response, and affect cell behaviour. The observation that cells are able to follow gradients of mechanical stiffness dates back to Lo *et al.* whom first reported that fibroblasts tend to move from softer to the stiffer regions of a matrix-coated substrate [11]. This phenomenon was coined as durotaxis after Latin *durus* (hard) and Greek *taxis* (regular arrangement). Since then, durotaxis or globally renowned as mechanotaxis, has been the object of numerous studies in the last two decades. However, although the effects of mechanical guidance underlying a broad diversity of functions on cells are becoming well known, experimental evidence is still scattered and mechanisms remain largely unknown.

22.2. THEORETICAL MECHANISMS OF DUROTAXIS

The mechanisms underlying durotaxis, as abovementioned, are still poorly understood, but overall assume the involvement of active sensing and locomotion of the cell. Various theoretical suggestions on how the mechanism of durotaxis occurs have been proposed. One mechanosensing hypothesis theorises that cells move through the generation of traction by a “motor-clutch” mechanism with a centripetal flow of actin polymerization towards the centre of the cell which moves forward the cell's leading edge. Upon formation of every new pair of ligand-receptor bonds, a traction-bearing structure is either activated or anchored at the intracellular domain of the receptor, with focal adhesions on rigid ECM exerting high force and focal adhesions over soft ECM exerting low forces, as schematized in Figure 2. Sensing mediated by focal adhesions and actin-containing filopodia in front of the leading edge allow cells to determine physical characteristics of the substrate before moving and to avoid the formation of mature focal adhesions on soft substrates which would lead to backtracking of the cell. Stiffer substrates cause tension to develop at nascent adhesions, promoting the maturation of focal adhesions and allowing extension/spreading of the cell. On the other hand soft substrates hampers this increase of tension inhibiting maturation into focal adhesions, which then promotes the retraction of filopodia through myosin II-dependent contractile forces.

Figure 2. Schematization of focal adhesions on stiffness gradient. On soft substrates cells do not develop abundant stress fibres and generate smaller forces, making adhesion sites on these regions less stable. In this way, a cell moving on a substrate with a stiffness gradient relocates towards the stiffer region.

Consequently, an asymmetry in traction force is generated leading to directed cell movement towards the more rigid substrate. Thus, forces and deformations, which occur through ECM linked proteins, modulate the dynamic flow of actin cytoskeleton during the durotactic movement [12].

Another theory proposed by Plotnikov and Waterman, hypothesises that cells attempt to maintain constant substrate deformation during migration [13,14]. This theory proposes that cell migration behaviour is mediated by the tugging motion on focal adhesions, migrating faster when the stiffness differences between the opposing ends of cells are greater [12]. In addition, cell migration on stiffer substrates involves higher forces to break cell to ECM bonds, which may explain the presence of pronounced stress fibers occurring in cells on stiff substrates such as plastic or glass.

An alternative theory has put forward that durotaxis is caused by the different levels of Ca^{2+} ions on the opposing extremities of the cell [15,16]. This theory sustains that the focal adhesions on higher stiffness support a more frequent contraction with consequential higher activation of stretch-mediated Ca^{2+} ion channels and cell migration towards regions of higher stiffness.

Although all these theoretical proposals are still under intense investigation, they remain the key basis theories for durotactic cell migration.

22.3. DIVERSITY OF MECHANOTACTIC CELL BEHAVIOUR

The mechanotactic behaviour of cells is cell-type dependant and context dependent [17-19]. Georges *et al.* reported that neurons and astrocytes react differently to the mechanical properties of the substrate. Astrocytes prefer stiff substrates, whereas they found that cortical neurons show more branched morphology on softer poly(acrylamide gels) (200 Pa vs 9000 Pa) [20]. Balgude and co-workers also demonstrated that neurite extension of chick dorsal root ganglia (DRG) neurons was higher in softer gels [21]. According to Flanagan's group, the formation of neurite branches was enhanced by softer substrates [22]. Ulrich *et al.* observed that glioma cells cultured on fibronectin-coated polymeric ECM with varied but defined mechanical rigidity exhibited altered cell morphology and cytoskeletal organization. These authors showed that glioma cells cultured on softer substrates showed a decreased spreading area, disappearing stress fibres and focal adhesions. Interestingly, all evaluated glioma cell lines cultured on the softest substrates were rounded but viable with cortical rings of F-actin and punctuate vinculin-positive focal complexes, and with no indication of apoptosis [23]. Additionally studies reported by Leach *et al.*, implied that there is an optimal substrate stiffness for neuronal cells that is between 10 Pa and 200 Pa [24].

Difference of cell functions, such as cell spreading, growth, and differentiation, were also observed to be modulated by the mechanic properties of the substrate. Pelham *et al.* reported that cells on flexible substrates showed reduced spreading and increased rates of motility compared to cells on rigid substrates [25]. Wang and his group observed cell proliferation to be increased on substrates of higher mechanical stiffness whereas the rate of apoptosis was increased on more flexible substrates [26]. Through investigating angiogenesis

in vitro, Vailhe and co-workers demonstrated that the formation of capillary-like structures was influenced by the rigidity of the fibrin gels utilized [27]. Similarly Deroanne's group showed that cell differentiation was affected by the mechanical properties of the substrate. In fact decreasing the substrate rigidity increased the number of endothelial cells to switch to a tube-like pattern [28].

22.4. INFLUENCE OF ECM STIFFNESS ON CELLS

As abovementioned, cells connect and interact with their surrounding environment sensing and responding to its physical, mechanical environment, through cues from adjacent cells, external stress, and the ECM. The ECM is fundamental as it provides cells structural support as well as acting as an external cell-signalling region, being mostly composed of adhesive glycoproteins, proteoglycans, glycosaminoglycans and fibrous proteins that also incorporates biologically active molecules and growth factors. This process by which cells can sense rigidity of the ECM is referred to as mechanotransduction where the mechanical signals regulate and feedback the cytoskeletal reorganization and actomyosin contractility [29]. Cells, in fact, constantly survey the mechanical properties of their environment not only adhering to the matrix but also exerting a “pulling” force on the matrix substrate, which deforms the matrix in accordance with its elastic properties [30]. Forces that originate in the cytoskeleton are transmitted across the cell and eventually to ECM through specialized adhesion sites (focal adhesions). Focal adhesions are protein complexes connecting the cytoskeleton to the ECM at the sites of integrin binding. These complexes appear to act as plausible key signalling candidates in the translation of mechanical cues from the extracellular environment into biochemical signals inside the cell [31]. The integrin binds to different ECM specific ligands (fibronectin, collagen, laminin or vitronectin), hence, physically connecting the actin cytoskeleton to the ECM and influencing the downstream signalling events (including the activation of extracellular signal-regulated kinases (ERK), mitogen-activated protein kinases (MAP) and focal adhesion kinases (FAK)) which determines the final cell behaviour and fate [32,33]. Cells' responses to extracellular cues are therefore strongly linked to the myosin activity in the cytoskeleton, whereas the organization of the actin cytoskeleton is controlled by mechanical and geometrical properties of the surrounding matrix. Upon integrin activation, different cytoskeletal proteins like actin, talin and paxillin and signalling molecules such FAKs are connected and structured at the focal adhesion sites [34]. On stiff substrates which cannot deform under contraction, cells assume an unnatural flattened morphology, with increased proliferation rates and have also shown differences in cancer drug sensitivities [35,36]. This was explained by considering that stiffer substrates induce higher levels of expression of adhesion molecules (*i.e.* integrins), to improve adhesion [37]. Moreover, an increase in the synthesis of cellular structural proteins, such as

actin was observed inducing different expression profiles [38]. On the other hand, cell spread area was shown to decrease with decreasing substrate stiffness and cells observed on soft substrates assumed a more rounded morphology with less focal adhesions and actin fibres, deforming in concurrence with the deformation of the surrounding soft matrix (Figure 3) [39].

Figure 3. Cells response to extracellular cues is strongly linked to myosin activity in the cytoskeleton, whereas the organization of the actin cytoskeleton is controlled by mechanical and geometrical properties of the surrounding matrix. Cells adhere to the substrate through mechano-responsive molecular complexes, such as integrin-mediated adhesions, and locally and rapidly adapt to varying forces exerted at the cell-substrate interface.

Not all cell types display mechanosensing behaviour. Principally tissue forming cells including fibroblasts, mesenchymal stem cells, endothelial cells and epithelial cells were demonstrated to differ in behaviour with increased spreading and traction forces and decreased motility on stiffer substrates [16,18,39-41]. Fibroblasts and endothelial cells showed an intensification of spreading and a more increased assembly of their cytoskeleton into actin stress fibres and focal adhesions at ~3 kPa. On the other hand pre-osteocytes re-organize their cytoskeleton and show increase spreading at ~60 kPa while neutrophils appeared to be insensitive to stiffness changes [18,42]. Stem cell differentiation was also shown to be influenced by stiffness of the ECM [43,44]. Klein investigated cell cycle progression regulated by ECM stiffness in endothelial cells through the activation of the small guanine triphosphatase (GTPase) RAC1 [45]. ECM stiffness was also showed to influence gene expression and cell fate. Engler and coworkers induced mesenchymal stem cells to differ in bone differentiation in stiff environments, whereas into adipocyte differentiation on soft substrates [40]. Analogous observations with skeletal muscle stem cells and with adult neural stem cells were reported

[46,47]. Furthermore proteins of the hippo pathway were shown to be associated in the transcriptional effects of ECM stiffness and cytoskeletal organization, influencing the stiffness-dependent regulation of ECM gene expression and cell cycle progression [48].

As mechanosensitivity is associated to the cells' ability to rearrange adhesion ligands and to apply traction forces on the substrate, substrate mechanics and adhesiveness should be regarded as coupled variables. Peyton and Putnam reported a biphasic dependence of cell migration speed on ECM stiffness. In their study, the optimal stiffness at which cell migration speed is maximized was found to depend on the density of immobilized ECM ligands [49]. Investigating spreading of smooth muscle cells (SMCs) on collagen-coated poly(acrylamide gels), Engler's group showed that the cytoskeletal organization of muscle cells depends on the stiffness of the substrate on which they are grown with optimal substrate stiffness closest to that of relaxed muscle bundles [50]. They showed that matrix compliance and ligand density are highly coupled variables that determine mean cell responses. The same group stressed the importance of substrate stiffness on cellular motility for anchorage dependent cells [29]. Anchorage dependent, contractile cells show an increase in cellular adhesions and a more organized cytoskeleton with increasing stiffness of the substrate on which they are grown. This is explained considering the elasticity of their natural environment. In fact *in vivo* muscle and skin is much stiffer than the brain and the spinal cord and for this reason neurons prefer to migrate towards soft substrates, whereas muscle cells and fibroblasts favour stiffer substrates. Durotaxis therefore, occurs as a result of "positive feedback" of cells-substrate reinforcement as cell actively exerts forces on their ECM micro-environment through focal adhesions. Cells subject to durotaxis, can mediate signal transduction as ECM bound proteins or proteins linked to scaffolds connected to the ECM undergo conformational modifications as a result of changes to the ECM [51,52]. Scaffolding proteins allow in fact the linkage between integrins and actin cytoskeleton, which will form the ECM-integrin-cytoskeleton connections which develop to focal complexes [53]. In order for the focal complexes to mature to focal adhesions, and to connect to stress fibres, forces must be generated by the cell itself through its contractile machinery, but also from the surrounding environment [29]. More specifically, mechano-sensitive ion channels present near focal adhesion are activated as cells exert pulling forces [54-56]. This leads to an influx of extracellular calcium, increased level of acto-myosin contractility and reinforcement of the cell-substrate adhesions with clustering of focal adhesion complexes [57].

Concurrently, various proteins such as focal adhesion kinase, paxillin, vinculin and talin are recruited to reinforce the adhesion (Figure 4) [58,59]. As the local stiffness of the microenvironment increases, a solitary cell will consequently form a stronger adhesion on its substrate. Likewise, in the presence of a

gradient of substrate rigidity, cell will have a tendency to form stronger adhesions on stiffer ECM regions.

Figure 4. Fluorescent images of NIH 3T3, cultured for 24 h, stained for vinculin in green, plated on square-patterned substrates and pillars in pits for 24 h. DNA is blue and actin is red. The figures outline the stiff regions, caused by the presence of the square patterns underneath the membrane, whereas the regions surrounding the pillars are soft. The force is transmitted to the extracellular environment at cell-matrix contacts and actively generated by myosin molecular motors interacting with actin filaments. Scale bar: 20 μm .

Although many important advances have been acknowledged not much information about the diverse pathways activation and signal transduction is known. Hence, more investigations on the the role of the localization of integrin-modulated signal molecules in the regulation of cell differentiation by ECM elasticity is also an important target for future work.

22.5. DIVERSITY OF MECHANICAL FEATURES OF THE SUBSTRATE

Over the past years different methods have been used to create mechanical heterogeneities on substrates. So far, purely elastic poly(acrylic acid) (PAA) hydrogels as well as thin poly(acrylamide) gels have been widely used for mechanotaxis means [22,25,59]. Photocurable poly(acrylamide) gels are chemically cross-linked gels formed by the polymerization of acrylamide with a cross-linking agent, usually *N,N'*-methylenebisacrylamide. These gels are

widely used as a compliant substrate for mechanotaxis although the unpolymerized acrylamide monomer is highly toxic and often results in loss of cell viability. Poly(dimethylsiloxane) (PDMS) commonly used in biomedical applications and in membrane technology for its biocompatibility, mechanical compliance, chemical inertness, and remarkable flexibility has also been used for prototyping substrates for mechanotaxis means [60-63]. Moreover these elastomers may have an elastic moduli able to effectively cover the entire range of soft tissue elastic moduli from approximately 1 kPa to > 1 MP [64-66]. This is advantageous as at the tissue-scale range the stiffness of cell microenvironment displays high variation within the body. Between different tissues, ECM rigidity often varies over several orders of magnitude, *e.g.*, brain (260–490 Pa), liver (640 Pa), kidney (2.5 kPa), skeletal muscle (12–100 kPa) and cartilage (950 kPa) [67]. Tissue variation can also be triggered by pathological factors such as malignant tumours, which are stiffer than the surrounding healthy tissue [68].

Differences of elasticity have been with various approaches. Ohashi and colleagues showed that cells develop stress fibres in response to nonuniform strain and development of these stress fibres eventually leads to a shift in the location of the cell nucleus [69]. The monotonically increasing strain gradient was obtained by embedding glass within a silicone elastomer and stretching the substrate. The lowest strain region, close to the glass-elastomer border showed no signs of cellular elongation or orientation; however cells within the region of high strain aligned perpendicular in direction of stretch. They also showed a strain dependent formation of stress fibres exists which led to cell remodelling due to the strain gradient. Similarly cells also tend to have more pronounced alignment if microgrooves in the substrate are perpendicular to stretch than if they are parallel to the direction of stretch [70]. Loesberg work showed that mechanical loading played a secondary role in fibroblast phenotype modulation while substrate topography was the primary influence.

Kuntanawat and co-workers simply modified the thickness of the film, altering the apparent elasticity of the substrate by variation in height of a thin poly(acrylamide) gel [71]. The thicker the gel, the less influence has the mechanical property of the (hard) substrate, thus the softer the surface showing that cells migrated along the gradient. A method reported used to generate patterns of elasticity is through the control of the polymerization sites through photomasks or laser beams. Nemir *et al.* used a base polymer and soaked the solid gel with a solution containing a different polymer precursor. The precursor penetrated the network of the gel and was polymerized *via* UV-curing at determined positions with the aid of a photomask. The unreacted precursor was then removed, leaving areas composed only of the base polymer [72]. A similar approach was used by Hahn which used a two-photon absorption (TPA) photolithography to pattern bioactivity bioactive features into optically transparent, photoactive materials by diffusing a second precursor inside a base polymer and removal of the unreacted pre-polymer

[73]. Kloxin and co-workers demonstrated to be able to create gradients in crosslinking density with a predictable tuning of the gel structure in real-time with low-intensity, long-wavelength UV light. They used a UV-degradable cross-linker, undoing the network at the positions affected by the laser [74]. A different strategy was followed by Chou and colleagues. The pattern was created by filling the channels of a topographically patterned sample with a second polymer of different composition [75].

However one must take into account that the chemistry of the precursors is different and may be sufficient to form a chemical gradient which can override the mechanical one. Patterns of elasticity can be prepared by coating solid structures with a relatively soft film. To date, the work of Cortese and coworkers, has showed that a single material has been able to influence cell behaviour [6,76]. PDMS substrates were developed where the elastic modulus could be easily and independently tuned to mimic soft tissues over a three order-of-magnitude range. The pattern was created by utilizing the contrast between air chambers and the geometry of the underlying bulk substrate. Topographic patterns of PDMS with desired geometry were fabricated and covered with a thin elastic film, creating air pockets between the patterned structures (Figure 5).

Figure 5. Schematization of the fabrication process of the thin membrane on the substrate. a) A PDMS patterned substrate was created by pouring a pre-polymer of PDMS on a pre-patterned SU8 master substrate b) Bonding of substrate and a thin PDMS membrane, c) the final substrate with membrane for mechanotaxis purpose was obtained.

The thin membrane PDMS film was studied in various mass to mass ratios to create low stiffness gels. Moreover by varying the mechanical rigidity of the film the elasticity could be finely tuned as well as cell behaviour [6]. To investigate the conditions to induce mechanotaxis, different PDMS membrane thin films with different surface elasticity conditions were prepared by changing the base to curing ratios *i.e.* the degrees of crosslinking and dilution with heptane [6,76]. The embedded structures affect the elasticity of determined areas of the substrate, while others remain unaffected (either no

solid structure is located underneath or the film thickness is high enough to hide the effect of the structure). When cells move towards the high Young's modulus area of the substratum, the cells would extend their lamellipodia and move directly onto the rigid part of the substrate. In contrast, when cells migrate from stiffer substrates (*i.e.* lines, Figure 6) the cells would move up and down the mechanical interface and cross over onto the stiff regions without residing on the softer side. Thus, mechanical interactions between a cell and its underlying substratum play a crucial role in modulating cell motility.

Figure 6. Time-lapse phase contrast images of human fibroblasts cells moving on the membrane. The darker lines are the patterned grooves underneath the membrane. In this sequence of images, cells feel and respond to the substrate clearly moving along the ridges of the lines on the harder regions. Attention has been focused with the arrow on a particular cell, moving rapidly towards the top. Images were obtained approximately 1 h after seeding cells on the substrate, and the interval between each image was about 30 min. Scale bar: 100 μm . Figure is adapted from ref. [6].

22.6. CELL'S RESPONSE TO FORCE

Rigidity sensing and durotaxis responses can be quantified by measuring traction forces while cells are simultaneously interacting with substrates of different rigidities. The concept of durotaxis is even more complicated by the cell's response to force. Not only the cell detects externally applied force but it must respond appropriately to this force by actively exerting force on the substrate by deformation of the substrate. This process as a positive feedback loop, requires cell migration to mediate the function of durotaxis both actively and passively simultaneously. An approach to measure these forces has been developed using substrates composed of flexible arrays of micropost arrays [77-81]. Micropost arrays present a powerful topographic technology to measure the cellular traction forces on the substrate. Specifically, when cells are seeded onto arrays of identical circular microposts, they spread over several posts (Figure 7) and can physically pull at the tops of the posts, resulting in visible displacements. The deflection of each post provides a direct measurement of the local force exerted by the attached cells independently of the forces acting on the neighbouring posts. The pillars simply act as independent springs and the linear theory of elasticity gives their deflection (Figure 7).

Figure 7. Micropost array. (a) Schematic illustration of a cell seeded on a microtopographic stiffness gradient with an enlarged view of individual microposts with increasing radii (r) and equivalent interpost spacing (l). (b) Micropost cantilever model. The linear stiffness at the top of the micropost is derived from its geometric and material properties, including the Young's modulus (E), shear modulus (G), shear coefficient (k), micropost height (H), and micropost radius (r). A force applied at the top of the micropost (F), parallel to the substrate, will result in a displacement at the top of the micropost (δ). (c) Physically pull of the tops of the microposts by the cell, resulting in visible displacement of the post. Figure is adapted from ref. [80].

For a cylinder of radius r and length H bent by the application of a force F , it leads to the following formula:

$$F = k_{\text{bend}} \delta = \left(\frac{3\pi k E G r^4}{4k G H^3 + 3E H r^2} \right) \delta$$

where E , G , k , k_{bend} and δ are, respectively, the Young modulus, the shear modulus, the shear coefficient, the spring constant and the deflection of the post. A very interesting work was presented by Sochol and co-workers. Their micropost arrays of varying anisotropy showed an optimize unidirectional control of cell migration through dual axis durotaxis cues restricting movement in the lateral direction in addition to promoting migration in the direction of increasing micropost stiffness. Additionally, higher gradient strength was found to enhance this directional response [82].

Figure 8. Scanning electron microscopy image of cells spreading on micropillar surface of two different diameters, where micropillars with larger diameter are stiffer (A). Cells positioned at the interface of the stiff and soft micropillar interface preferentially migrated and resided onto the stiffer surface (B). Figure is adapted from ref. [83].

Trichet's group demonstrated that when fibroblast cells are grown on array of anisotropic micro-pillars presenting soft and stiff micropillars, these cells have a tendency to migrate towards and reside on the stiff region (Figure 8) [83]. These studies suggest that the cytoskeleton, specifically actomyosin contractility, is crucial for rigidity sensing during durotaxis.

In conclusion, the ability to measure traction forces while cells interact with substrates of different rigidities is a key step to unravelling the mechanism underlying durotaxis. Micropillared arrays enable high control over positioning and geometry using microfabrication and provide a powerful tool for investigating the cellular response to mechanical properties of the substrate. However, more studies are indispensable to investigate the presence of cell-cell interaction, as cells integrate polarizing mechanotransductive cues from not only the substrate but also from neighboring cells. It has been shown in fact that collective migration in epithelial sheets enhanced transmission of mechanical force signals transmitted through cell-cell adhesions [84].

Therefore cells combine mechanical cues from both the substrate, as in the form of rigidity, and contacting cells associated to physical forces exerted at cell-cell contacts.

22.7. OVERRIDING ROLE OF CHEMOTAXIS OR MECHANOTAXIS?

An important experimental question that should be investigated is: does chemotaxis override durotaxis during cell migration? Although the molecular mechanisms to detect external stimuli of durotaxis are not identical to that of chemotaxis, there might exist common downstream signalling pathways that drive polarization and migration [52]. Similarly to chemotaxis, cells undergoing durotaxis can sense physical forces and transduce them into biochemical signals. What distinguishes the two phenomena is that durotaxis does not require biochemical transduction to direct cellular motion. Knowledge of the parameters of chemotaxis and durotaxis would therefore help in the design of ECM scaffold stiffness.

Understanding the integrated mechanotactic responses of cells to the microenvironment is necessary in order to predict and control the behaviours of cells for therapeutic applications. In particular, understanding how to regulate the cell shape and functions, controlling the signals coming from ECM and the external forces are the primal tasks in tissue engineering as well as in development of functional biomaterials. Then, providing insight into what, where and when a cell senses ECM cues and activates its biochemical responses is fundamental to understand how such cues can be incorporated into new, three-dimensional scaffolds to treat diseases. Besides, insight of cell motility *per se* has important consequences for improving clinical treatments as therapeutic interventions that either interferes with mechanotransductive signalling or mechanical remodelling. These considerations are especially important for example in the development of anti-cancer therapies, which might be able to target aspects that are dysregulated in this kind of disease. Nonetheless, the role of mechanotaxis and its controlling mechanisms are still under extensive study.

An important step in research for cellular response to mechanical gradients is the design of a substrate that can control directional movement of cells maintaining unvaried the physical and chemical properties to avoid undesirable responses. Thus, to fully understand the role of mechanics on cell function it would be ideal to tailor the elastic modulus without altering other material properties such as surface energy, chemistry and roughness. This type of engineered substrate is useful for studying separately the chemical and mechanical stimuli contributing to the time-dependent migration of different cell types.

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REFERENCES

1. R.R. Kay, P. Langridge, D. Traynor, O. Hoeller. *Nat. Rev. Mol. Cell. Biol.* **9** (2008) 455.
2. F. Pagani, R.C. Paolicelli, E. Murana, B. Cortese, S. Di Angelantonio, E. Zurolo, E. Guiducci, T.A. Ferreira, S. Garofalo, M. Catalano, G.D'Alessandro, A. Porzia, G. Peruzzi, F. Mainiero, C. Limatola, C.T. Gross, D. Ragozzino. *Front. Cell. Neurosci.* **9** (2015) 111.
3. B. Cortese, I.E. Palamà, S.D'Amone, G. Gigli. *Integr. Biol.* **6** (2014) 817.
4. B. Cortese, M.O. Riehle, S.D'Amone, G. Gigli. *J. Coll. Inter. Sci.* **394** (2013) 582.
5. B. Cortese, C. Piliago, I. Viola, S. D'Amone, R. Cingolani, G. Gigli. *Langmuir* **25** (2009) 7025.
6. B. Cortese, G. Gigli, M.O. Riehle. *Adv. Funct. Mater.* **19** (2009) 2961.
7. M.J. Dalby, N. Gadegaard, R.O.C. Oreffo. *Nat. Mat.* **13** (2014) 558.
8. M.J. Dalby, N. Gadegaard, R. Tare, A. Andar, M.O. Riehle, P. Herzyk, C.D.W. Wilkinson, R.O.C. Oreffo. *Nat. Mat.* **6** (2007) 997.
9. A.D. Bershadsky, N.Q. Balaban, B. Geiger. *Annu. Rev. Cell Dev. Biol.* **19** (2003) 677.
10. B. Geiger, J.P. Spatz, A.D. Bershadsky. *Nat. Rev. Mol. Cell. Biol.* **10** (2009) 21.
11. C.M. Lo, H.B. Wang, M. Dembo, Y.L. Wang. *Biophys. J.* **79** (2000) 1.
12. C.E. Chan, D.J. Odde. *Science* **322** (2008) 1687.
13. S.V. Plotnikov, A.M. Pasapera, B. Sabass, C.M. Waterman. *Cell* **151** (2012) 1.
14. S.V. Plotnikov, C.M. Waterman. *Curr. Opin. Cell Biol.* **25** (2013) 619.
15. T. Mammoto, A. Mammoto, D.E. Ingber. *Annu. Rev. Cell Dev. Biol.* **29** (2013) 27.
16. T. Kobayashi, M. Sokabe. *Curr. Opin. Cell Biol.* **22** (2010) 669.
17. R.J. Young, P.A. Lovell. *Introduction to Polymers, 3rd Ed.*, CRC Press, USA, 2011, p. 469.
18. T. Yeung, P.C. Georges, L.A. Flanagan, B. Marg, M. Ortiz, M. Funaki, N. Zahir, W. Ming, V. Weaver, P.A. Janmey. *Cell Motil. Cytoskeleton* **60** (2005) 24.
19. S. Nemir, J.L. West. *Ann. Biomed. Eng.* **38** (2010) 2.
20. P.C. Georges, W.J. Miller, D.F. Meaney, E.S. Sawyer, P.A. Janmey. *Biophys. J.* **90** (2006) 3012.
21. A.P. Balgude, X. Yu, A. Szymanski, R.V. Bellamkonda. *Biomaterials* **22** (2001) 77.
22. L.A. Flanagan, Y.E. Ju, B. Marg, M. Osterfield, P.A. Janmey. *NeuroReport* **13** (2002) 2411.
23. T.A. Ulrich, E.M. de Juan Pardo, S. Kumar. *Cancer Res.* **69** (2009) 4167.
24. J.B. Leach, X.Q. Brown, J.G. Jacot, P.A. Dimilla, J.Y. Wong. *J. Neural. Eng.* **4** (2007) 26.
25. R.J. Pelham, Y.L. Wang. *Proc. Natl. Acad. Sci.* **94** (1997) 13661.
26. H.B. Wang, M. Dembo, Y.L. Wang. *Am. J. Physiol. Cell Physiol.* **279** (2000) C1345.
27. B. Vailhé, X. Ronot, P. Tracqui, Y. Usson, L. Tranqui. *In Vitro Cell. Dev. Biol. Anim.* **33** (1997) 763.

28. C.F. Deroanne, C.M. Lapiere, B.V. Nusgens. *Cardiovasc. Res.* **49** (2001) 647.
29. D.E. Discher, P. Janmey, Y.L. Wang. *Science* **310** (2005) 1139.
30. C.S. Chen. *J. Cell. Sci.* **121** (2008) 3285.
31. D.E. Ingber. *Faseb J.* **20** (2006) 811.
32. E. Ruoslahti. *Adv. Cancer Res.* **76** (1999) 1.
33. M.E Hemler, R.R. Lobb. *Curr. Opin. Hematol.* **2** (1995) 61.
34. D. Riveline, E. Zamir, N.Q. Balaban, U.S. Schwarz, T. Ishizaki, S. Narumiya, Z. Kam, B. Geiger, A.D. Bershadsky. *J. Cell Biol.* **153** (2001) 1175.
35. L. David, V. Dulong, D. Le Cerf, L. Cazin, M. Lamacz, J.P. Vannier. *Acta Biomater.* **4** (2008) 256.
36. J.L Horning, S.K. Sahoo, S. Vijayaraghavalu, S. Dimitrijevic, J.K. Vasir, T.K. Jain, A.K. Panda, V. Labhasetwar. *Mol Pharm.* **5**(2008) 849.
37. J. Du, X. Chen, X. Liang, G. Zhang, J. Xu, L. He, Q. Zhan, X.-Q. Feng, S. Chien, C. Yan. *Proc. Natl. Acad. Sci. U.S.A.* **108** (2011) 9466.
38. P.A. Janmey, R.T. Miller. *J. Cell. Sci.* **124** (2011) 9.
39. M. Dembo, Y.L. Wang. *Biophys. J.* **76** (1999) 2307.
40. A.J. Engler, S. Sen, H.L. Sweeney, D.E. Discher. *Cell* **126** (2006) 677.
41. R.J. Pelham, Y.L. Wang. *Biol. Bull.* **194** (1998) 348.
42. H.J. Kong, T.R Polte, E. Alsberg, D.J. Mooney. *Proc. Natl Acad. Sci. U.S.A.* **102** (2005) 4300.
43. F. Watt, W. Huck. *Nat. Rev. Mol. Cell Biol.* **14** (2013) 467.
44. P. Lu, V. Weaver, Z. Werb. *J. Cell Biol.* **196** (2012) 395.
45. D.E.A. Klein, L. Yin, D. Kothapalli, P. Castagnino, F.J. Byfield, T. Xu, I. Levental, E. Hawthorne, P.A. Janmey, R.K. Assoian. *Curr. Biol.* **19** (2009) 1511.
46. P.M. Gilbert, K.L. Havenstrite, K.E. Magnusson, A. Sacco, N.A. Leonardi, P. Kraft, N.K. Nguyen, S. Thrun, M.P. Lutolf, H.M. Blau. *Science* **329** (2010) 1078.
47. K. Saha, A.J. Keung, E.F. Irwin, Y. Li, L. Little, D.V. Schaffer, K. E. Healy. *Biophys. J.* **95** (2008) 4426.
48. S. Dupont, L. Morsut, M. Aragona, E. Enzo, S. Giulitti, M. Cordenonsi, F. Zanconato, J. Le Digabel, M. Forcato, S. Bicciato, N. Elvassore, S. Piccolo, *Nature* **474** (2011) 179.
49. S.R. Peyton, A.J. Putnam. *J. Cell. Physiol.* **204** (2005) 198.
50. A.J. Engler, M.A. Griffin, S. Sen, C.G. Bönnemann, H. Lee Sweeney, D.E. Discher, *J. Cell Biol.* **166** (2004) 877.
51. D.E. Discher, P. Janmey, Y.L. Wang. *Science* **310** (2005) 1139.
52. P. Roca-Cusachs, T. Iskratsch, M.P. Sheetz. *J. Cell Sci.* **125** (2012) 3025.
53. P. Roca-Cusachs, R. Sunyer, X. Trepac. *Curr. Opin. Cell Biol.* **25** (2013) 543.
54. B. Geiger, A. Bershadsky, R. Pankov, K.M. Yamada. *Nat. Rev. Mol. Cell Biol.* **2** (2001) 793.
55. H. Wolfenson, A. Bershadsky, Y.I. Henis, B. Geiger. *J. Cell. Sci.* **124** (2011) 1425.
56. F. Sachs. *Mol. Cell. Biochem.* **104** (1991) 57.
57. J. Arnadóttir, M. Chalfie. *Ann. Rev. Biophys.* **39** (2010) 111.
58. C. Grashoff, B.D. Hoffman, M.D. Brenner, R. Zhou, M. Parsons, M.T. Yang, M.A. McLean, S.G. Sligar, C.S. Chen, T. Ha, M.A. Schwartz. *Nature* **466** (2010) 263.
59. A. Engler, L.e Bacakova, C. Newman, A. Hategan, M. Griffin, D. Discher, *Biophys. J.* **86** (2004) 617.
60. J.C. McDonald, G.M. Whitesides. *Acc. Chem. Res.* **35** (2002) 7.
61. C.F. Adams, A.J. Paul. *J. Crustacean Biol.* **19** (1999) 1.

62. Y. Zhao, C.C. Lim, D.B. Sawyer, R. Liao, X. Zhang. *J. Micromech. Microeng.* **15** (2005) 9.
63. A. Gadre, M. Kastantin, S. Li, R. Ghodssi, *Proc. Int. Semiconductor Device Research Symp.* Washington DC, USA, 2001, p. 186.
64. X.Q. Brown, K. Ookawa, J.Y. Wong. *Biomaterials* **26** (2005) 3123.
65. E. Gutierrez, A. Groisman. *PLoS One* **6** (2011) e25534.
66. P.Y. Wang, W.B. Tsai, N.H. Voelcker. *Acta Biomater.* **8** (2012) 519.
67. I. Levental, P.C. Georges, P.A. Janmey. *Soft Matter.* **3** (2007) 299.
68. S. Kumar, V.M. Weaver. *Cancer Metastasis Rev.* **28** (2009) 113.
69. T. Ohashi, M. Masuda, T. Matsumoto, M. Sato. *Clin. Hemorheol. Microcirc.* **37**(2007) 37.
70. W.A. Loesberg, X.F. Walboomers, J.J.W.A. van Loon, J.A. Jansen. *J. Biomed. Mat. Res.* **75A** (2005) 723.
71. P. Kuntanawat, C. Wilkinson, M. Riehle. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* **146** (2007) S192.
72. S. Nemir, H.N. Hayenga, J.L. West. *Biotechnol. Bioeng.* **105** (2010) 636.
73. M.S. Hahn, J.S. Miller, J.L. West. *Adv. Mater.* **18** (2006) 2679.
74. A.M. Kloxin, M.W. Tibbitt, A.M. Kasko, J.A. Fairbairn, K.S. Anseth. *Adv. Mater.* **22** (2010) 61.
75. S.-Y. Chou, C.-M. Cheng, P.R. LeDuc, *Biomaterials* **30** (2009) 3136.
76. I. E. Palamà, S. D'Amone, A. M. L. Coluccia, M. Biasiucci, G. Gigli, *Integr. Biol.* **4** (2012) 228.
77. F. Binamé, G. Pawlak, P. Roux, U. Hibner. *Mol. Biosyst.* **6** (2010) 648.
78. W.C. Wei, H.H. Lin, M.R. Shen, M.J. Tang. *Am. J. Physiol. Cell Physiol.* **295** (2008) C1579.
79. N.J. Sniadecki, C.S. Chen. *Methods Cell Biol. – Cell Mechanics* **83** (2007) 313.
80. L. Tan, J. Tien, D.M. Pirone, D.S. Gray, K. Bhadriraju, C.S. Chen. *Proc. Natl. Acad. Sci. U.S.A.* **100** (2003) 1484.
81. I.K. Lin, Y.-M. Liao, Y. Liu, K.-S. Ou, K.-S. Chen, X. Zhang. *Appl. Phys. Lett.* **93** (2008) 251907.
82. R.D. Sochol, A.T. Higa, R. Janairo, S. Li, L. Lin. *Soft Matter.* **7** (2011) 4606.
83. L. Trichet, J. Le Digabel, R.J. Hawkins, S.R.K. Vedula, M. Gupta, C. Ribault, P. Hersen, R. Voituriez, B. Ladoux. *Proc. Natl. Acad. Sci. U.S.A.* **109** (2012) 6933.
84. M.R. Ng, A. Besser, G. Danuser, J.S. Brugge. *J. Cell Biol.* **199** (2012) 545.

NANOMEDICAL APPLICATIONS OF GRAPHENE AND GRAPHENE OXIDE

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23.1. INTRODUCTION

Typical carbon based nanomaterials such as graphene and its derivatives, fullerene and carbon nanotubes, possess many interesting physical and chemical properties that are useful in biomedical and biological applications [1-3]. As a result, graphene-based materials have attracted considerable attention in the past few years. Graphene and graphene oxide (GO) have been extensively explored as promising biomaterials for biomedical applications due to their unique properties: two-dimensional planar structure, large surface area, chemical and mechanical stability and good conductivity. Since its discovery in 2004, graphene, a single-atom thick and a two dimensional arrangement of conjugated sp^2 carbons in a honeycomb structure, has been studied extensively in the biotechnology field owing to its multivalent functionalization and efficient surface loading with various biomolecules. A high surface area-to-volume ratio and excellent chemical versatility make this special material an ideal candidate for the development of a new generation of nanomedicines with applications encompassing the detection of biomarkers to imaging and cancer therapy. GO, which is highly oxidized graphene with number of carboxyl, hydroxyl and epoxide groups on its surface and edges, can load various drug molecules *via* π - π stacking, hydrophobic or electrostatic interactions and hydrogen bonding. GO can be easily functionalized with various hydrophilic macromolecules which can improve its biocompatibility and regulate its properties in biological systems or with targeting ligands or active agents for selective or controlled drug delivery.

Graphene sheets were first separated from graphite in a process of mechanical exfoliation of graphite by using adhesive tape. Using this process, single-layer graphene may be obtained. However, this approach yields only small quantities of graphene that are useful only for fundamental study. Subsequently, large-area graphene films composed of a single or few layers have been produced by chemical vapor deposition (CVD) on metal substrates. This method is accomplished by capturing a hydrocarbon precursor *via* surface chemical adsorption by catalytic decomposition of hydrocarbons on transition metals such as Pt, Ni, Ru, Rh, Ir or Cu [8]. Another method to obtain graphene sheets is the growth of graphitic layers by the sublimation of Si from SiC substrates. This approach has been known since 1975, when Bommel *et al.* first reported graphite formation on the (0001) polar plane of SiC [9]. In 2007, de Heer *et al.* reported the fabrication of an epitaxial graphene down to one or two layers on the (0001) face of a 6H-SiC wafer using a thermal decomposition method [10]. Although epitaxial graphene and CVD-grown graphene are high-quality materials, transfer to another substrate is necessary, which limits the versatility of these processes in a wide range of applications. The last method of graphene synthesis is the creation of a colloidal suspension made of graphite and its derivatives. So far, oxidative exfoliation *via* the Hummers method is the

most popular method for the generation of GO [11]. It involves oxidative exfoliation of graphite using a mixture of strong oxidants. The prepared GO, with a large number of functional groups on its surface, can be further reduced to obtain reduced graphene oxide (RGO) by reacting with reducing agents such as N_2H_4 [12]. Graphene oxide can be also produced by the Brodie [13] and Staudenmaier [14] methods, which require the utilization of strong oxidants. The level of the oxidation can be varied depending on reaction conditions and the type of graphite precursor. This chemical approach is scalable, enables high-volume production of graphene or GO that can be easily chemically functionalized and used for a wide range of applications. The most common methods of graphene production are presented in Figure 1.

Figure 1. Schematic representation of common methods used for graphene production

23.2. BIOCOMPATIBILITY OF GRAPHENE AND GRAPHENE OXIDE

In order to use graphene and GO in clinical applications, it is necessary to verify their biocompatibility and toxicity by performing extensive *in vitro* and *in vivo* studies using cells and animal models. The first report on the interaction between GO and human dermal fibroblasts (HDF) was presented in 2011 by Wang *et al.* [15]. They examined fibroblasts cultured with different doses of GO for various periods of time (from 1–5 days) and showed that GO at a dose below $20\text{ }\mu\text{g mL}^{-1}$ was not toxic to the cells; however, a dose of more than $50\text{ }\mu\text{g mL}^{-1}$ led to obvious cytotoxicity and a decreased cell survival rate, observed as floating cells and apoptosis. This study showed that GO was internalized by cells and was mainly located inside the cytoplasm, as well as in lysosomes, mitochondria, and the endoplasm. Furthermore, as the culture time increased, the amount of GO inside the cells increased accordingly. GO appeared as black dots scattered throughout the cytoplasm and around the nucleus, although a few GO particles were located inside the nucleus. Next, the authors studied the effects of GO on cell adhesion proteins and found that cell adhesion decreased markedly with an increase in the GO concentration and culture time. HDF cells treated with GO at doses of $100\text{ }\mu\text{g mL}^{-1}$ and $20\text{ }\mu\text{g mL}^{-1}$ exhibited morphological changes characteristic of apoptosis after 24 h and 72 h, respectively, such as membrane vesicles, fragmentation, unclear cell boundaries and the formation of nodular structures encapsulating GO.

Chang *et al.* [16] also performed *in vitro* studies with GO. They examined the effects of GO on morphology, viability, mortality and membrane integrity of the lung cancer cell line A549. According to these studies, GO does not enter A549 cells and exerts no obvious cytotoxicity. However, it can cause dose-dependent oxidative stress in cells and induce a slight loss of cell viability at high concentrations ($200\text{ }\mu\text{g mL}^{-1}$). They found that the size of GO was another factor that influenced A549 viability, since GO with a smaller particle size induced more viability loss than larger GO particles. Hu *et al.* [17] excluded oxidative stress as a reason for GO toxicity. They performed *in vitro* tests to assess the effect of fetal bovine serum (FBS) on the cytotoxicity of GO. They showed that, at low concentrations of FBS (1 %), human cells were sensitive to the presence of GO and showed concentration-dependent cytotoxicity. Interestingly, the cytotoxicity of GO was greatly mitigated at 10 % FBS. Hence, the authors proposed that the cytotoxicity of GO nanosheets occurred as a result of physical damage to the cell membrane, which was induced by direct interactions between the cell membrane and GO nanosheets. This effect was largely attenuated when GO was incubated with FBS due to the high protein adsorption ability of GO. The cytotoxicity occurred mostly during the initial contact stage between GO and cells and was independent of exposure duration. As oxidative stress is a time-dependent process, it did not contribute to GO toxicity.

Ruiz and co-authors [18] studied the role of GO films on mammalian cell (colorectal adenocarcinoma, HT-29) attachment and proliferation. They compared the attachment and growth of mammalian cells on glass slides coated with 10 μg of GO to cells on uncoated glass slides. The results showed that the mammalian cells attached more efficiently to the GO-coated glass slides and grew, indicating that GO promotes cell attachment and proliferation.

Liao and co-workers [19] compared the cytocompatibility of GO with that of RGO using adherent skin fibroblasts. They found that RGO was more toxic to adherent cells than GO, probably due to faster sedimentation and the formation of more compact aggregates of graphene, as compared to GO, during 24 h of static aging; this greatly inhibited nutrient availability and the growth of human skin fibroblasts. Our group investigated the cytocompatibility of GO and RGO on mice fibroblasts (line L929) [20]. We examined the influence of the concentration of the nanomaterial and the dispersant used to stabilize the suspension (sodium deoxycholate (DOC), poly(ethylene glycol) (PEG) and the co-polymer Pluronic P123). The results showed that the dispersant used to stabilize the suspension, the type of material and its concentration significantly influenced cell toxicity. The highest viability of the cells was observed when both materials were dispersed in PEG. Both materials showed relatively good cytocompatibility when the concentration was between 3.125 $\mu\text{g mL}^{-1}$ and 12.5 $\mu\text{g mL}^{-1}$. Increasing the concentration of the nanomaterial reduced the cell viability. A comparison of the cytocompatibility of GO and graphene was also performed by the Liao group [19]. They performed an *in vitro* study on the blood compatibility of GO and graphene particles of various sizes and oxygen content suspended in human red blood cells (RBCs) by assessing hemolysis. Both GO and graphene showed dose-dependent hemolytic activity on RBCs. In the case of GO, the extent of exfoliation and the particle size had critical effects on the degree of hemolysis. Smaller GO particles exhibited higher hemolytic activity than larger GO particles. Compared to individually dispersed GO sheets with a higher surface oxygen content, aggregated graphene showed lower hemolytic activity. The authors proposed that the disruption of the RBC membrane was attributed to the strong electrostatic interactions between negatively charged oxygen groups on GO and the graphene surface and positively charged phosphatidylcholine lipids, which are present on the RBC outer membrane. They also found that covering GO sheets with chitosan eliminated their hemolytic activity. A different method of graphene modification was investigated by Santos *et al.* [21] using poly(N-vinylcarbazole) (PVK); these authors showed that the obtained nanocomposite exhibited lower cytotoxicity compared to unmodified graphene. Cheng and co-workers [22] confirmed the high hemolytic activity of GO and proposed its reduction with dopamine and simultaneous adhesion to RGO by one-step pH-induced polymerization to polydopamine (pRGO) and further functionalization with heparin (Hep-g-pRGO) and protein (BSA-g-pRGO). They found that the obtained composites exhibited lower

hemolysis ratios and no visible hemoglobin release was observed. After the reduction of GO and functionalization with a biopolymer, the amphiphilic structure of GO was destroyed, and the highly concentrated surface charge of GO vanished; thus, the resulting RGO had difficulty interacting with or partitioning into lipid bilayers, and hemolysis was greatly suppressed. Furthermore, the grafted biopolymer increased the electrostatic repulsion between RGO and the lipid bilayer of the red blood cell membrane, which might further inhibit strong cell interactions and suppress hemolysis. These authors further examined the morphology of human umbilical vein endothelial cells (HUVECs) cultured with the as-prepared composites or with GO reduced with hydrazine. Figure 2 presents the morphology of the cells analyzed by scanning electron microscopy. The best morphology was observed for the cells exposed to Hep-g-pRGO while hydrazine-RGO exhibited the highest toxicity. The cells treated with hydrazine-RGO could hardly attach onto the substrate and showed very few plasmodesma. Furthermore, some RGO particles were adhered to the cells, which might have caused serious adverse effects on the viability of the cells.

Figure 2. Scanning electron microscopy images of HUVECs cultured with pRGO (B-1), Hep-g-pRGO (B-2), BSA-g-pRGO (B-3) and hydrazine-RGO (B-4) at a concentration of 100 mg mL^{-1} for 24 h on glass supports. The black scale bar represents $2 \mu\text{m}$ [22].

Singh *et al.* [23] examined the effect of amine modification of graphene on platelet reactivity. They revealed that amine-modified graphene had no

stimulatory effect on human platelets, nor did it induce pulmonary thromboembolism in mice following intravenous administration. Moreover, it did not evoke the lysis of erythrocytes. These results differ remarkably from other observations with GO and RGO. They suggested that these effects arose from profound physical interactions between GO sheets and platelets. GO, which exhibits substantial interaction with cells, elicited activation-specific platelet responses including aggregation, a rise in intracellular calcium / reactive oxygen species and enhanced protein tyrosine phosphorylation, associated with extensive pulmonary thromboembolism. In contrast, NH_2 -modified graphene was inert toward cells, did not induce platelet stimulation, and had no demonstrable thrombogenicity *in vivo*. Negatively charged graphene sheets physically interacted with platelets and activated them, while positively charged sheets did not. As a direct interaction between nanomaterials and cells modulates critical cell signaling pathways and contributes to observed toxicity, the modification of surface charge with the ensuing diminished effect on nanomaterial cell interaction can bring about a significant attenuation in toxicity. Another method for enhancing GO biocompatibility is functionalization with nanoparticles. In our previous study [24], we showed that GO modification with magnetite nanoparticles resulted in improved biocompatibility with mouse fibroblasts (L929) compared to unmodified GO. A summary of these *in vitro* studies is presented in Table 1.

Jiao *et al.* reported on a metabolomics approach to investigate metabolic responses in graphene treated HepG2 cells [25]. They found that increasing the graphene concentration resulted in a decrease in creatine, which is an intracellular energy intermediate that plays a key role in safeguarding cellular energy storage and transmission. In contrast, graphene up-regulated the N^2 -(D-1-carboxyethyl)-arginine metabolite. Arginine has been found to be involved in urea cycle, an important metabolic pathway in which toxic ammonia produced from amino acid metabolism is converted into urea. This study also showed that graphene caused protein unfolding or mis-folded protein accumulation, resulting in increased energy requirements for protein synthesis, which was consistent with arginine and proline metabolism.

In vivo studies have confirmed the dose-dependent toxicity of GO. Wang *et al.* [15] investigated the influence of GOs on mice and showed that GO at low (0.1 mg) and moderate (0.25 mg) doses did not exhibit obvious toxicity to mice; conversely, a high dose (0.4 mg) induced chronic toxicity, as 4 out of 9 mice died due to lung granuloma formation. It was also shown that GO was mainly located in the lung, liver, spleen, and kidney, and was inefficiently cleared by the kidney. Zhang and co-authors [26] also studied the distribution and biocompatibility of GO in mice and showed that it was predominantly deposited in the lungs, where it was retained for a long period of time. Moreover, GO exhibited prolonged blood circulation and low uptake in the reticuloendothelial system (RES). When the mice were exposed to GO at a dose of 1 mg kg^{-1} for 14 days, no pathological changes were observed in the

examined organs. However, when the dose was increased to 10 mg kg⁻¹, the authors observed pathological changes, including inflammatory cell infiltration, pulmonary edema and granuloma formation in the lungs of mice.

Table 1. Review on *in vitro* studies performed with graphene oxide, graphene and their modified forms

Ref.	Material	Cell line	Results
Wang <i>et al.</i> [15]	GO	Human fibroblasts (HDF)	Dose-dependent toxicity, cytotoxic at a dose above 50 $\mu\text{g mL}^{-1}$; GO mainly located inside cytoplasm such as in lysosomes, mitochondria and the endoplasm.
Chang <i>et al.</i> [16]	GO	Human lung cancer cells (A549)	Dose- and size-dependent toxicity, cytotoxic at a dose of 200 $\mu\text{g mL}^{-1}$; smaller sizes resulted in higher toxicity.
Hu <i>et al.</i> [17]	GO	Human lung cancer cells (A549)	Concentration-dependent toxicity; cytotoxicity of GO as a result of physical damage to the cell membrane, induced by direct interactions between the cell membrane and GO nanosheets; cytotoxicity was independent of the duration of exposure.
Ruiz <i>et al.</i> [18]	GO	Human colorectal adenocarcinoma cells (HT-29)	Enhanced growth of mammalian cells on GO-coated glass slides, indicating that GO promotes cells attachment and proliferation.
Liao <i>et al.</i> [19]	GO and RGO	Adherent human skin fibroblasts (CRL-2522)	RGO more toxic than GO.
Wojtoniszak <i>et al.</i> [20]	GO and RGO dispersed in PEG, DOC and Pluronic	Mouse fibroblasts (L929)	Dose- and dispersant-dependent cytotoxicity; excellent

Ref.	Material	Cell line	Results
			biocompatibility of GO/PEG was observed.
Liao <i>et al.</i> [19]	GO and graphene	Human red blood cells	Dose and size-dependent toxicity; GO with smaller particles showed greater hemolytic activity; graphene showed better biocompatibility than GO; covering GO with chitosan decreased its toxicity.
Santos <i>et al.</i> [21]	Graphene functionalized with PVK	Human fibroblasts (NIH 3T3)	The nanocomposite exhibited lower cytotoxicity compared to unmodified graphene.
Cheng <i>et al.</i> [22]	GO reduced with dopamine and functionalized with heparin	Human blood cells and human umbilical vein endothelial cells	Suppressed hemolysis ratios and no visible hemoglobin release.
Singh <i>et al.</i> [23]	Amine-modified graphene	Platelets from human blood	The material had no stimulatory effect on human platelets nor did it induce pulmonary thromboembolism, or the lysis of erythrocytes.
Urbas <i>et al.</i> [24]	GO/Fe ₃ O ₄	Mouse fibroblasts (L929)	GO modification with Fe ₃ O ₄ resulted in enhanced cytocompatibility.

In vivo studies confirmed the improved biocompatibility of GO following modification of its surface. For instance, Duch *et al.* [27] administered solutions of aggregated graphene, Pluronic dispersed graphene and GO directly into the lungs of mice and investigated the toxicity of these nanomaterials. The introduction of GO resulted in severe lung injury that persisted for more than 21 days after administration. In cultured alveolar macrophages and epithelial cells, GO increased the generation of mitochondrial reactive oxygen species by participating in redox reactions with components of the mitochondrial electron transport chain, thus activating inflammatory and apoptotic pathways. In contrast, this toxicity was significantly reduced in the case of pristine graphene after liquid phase exfoliation and was further minimized when the unoxidized graphene was well-dispersed with the block copolymer Pluronic. The authors suggested that the covalent oxidation of graphene is a major contributor to its pulmonary toxicity and proposed that the dispersion

of pristine graphene in Pluronic provides a method for safe handling and potential biomedical application. Yang *et al.* [28] studied the *in vivo* behavior and long-term toxicity of GO and PEGylated GO derivatives after oral and *intraperitoneal* (i.p.) injection. They showed that PEGylated GO derivatives after oral administration could not be adsorbed by organs and were rapidly excreted. In contrast, PEGylated GO derivatives could be engulfed by phagocytes in the RES system after i.p. administration, in a size- and surface coating-related manner. Despite the long-term retention of i.p. injected GO and PEGylated GO in the mouse body, no significant toxicity was noted. The authors did not observe the formation of granulomas in mice injected with a high doses of GO or PEGylated GO. These results suggest that the *in vivo* behaviors and toxicology of nanomaterials, including graphene, are closely associated with their surface coating, size and route of administration. Chong *et al.* [29] examined the *in vitro* and *in vivo* toxicity of graphene quantum dots (GQD) with sizes varying from 3–5 nm. The material did not induce apoptosis or necrosis in HeLa cells at a concentration of 160 mg mL⁻¹, and the degree of apoptosis was not related to the dose of GQD. PEGylated GQD labeled with Cy7 were intravenously or intraperitoneally injected into Balb / c mice bearing 4T1 murine breast cancer tumors. The authors found that the material preferentially accumulated in the kidneys and at tumor sites compared with the control group, and leaked from the kidneys very quickly. Furthermore, after the injection of GQD-PEG (20 mg kg⁻¹) to mice every day for 14 days, the main organs did not display any clear differences with the control group. The researchers suggested that the very high biocompatibility of GQD originates from the small particle size and high oxygen content compared to the more widely used PEGylated GO. In a later study, Qu *et al.* [30] reported that the addition of a non-ionic surfactant, Tween 80, to the GO suspension hindered GO accumulation in the lungs of treated mice; however, the Tween 80-modified GO tended to accumulate more in the liver compared to the pristine GO suspension. The reason for this difference presumably resides in the first filtration of GO by the lungs as the particles circulate in the blood. Subsequently, the remaining GO in the circulation might be cleared by the liver, leading to greater GO deposition in the liver when GO was suspended in PBS compared to when GO was suspended in Tween 80. Therefore, the accumulation of GO in the liver was likely subject to the dynamics of filtration by the lung. In the liver, most GO aggregates were localized within Küppfer cells (macrophages) and no GO was found in hepatocytes (Figure 3), which highlights the important role of mononuclear phagocytic system (MPS) in clearing GO from the circulation. Moreover, the authors did not find GO in the kidneys of mice given either type of GO, suggesting that GO aggregates and agglomerates were not able to penetrate the glomerular basement membrane when passing through the kidneys. This was probably attributable to the inability of larger particles, such as GO, to cross the basement membrane [30].

Figure 3. Representative histological images of lungs from mice treated with GO suspended in PBS (GO1) and PBS containing Tween 80 (GO2). Hematoxylin and eosin stains of lungs tissues of control mice treated with PBS containing 1 % Tween 80 (a, d), GO1 (b, e) and GO2 (c, f). Arrows show GO aggregations [30].

Lin *et al.* [31] examined the *in vitro* toxicity (in human retinal pigment epithelium (RPE) cells) and *in vivo* biocompatibility of hydroxylated graphene (G-OH) administered to Zelanian rabbits. They found that an amount of 10, 20 and 50 $\mu\text{g mL}^{-1}$ of G-OH did not cause RPE cell apoptosis and DNA damage within 48 h while a minor degree of cell apoptosis and DNA damage were observed when cells were exposed to more than 50 $\mu\text{g mL}^{-1}$. The expression of both caspase-3 and p53 showed increasing trends that were both dose- and time-dependent. Comet and highly reactive oxygen species (ROS) assays also exhibited dose-dependent increasing trends, although no time-dependent change was observed. Possible genotoxicity induced by G-OH might have occurred when the concentration of G-OH was higher than 100 $\mu\text{g mL}^{-1}$. They also found that *intravitreal* injection of G-OH caused few changes to eyesight-related functions such as intraocular pressure, electroretinography and retinal structures. Figure 4 presents transmission electron microscopy (TEM) images of ARPE-19 cells exposed to G-OH for various periods of time. When cells were exposed for 24 h, G-OH was mainly located around the cells. No visible damage or changes to cell morphology were observed, although liquor bubble was increased and expanded. Increased amounts of phagocytic vacuoles with G-OH and G-OH inside karyotheca were observed when cells were exposed for 48 h. No G-OH inside the cell was visible when the incubation time was increased to 72 h. G-OH was released from the cell while the nucleus and organelles remained intact. This finding suggested that penetration of G-OH into and out of the cytoplasm by means of endocytosis and exocytosis did not damage cell membranes [31].

Figure 4. TEM images of APRE-19 cells treated with G-OH for different periods of time [31]

Hu *et al.* [32] investigated the effect of graphene on *in vivo* metabolism in wheat roots. They found that graphene upregulated the content of ribose, which contributed negatively to the number of wheat roots; this could be a result of cell dysfunction. Functionalization of graphene with humic acid (HA) resulted in an enhancement in the anabolism of inositol and an increase in the number of wheat roots, suggesting that HA protects the function of cell membranes exposed to graphene. Their metabolic analysis confirmed that the products of phosphoenolpyruvic acid catabolism, *i.e.* phenylalanine and lanostane, increased the number of wheat roots. For graphene analysis, an enhancement in the content of alkane influenced the biosynthesis of downstream fatty acids and reduced cell membrane fluidity and permeability. Graphene–HA promoted lysine anabolism and the number of wheat roots compared with the blank and graphene. Graphene and graphene–HA, respectively, enhanced and inhibited the biosynthesis of cadaverine, which is a toxic diamine produced by the decarboxylation of lysine in plants during decomposition. Compared with control results, graphene and graphene–HA enhanced and inhibited the biosynthesis of gluconic acid, respectively. Thus, HA may regulate the direction of glucose metabolism fluxes. The content of mannose, which plays osmoprotective and antioxidant roles in extrinsic stress, was up- and downregulated by graphene–HA and graphene, respectively. Graphene and graphene–HA reconstituted the metabolic flux of glycometabolism, fatty acids, amino acids and the tricarboxylic acid cycle, which affected the biosynthesis of chlorophyll. Another study by Hu [33] revealed that GO greatly amplifies the phytotoxicity of arsenic (As), a

widespread contaminant, in wheat, causing a decrease in biomass and root numbers and increased oxidative stress, which are thought to be regulated by metabolism. Compared with the control / As-only / GO-only treatments, AsGO inhibited carbohydrate metabolism, except for galactofuranoside. The inhibition of carbohydrate metabolism was linked to the insufficiency of energy supplementation, which was consistent with growth inhibition. Carbohydrate metabolism is also considered to be an indicator of osmoprotection. Thus, the results indicate that AsGO increased cellular electrolyte leakage. AsGO enhanced amino acid and secondary metabolism, including that of leucine, glycine, phenylalanine, naphthalene and octadecamethyl-cyclononasiloxane, events that are associated with the cellular response to stress. AsGO significantly enhanced the accumulation of a saturated fatty acid (alkane), which was related to a reduction in membrane fluidity, which most likely led to membrane structural damage. AsGO also disturbed the urea cycle and induced the accumulation of amino acids, such as lysine, threonine, asparagine and isoleucine. Specifically, the pathway of nitrogen storage was altered.

23.3. GRAPHENE AND GRAPHENE OXIDE IN TARGETED DRUG DELIVERY

Chemotherapy is commonly used in the treatment of various types of cancers, although the delivery of an effective dose of conventional chemotherapeutic drugs to tumors is challenging due to the presence of multiple barriers. The construction of new and effective drug delivery systems (DDS) with the ability to improve the therapeutic profile and efficacy of therapeutic agents is one of the most challenging issues faced by medicine nowadays. The ideal DDS should exhibit several properties for efficient cancer therapy, *e.g.* targeted delivery of the drug (precisely into tumor cells without damaging healthy tissue), efficient loading of the anticancer drug and controlled release of the drug at the required dosage. In order to achieve precise, controlled drug delivery, drug carriers based on nanomaterials are being increasingly investigated because of their unique structures and tunable properties.

One-atom thickness and two-dimensional planes provide graphene with a large specific surface area for the immobilization of a large number of substances, including a wide range of nanoparticles, biomolecules, fluorescent dyes and drugs. Among various subtypes of graphene-based materials, GO has attracted considerable attention and has been widely investigated in the realm of nanomedicine. Aromatic anticancer drugs can be loaded non-covalently onto GO *via* π - π stacking and/or van der Waals interactions. Moreover, functional groups such as epoxide, hydroxyl and carboxyl [34] at the basal plane and edges of GO enables the formation of strong hydrogen bond interactions and covalent grafting with various drugs.

Pristine graphene is highly hydrophobic and water insoluble due to the strong π - π interactions between graphene sheets. GO and its chemically converted derivatives form stable suspensions in pure water, but they generally tend to aggregate in saline or other biological solutions. In addition, many anticancer drugs exhibit low water solubility and poor bioavailability; moreover, the emergence of drug resistance in patients significantly decreases their therapeutic efficacy. Therefore, a variety of approaches have been elaborated to prepare water soluble graphene and to improve the stability of graphene, its derivatives and hybrids in buffer solutions and biological media. For example, synthetic polymers or biopolymers have been widely used to stabilize and improve the performance of GO and RGO as nanocarriers in nanomedicine [35-37]. Numerous attempts have been made to build graphene-based nanocarrier systems with good water solubility with the usage of PEG [38-40], a hydrophilic biocompatible polymer extensively conjugated with various nanomaterials in order to reduce their non-specific adsorption to biological molecules and cells, and to improve their biocompatibility and *in vivo* pharmacokinetics for better tumor targeting. To investigate the biodistribution and clearance of PEGylated graphene, hematoxylin and eosin stained images of several organs from mice in the control and treated groups 3, 7, 20, 40 and 90 days after the injection of nanographene sheets-PEG (NGS-PEG) at the dose of 20 mg kg^{-1} were collected [38]. No significant toxic effect on the organs was observed at various time points after the injection of NGS-PEG, except for some brown-black spots in the liver and spleen noted at early time points. Overall, there were no apparent histopathological abnormalities or lesions in the treated groups at the tested NGS-PEG dose (Figure 5).

Figure 5. Representative hematoxylin and eosin stained images of major mouse organs: liver, spleen, and kidney collected from control untreated mice and NGS-PEG injected mice at various time points post-injection. The dose of NGS-PEG was 20 mg kg^{-1} . No obvious organ damage or lesions were observed for NGS-PEG treated mice [38].

The first reports on applying this polymer to enhance the performance of graphene materials comes from the Dai group [41,42]. Both publications present the preparation of biocompatible nanoGO conjugated with six-armed PEG-amine stars *via* carbodiimide catalyzed amide formation. It was confirmed that the enhancement of aqueous stability to the nanoGO in buffer solutions and other biological environments can be achieved by this process. The first publication also presents studies on the conjugation of NGO-PEG hybrid with SN38, a camptothecin (CPT) analogue. The study revealed high potency regarding cancer cell damage, similar to that of free SN38 in an organic solvent. The second publication described work on loading doxorubicin (DOX) onto ultrasmall nanoGO sheets functionalized with PEG. One of the latest reports [43] proposed a new DDS based on PEGylated GO loaded with paclitaxel (PTX) tested on human lung cancer A549 and human breast cancer MCF-7 cells. GO-PEG-PTX presented higher cytotoxic effect on both cells in comparison with free PTX in time- and concentration-dependent manner. One of the first reports on improving the aqueous stability of graphene / GO-based nanocarrier systems by coating with natural polymers other than PEG was published by Bao *et al.* [44], who confirmed the enhanced aqueous solubility and biocompatibility of chitosan-functionalized GO. Additionally, Zhang *et al.* [45] used dextran to achieve a similar goal. Recently, in order to improve the colloidal stability of graphene, the group of Maity [46] covered nanosheets with a carbohydrate coating composed of a mixture of chitosan and dextran. The abundance of hydrophilic groups present in dextran and chitosan made the hybrid highly water soluble, while the presence of primary amine groups on chitosan enabled further covalent functionalization of the system. Similar to the roles of PEG, chitosan or dextran in graphene-based DDSs, poly(ethylene imine) [47,48], heparin [49,50], dimethylaminoparthenolide (DMAPT) [51] and starch (a linear polymer formed by α -1-4 glycosidic bonds between D-glucose units) [52] can also be used. Wojtoniszak *et al.* [53] found that the dispersion of GO covalently conjugated with methotrexate (MTX) in biocompatible polymers (PEG and poly(styrenesulfonate) (PSS)) resulted in an extension of the MTX release time in comparison to the suspension in phosphate buffered saline (PBS). The anti-proliferative activity of the anticancer drug bound to GO depended on the dispersant used to stabilize the suspension. Recently, cyclodextrin and cyclic oligosaccharides have also been used in DDS [54] in order to prevent the intermolecular aggregation of GO sheets in water, which then facilitates the disruption of a pristine GO sheet into smaller components during sonication. The targeting ability of the system was achieved due to the presence of hyaluronated adamantane (HA-ADA) chains, since the HA skeleton can precisely recognize the HA receptor expressed by tumor cells during cancer metastasis.

It has been established that the size of the nanocarrier plays an important role in controlling the *in vivo* fate [55] and in overcoming the barriers of the reticuloendothelial system to transport anticancer drug molecules deeply into

tumor cells by means of intravenous injection [56]. Nanomaterials with a size smaller than 100 nm are now considered more suitable for extravasation from the bloodstream into tumors through the enhanced permeation and retention (EPR) effect [57]. It is believed that nanocarriers with a size smaller than 100 nm can enhance the therapeutic effect, so they have been the focus of considerable research efforts in recent years. For example, Zhao *et al.* [58] presented a DDS based on biocompatible GO nanoparticles (GON) prepared under strong oxidizing conditions and with a prolonged oxidation time. The material exhibited a nearly spherical shape with a size of *cca.* 43 nm. It was then conjugated onto a functional polymer synthesized from PEGylated alginate brushes (ALG-PEG) and cytamine (Cy), forming a biocompatible, 3-D nanoscaled nanocarrier for the efficient loading and triggered release of DOX. The functional polymer Cy-ALG-PEG was grafted onto GON *via* reduction-sensitive disulfide bonds, so the nanocarrier was responsive to the cleavage of the disulfide bond to detach the Cy-ALG-PEG polymer moieties under reducing conditions. Once being taken up by tumor cells, the Cy-ALG-PEG brushes detached to release the loaded DOX. The majority of researchers focused on exploring the potential applications of graphene in the field of drug delivery have investigated graphene / GO sheets with a size less than 100 nm [41,51,54].

We can differentiate two targeting approaches: passive strategies based on EPR and active strategies that take advantage of the overexpression of receptors on cancer cells. Therefore, nanomaterials with targeting moieties, such as antibodies, peptides, and other ligands, are able to recognize and bind to tumor tissue through specific interactions between targeting moieties and receptors on the tumor. For example, Zhang *et al.* [59] built a nanocarrier for mixed anticancer drugs, *i.e.* DOX and CPT, based on folic acid (FA) covalently grafted to nanoscale GO (NGO) functionalized with sulfonic acid groups. The sulfonic acid groups rendered NGO stable in physiological solutions, while the FA moieties allowed it to specifically target MCF-7 cells, *i.e.* human breast cancer cells with FA receptors. It was demonstrated that FA-NGO loaded with the two anticancer drugs showed specific targeting to MCF-7 cells and remarkably high cytotoxicity compared to NGO loaded with either DOX or CPT only. The Maity group [46] also used FA to provide targeting specificity in their graphene-based nanocarrier with a carbohydrate coating for both hydrophobic and hydrophilic drugs such as paclitaxol, CPT, DOX, curcumin. Yang *et al.* [60] added FA-modified β -cyclodextrin to their graphene-based nanocarrier for DOX as a target unit. The research team of Robinson [61] applied a targeting peptide composed of the Arg-Gly-Asp (RGD) motif to nano-rGO, which provided selective cellular uptake in U87MG cancer cells and highly effective photoablation of cells *in vitro*. Sun *et al.* [42] loaded DOX onto nano-GO with high capacity. Antibody-guided targeting selectively transported the anticancer drug into specific cancer cells. Despite the well-established good performance of the abovementioned targeting moieties, they have also exhibited some

drawbacks. The usage of FA is economically preferable, but its poor water solubility limits its wide application. Peptides have limited target cell types, while the main disadvantage of applying antibodies is their cost. On the other hand, the hyaluronic acid (HA) moiety, which also exhibits specific recognition of receptors overexpressed on the surface of various tumor cells, possesses better water-solubility and stability. It is also preferable from the economical point of view and ubiquitously available. In a study presented by Song *et al.* [62], a system has been developed based on the conjugation of HA to GO *via* hydrogen bonding and loading with DOX. *In vitro* studies (cellular uptake and cytotoxicity assays) on model cells revealed that the HA-GO-DOX system transported DOX specifically to target HepG2 cells, and then efficiently inhibited their proliferation. Simultaneously, the viability of control cells remained high, suggesting a reduction in the side effects of anticancer drug on normal tissue. The tumor inhibition rates of HA-GO-DOX, GO-DOX and free DOX were evaluated in an *in vivo* study using an H22 hepatic cancer cell-bearing mouse model. The studies showed that the highest anticancer efficacy was exhibited by the hybrid developed with HA. HA was also applied in DDS by the group of Jung [63], who constructed a nanoGO-hyaluronic acid (NGO-HA) conjugate loaded with epirubicin, a cancer drug favored over DOX, by π - π stacking. *In vitro* tests confirmed the pH-dependent drug release and target-specific anti-cancer effect of the complex on B16F1 cells. The enhanced release of epirubicin under acidic conditions might be beneficial for the target-specific intracellular delivery of cancer drugs.

One of the most promising directions in targeted drug delivery is the construction of magnetic-functionalized graphene-based hybrids loaded with anticancer drugs. The first report in this field was published by the group of Yang [64], who developed a hybrid of GO with Fe₃O₄ nanoparticles by a chemical deposition method and conjugated it with DOX hydrochloride (DXR). High drug loading and a large amount of Fe₃O₄ nanoparticles deposited on the graphene sheets were obtained simultaneously. This system exhibited superparamagnetic properties, it tended to congregate at acidic pH values and could be redispersed at basic pH values. The GO-Fe₃O₄ hybrid and GO-Fe₃O₄-DXR moved under the influence of an external magnetic field after congregating under acidic conditions. The observed pH-triggered controlled magnetic behavior allows us to consider this material as a promising candidate for targeted drug delivery purposes. Ma *et al.* [65] also synthesized a hybrid based on GO and iron nanoparticles for magnetically targeted drug delivery. Additionally, they functionalized it with PEG to acquire high stability in physiological solutions. As in the previous report, the hybrid was loaded with DOX. Pluronic F127 [66] and poly(ethylene imine) [44] have also been studied as agents to provide physiological dispersivity and stability in such systems. A very interesting approach based on combining the therapeutic effect of two anticancer drugs, CPT and methotrexate (MTX), loaded onto an ultrafine GO-magnetic nanoparticle nanocomposite was recently presented by Shen *et*

al. [67]. The *in vivo* assay conducted on male S-180 sarcoma-bearing Balb/c mice showed specific accumulation capacity to the tumor site by the induction of an external magnetic field (Figure 6). Increased drug and magnetic nanoparticle accumulation in that field and the consequent dual-drug synergistic effect and hyperpyrexia significantly contributed to an increase in the inhibition of tumor cell growth and the induction of necrosis. A pH-dependent drug release pattern was observed, in which the anticancer drugs MTX and CPT were released around tumor tissues.

Figure 6. (A) Typical *in vivo* images of male S-180 sarcoma-bearing Balb/c mice treated with magnetic field at defined time periods (0, 1, 2, 3, and 4 h) after intravenous injection of the RhB-labeled MTX@uGO-COOH@MNP@OA@CPT composite. The tumor sites are indicated by red circles. Color coding of fluorescence indicates high levels of fluorescence in red and low levels in blue. Excitation = 570 nm, emission = 650 nm. (B) Representative microscopic image of histochemical analysis of tumor tissue induced with a magnetic field for 4 h after intravenous administration (H&E and Prussian blue stain, 40×) [67].

Wang *et al.* [68] improved the delivery efficacy and targeting of the drug carrier based on GO-Fe₃O₄ by conjugating the hybrid with FA *via* chitosan as a bridge, followed by loading with DOX. This is an example of the successful combination of active targeting (FA) and passive targeting (magnetic nanoparticles) strategies in drug delivery. Chitosan enhanced the stability and biocompatibility of the complex and also encapsulated and controlled the release of drug molecules. Studies revealed high loading efficiency, prolonged release rate and a pH-dependent mechanism of drug release; pH-activated drug release was also observed by Fan *et al.* [69] and Wang *et al.* [70], who presented the preparation of DDSs based on magnetic-functionalized graphene and 5-fluorouracil (5-FU). Xu *et al.* [71] investigated a similar DDS with levofloxacin (LOFX) and Chen *et al.* [72] described the construction of multifunctional stimuli-responsive nanosystems.

Recent advances in the field of designing graphene-based nanocarriers dedicated for drug delivery applications have led to opening up exciting opportunities for the future and the broad usage of this kind of nanomaterial in real clinical conditions.

23.4. GRAPHENE AND GRAPHENE OXIDE IN PHOTODYNAMIC AND PHOTOTHERMAL THERAPY

Photothermal (PTT) and photodynamic (PDT) therapies are emerging non-invasive modalities for the treatment of various cancers. PDT involves the utilization of a photosensitizer (PS) which is excited with light of appropriate wavelengths, then emits fluorescence via the relaxation of the excited-singlet-state PS back to the ground state. PS can transfer the absorbed photon energy to oxygen in the surrounding tissue. The generation of ROS such as singlet oxygen and free radicals can oxidize cellular and sub-cellular compartments, resulting in irreversible damage to tumor cells [73,74]. Unlike photosensitizers, a PTT agent absorbs the light, which is then transformed into heat and transferred to the intercellular environment, which induces localized hyperthermia [75].

The first report on graphene utilization in PTT therapy was presented by Young *et al.* [76]. They examined nanographene sheets (NGS) functionalized with PEG conjugated *via* amide formation. The material exhibited high solubility and stability in physiological solution and displayed strong near infrared (NIR) radiation absorption. Moreover, it showed efficient tumor passive targeting and relatively low retention in the RES, which was hypothetically caused by the unique two-dimensional shape, small size (10–50 nm) and biocompatible PEG coating that favored the EPR effect of NGS. Therefore, an *in vivo* PTT therapy study was performed using mice bearing tumors (4T1). After exposure to the laser (808 nm), the surface temperature of tumor in NGS-PEG injected mice reached ~ 50 °C, compared to a ~ 2 °C rise for irradiated tumors in uninjected mice. All tumors of mice injected with NGS disappeared after 1 day and no tumor regrowth was noted over a course of 40 days, in contrast with control group where rapid tumor growth was observed. The *in vivo* results showed that PEGylated NGS is an excellent PTT therapy agent without exerting any noticeable toxic effects in treated mice.

Sahu *et al.* [77] functionalized nanoGO sheets (nanoGO) with Pluronic block copolymer and complexed them with methylene blue (MB) *via* electrostatic interactions. The nanoGO-MB hybrid showed pH responsive properties, as the release rate increased considerably under acidic conditions. This nanocomplex showed enhanced uptake of cancer cells compared to normal cells, and in the absence of light, it showed no major toxicity in cells. Intravenous injection of the composite into tumor bearing mice led to high tumor accumulation. PTT treatment resulted in a decrease in tumor growth but failed to abolish the

tumor. A slight decrease in tumor growth as compared to control group was observed after 10 min of laser exposure during PDT treatment. The combination of near-infrared (NIR) light-induced PDT therapy and subsequent PTT therapy led to complete ablation of the tumor as well as no regrowth of the tumor in 15 days, indicating the synergistic effect of dual phototherapy.

Jiang with co-workers [78] investigated graphene coated with tetrasulfonic acid tetrasodium salt copper phthalocyanine (TSCuPc) in combined *in vitro* PTT / PDT therapy. In this system, GR acted as a photosensitizer carrier and PTT agent, while TSCuPc acted as a hydrophilic PDT agent. *In vitro* cytotoxicity of PTT and PDT was determined with HeLa cells incubated in culture medium containing a series concentration of GR-TSCuPc for 24 h, and then irradiated with a 650 nm laser for 5 min. The cell viability results showed that the phototherapy effect of GR-TSCuPc was noticeably higher than that of free TSCuPc, indicating that combined non-invasive PTT / PDT exhibits better anticancer efficacy.

Golavelli *et al.* [79] immobilized a hydrophobic silicon naphthalocyaninebis (trihexylsilyloxy) (SiNc4) photosensitizer onto water dispersible magnetic and fluorescent graphene (MFG) *via* π - π stacking to yield the MFG-SiNc4 nanohybrid, using a non-covalent approach for single light-induced PTT / PDT. The phototherapeutic capability MFG-SiNc4 was further assessed in HeLa cells by monitoring cell viability under dark/light conditions. It was found that, under photoirradiation with a 775 nm laser, MFG-SiNc4 exerted a cytotoxic effect on HeLa cells. The PTT / PDT capabilities with appreciable cell killing efficacy was about 97.9 %; of this, 64.7 % was due to PDT and 33.2 % was due to PTT, suggesting that the decrease in cell viability by MFG-SiNc4 was mostly due to the toxicity induced by $^1\text{O}_2$ and other ROS. However, the inherent PTT capability of MFG, owing to its enhanced π -electrons and NIR adsorption, also contributed to some extent to the decrease in cell viability. Thus, immobilizing SiNc4 onto graphene facilitated the utilization of a single light source to simultaneously generate the PTT/PDT effect.

In another study, Yan and co-workers [80] designed a photo-theranostic platform based on sinoporphyrin sodium (DVDMS) photosensitizer-loaded PEGylated GO (GO-PEG-DVDMS) for combined PDT and PTT. After loading DVDMS onto GO-PEG an increase in optical absorption at 808 nm was observed with an increase in DVDMS loading. This resulted in the improved PTT effect of GO-PEG-DVDMS. Moreover, DVDMS anchoring onto GO-PEG did not inhibit the evolution of singlet oxygen. Interestingly, an *in vivo* study on PC9 tumor-bearing mice revealed that GO-PEG-DVDMS had highly efficient passive tumor targeting ability and a long retention time in the tumor tissue. It is worth noting that, during PTT treatment, all tumors treated with GO-PEG-DVDMS and laser irradiation (808 nm) were effectively ablated, leaving black scars at the original tumor sites; however, one week after treatment, the wounds had fully recovered. Furthermore, complete tumor regression was achieved by

intravenous injection of GO-PEG-DVDMS, at low injection dose of 1 mg kg^{-1} of GO-PEG and 2 mg kg^{-1} of DVDMS, in PDT treatment followed by PTT.

Zhen *et al.* [81] prepared FA-GO-PEG-fullerene (FA-GO-PEG/ C_{60}) nanohybrids for targeting photothermally enhanced PDT therapy. In this work, FA and PEG were conjugated onto GO *via* an imide linkage. Then, the FA-GO-PEG/ C_{60} nanohybrid was prepared by using nucleophilic addition. FA and PEG-functionalized graphene was used as nanocarrier of tumor-targeting C_{60} which offered improved HeLa cells uptake. After irradiation with a 532 nm laser, FA-GO-PEG/ C_{60} caused a marked decrease in cell survival and elevation in oxidative stress, which induced apoptotic death. Compared with PDT or PTT alone, the combined treatment with FA-GO-PEG/ C_{60} showed increased cell apoptosis and death, indicating a synergistic effect of combination phototherapy. Based on this study, the hypothetical mechanism of PTT and PDT combined therapy of FA-GO-PEG/ C_{60} is shown in Figure 7. The combination of graphene with fullerene C_{60} provides remarkable synergies in phototherapy. Firstly, the hybridization process prevents the restacking of graphene and the aggregation of C_{60} , which supply more surface active sites and increase the PDT efficiency of FA-GO-PEG/ C_{60} . Secondly, owing to the doping effect, the FA-GO-PEG/ C_{60} exhibits a broader absorption range and higher absorbance. Thirdly, the PTT effects of FA-GO-PEG/ C_{60} cause obvious cell damage and achieve synergistic enhancement of the phototherapy effects. Fourthly, the graphene carrier equipped with FA and PEG is specifically targeted to tumor cells, which significantly enhances the cellular uptake of FA-GO-PEG/ C_{60} . Once the FA-GO-PEG/ C_{60} is taken up by tumor cells, it increases in temperature and causes considerable intracellular ROS production under light irradiation. The photogenerated ROS and heating cause a marked decrease in cell survival and increased oxidative stress. Finally, the phototherapy effects of FA-GO-PEG/ C_{60} apparently involve the induction of apoptosis [81].

Figure 7. The hypothetical mechanism of synergistic enhancement of FA-GO-PEG/C₆₀ in combined PTT and PDT [81]

Rong *et al.* [82] confirmed that PEGylated GO is a good nanocarrier for photosensitizer molecules and enhances its cellular uptake. They loaded 2-(1-hexyloxyethyl)-2-devinyl (HPPH) onto GO-PEG to produce a GO-PEG-HPPH nanocomposite. The nanoplatform allowed for increased uptake of the HPPH into 4T1 murine mammary cancer cells through a more active endocytosis process compared to free HPPH, which accumulated through passive diffusion. Moreover, the cancer cells exposed to 671 nm laser for 3 min exhibited much lower viability when treated with GO-PEG-HPPH than with free HPPH and GO-PEG. *In vivo* study confirmed the accumulation of GO-PEG-HPPH in tumors, while a very small amount of the hybrid was observed in organs such as the liver and spleen, indicating high tumor selectivity. *In vivo* PDT study was carried out in 4T1 bearing mice intravenously injected with GO-PEG-HPPH or free HPPH at the same HPPH concentration. Mice were irradiated with a 671 nm laser 24 h after injection for 20 min. Tumors treated with GO-PEG-HPPH and irradiation were effectively ablated. However, the HPPH treated group showed negligible damage to the tumors and delayed tumor growth compared with the control groups. Importantly, the complex did

not exhibit toxicity to the treated mice, including no damage to the liver, spleen, kidney, heart or lung.

In another paper, Taratula with co-workers [83] developed novel low-oxygen graphene nanosheets (LOGr) chemically modified with poly(propyleneimine) dendrimers (LHRH) loaded with phthalocyanine (Pc) as a photosensitizer for imaging and PTT/PDT combination therapy. To evaluate the efficiency of the new nanoplatform as a PTT/PDT agent, the phototherapeutic effect of the LOGr-Pc-LHRH complex on ovarian cancer cells was examined. When the two treatments were combined under single 690 nm light irradiation, ovarian cancer cell viability was significantly reduced as compared with LOGr-Pc-LHRH (PTT) and LOGr-Pc-LHRH(PDT) treatment applied separately. Moreover, the cytotoxicity of LOGr-LHRH, Pc-LHRH, and LOGr-Pc-LHRH toward red blood cells was investigated using hemolysis studies and it was found that the nanoplatforms exhibited minimal hemolytic activity. The encapsulated photosensitizer, due to its very hydrophobic nature, was not able to escape from the dendrimer-based carrier in an intracellular environment, and hence could not directly induce toxicity.

Table 1. Summary on reviewed applications of graphene and GO, where DDS corresponds to DDS, TDDS–targeted DDS, PTT–PTT therapy, PDT–PDT therapy

Material	Application	Cell line	References
NGO-PEG-SN38	DDS	HCT-116, OVCAR-3, U87MG, MDA-MB-435, MCF-7 and MDA-MB-468 cancer cell lines	[41]
NGO-PEG-DOX	DDS	Raji and CEM	[42]
NGO-PEG-PTX	DDS	MCF 7	[43]
GO-CS-CPT	DDS	HepG2	[44]
GO-DEX-CS-Folate	DDS	HeLa	[46]
GO-PEI-UCNPs-DOX	DDS	MCF 7	[47]
GO-PEI-pDNA	DDS	HeLa	[48]
rGO-LHT7-DOX	DDS	KB cells and KB-bearing mice	[50]
G-COOH-PTL	DDS	Panc-1	[51]
Starch-functionalized graphene-HCPT	DDS	SW-620	[52]
GO-PEG-MTX and GO-PSS-MTX	DDS	MCF 7	[53]
CPT-GO-CD-HA-ADA	TDDS	MDA-MB-241	[54]
PEG-ALG-GON-DOX	TDDS	HepG2	[58]

Material	Application	Cell line	References
FA-NGO-DOX-CPT	TDDS	MCE-7	[59]
CD-ADA-PP-GO-DOX	DDS	HeLa-bearing mice	[60]
RGD- or RAD-functionalized GO	TDDS	U87 Mg	[61]
HA-GO-DOX	TDDS	H22-bearing mice	[62]
NGO-HA-DOX	TDDS	B16F1	[63]
GO-Fe-PEG-DOX	TDDS, PTT, MRI	4T1	[65]
MTX@u-GO-COOH-MNP@OA-CPT	TDDS, PTT	HepG2	[67]
G-Fe ₃ O ₄ -5-FU	TDDS	HepG2	[69]
GO-Fe ₃ O ₄ -5-FU	TDDS, MRI	-	[70]
GO-Fe ₃ O ₄ -MnO _x	TDDS, MRI	-	[71]
NGS-PEG	PTT	4T1-bearing mice	[76]
NGO-Pluronic-MB	PTT/PDT	HeLa-bearing mice and HeLa cells	[77]
Graphene-TSCuPc	PTT/PDT	HeLa cells	[78]
Fe-Graphene-fluoresceine-SiNC ₄	PTT/PDT	HeLa cells	[79]
GO-PEG-DVDMS	PTT/PDT	PC9-bearing mice	[80]
FA-GO-PEG/C ₆₀	PTT/PDT	HeLa cells	[81]
GO-PEG-HPPH	PDT	4T1-bearing mice	[82]
LOGr-Pc-LHRH	PTT/PDT	Ovarian cancer cells	[83]

23.5. SUMMARY

Extensive research into the toxicity and biocompatibility of graphene and GO has revealed multiple factors influencing their behavior in biological systems. Both *in vitro* and *in vivo* studies have shown the size- and dose-dependent toxicity of these nanomaterials. Another significant aspect is the concentration of oxygen-containing functional groups present on the surface of GO or RGO. For instance, blood toxicity studies have shown that graphene is more biocompatible than GO, which is a result of interaction between O-groups and blood cells. Functionalization of GO improves its biocompatibility, and different materials have been used for that purpose, for instance PEG, PVK, dopamine, heparin or amine modifiers. *In vivo* studies have revealed that the method of nanomaterial administration influences their behavior in living organisms. For example, PEGylated GO is rapidly excreted after oral administration, while after *intraperitoneal* administration it is engulfed by phagocytes in the RES and retained for a long period of time.

GO has been explored as an excellent nanocarrier of drugs by enhancing their cytotoxic effect. However, in order to prevent aggregation in a physiological environment, modification of the GO surface is necessary. The most commonly used materials are biocompatible polymers, such as PEG, chitosan, dextran and poly(ethylene imine). Another advantage of GO utilization as a nanocarrier of drugs is the opportunity to develop a targeted drug delivery system (TDDS). This is possible by functionalizing the nanocarrier with receptors, such as antibodies, peptides and other ligands that are able to specifically recognize tumor cells. Another TDDS is based on the functionalization of GO with superparamagnetic molecules, which enable the delivery of drugs under the influence of an external magnetic field. Furthermore, simultaneous functionalization of GO with an antitumor drug and magnetic particles has an effect on dual-drug synergism and hyperpyrexia, which significantly contributes to increased inhibition of tumor cell growth and the induction of necrosis.

Recent studies have shown that nanocarriers with sizes smaller than 100 nm can enhance the therapeutic effect. Particles of this size are most suitable for extravasation from the bloodstream into tumors due to the enhanced permeation and retention effect and increased tumor tissue permeation.

Graphene and GO are also appropriate nanocarriers of PDT therapeutic agents, and can enhance their anticancer activity. Simultaneously, they act as PTT agents able to absorb NIR irradiation, producing local hyperthermia resulting in the death of tumor tissues. Many reports have revealed that the combination of PDT and PTT in tumor tissues results in improved anticancer therapy.

Recent studies have demonstrated the great potential of graphene and GO usage in biomedical applications, especially in antitumor therapy, including targeted drug delivery and PDT/PTT therapies. However, the toxicology of these materials is still questionable and needs to be resolved before clinical use.

REFERENCES

1. C. Chung, Y.K. Kim, D. Shin, S.R. Ryoo, B.H. Hong, D.H. Min. *Acc. Chem. Res.* **46** (2012) 2211–2224.
2. N.S.W. Kam, T.C. Jessop, P.A. Wender, H.J. Dai. *J. Am. Chem. Soc.* **126** (2004) 6850–6851.
3. J. Shi, X. Yu, L. Wang, J. Gao, J. Zhang, R. Ma, R. Liu, Z. Zhang. *Biomaterials* **34** (2013) 9666–9677.
4. J. Liu, L. Cui, D. Losic. *Acta Biomater.* **9** (2013) 9243–9257.
5. A. Bianco, H.M. Cheng, T. Enoki, Y. Gogotsi, R.H. Hurt, N. Koratkar, T. Kyotani, M. Monthieux, C.R. Park, J.M.D. Tascon, J. Zhang. *Carbon* **65** (2013) 1–6.
6. Y. Li, H. Dong, Y. Li, D. Shi. *Int. J. Nanomedicine* **10** (2015) 2451–2459.
7. K.S. Novoselov, A.K. Geim, S.V. Morozov, D. Jiang, Y. Zhang, S.V. Dubonos. *Science* **306** (2004) 666–669.
8. H.C. Lin, Y.Z. Chen, Y.C. Wang, Y.L. Chueh. *J. Phys. Chem. C* **119** (2015) 6835–6842.
9. A.J. Van Bommel, J.E. Crombeen, A. Van Tooren. *Surf. Sci.* **48** (1975) 463–472.
10. W.A. de Heer, C. Berger, X. Wu, P.N. First, E.H. Conrad, X. Li, T. Li, M. Sprinkle, J. Hass, M.L. Sadowski, M. Potemski, G. Martinez. *Solid State Commun.* **143** (2007) 92–100.
11. W.S. Hummers, R.E. Offeman. *J. Am. Chem. Soc.* **80** (1958) 1339–1339.
12. S.F. Pei, H.M. Cheng. *Carbon* **50** (2012) 3210–3228.
13. B.C. Brodie. *Ann. Chim. Phys.* **59** (1860) 466–472.
14. L. Staudenmaier. *Ber. Dtsch. Chem. Ges.* **31** (1898) 1481–1487.
15. K. Wang, J. Ruan, H. Song, J. Zhang, Y. Wo, S. Guo, D. Cui. *Nanoscale Res. Lett.* **6** (2011) 8–15.
16. Y. Chang, S.T. Yang, J.H. Liu, E. Dong, Y. Wang, A. Cao, Y. Liu, H. Wang. *Toxicol. Lett.* **200** (2011) 201–210.
17. W. Hu, C. Peng, M. Lv, X. Li, Y. Zhang, N. Chen, C. Fan, Q. Huang. *ACS Nano* **5** (2011) 3693–3700.
18. O.N. Ruiz, K.A. Shiral Fernando, B. Wang, N.A. Brown, P.G. Luo, N.D. McNamara, M. Vangsness, Y.P. Sun, C.E. Bunker. *ACS Nano* **5** (2011) 8100–8107.
19. K.H. Liao, Y.S. Lin, C.W. Macosko, C.L. Haynes. *ACS Appl. Mater. Interfaces* **3** (2011) 2607–2615.
20. M. Wojtoniszak, X. Chen, R.J. Kalenczuk, A. Wajda, J. Łapczuk, M. Kurzewski, M. Drozdziak, P.K. Chuc, E. Borowiak-Palen. *Colloids Surf. B* **89** (2012) 79–85.
21. C.M. Santos, J. Mangadlao, F. Ahmed, A. Leon, R.C. Advincula, D.F. Rodrigues. *Nanotechnology* **23** (2012) 395101–395110.
22. C. Cheng, S. Nie, S. Li, H. Peng, H. Yang, L. Ma, S. Sun, C. Zhao. *J. Mater. Chem. B* **1** (2013) 265–275.
23. S.K. Singh, M.K. Singh, P.P. Kulkarni, V.K. Sonkar, J.J.A. Gracio, D. Dash. *ACS Nano* **6** (2012) 2731–2740.
24. K. Urbas, M. Aleksandrak, M. Jedrzejczak, M. Jedrzejczak, R. Rakoczy, X. Chen, E. Mijowska. *Nanoscale Res. Lett.* **9** (2014) 656–667.
25. X. Zhang, J. Yin, C. Peng, W. Hu, Z. Zhu, W. Li, C. Fan, Q. Huang. *Carbon* **49** (2011) 986–995.
26. M.C. Duch, G.R.S. Budinger, Y.T. Liang, S. Soberanes, D. Urich, S.E. Chiarella, L.A. Campochiaro, A. Gonzalez, N.S. Chandel, M.C. Hersam, G.M. Mutlu. *Nano Lett.* **11** (2011) 5201–5207.

27. K. Yang, H. Gong, X. Shi, J. Wan, Y. Zhang, Z. Liu. *Biomaterials* **34** (2013) 2787–2795.
28. Y. Chong, Y. Ma, H. Shen, X. Tu, X. Zhou, J. Xu, J. Dai, S. Fan, Z. Zhang. *Biomaterials* **35** (2014) 5041–5048.
29. G. Qu, X. Wang, Q. Liu, R. Liu, N. Yin, J. Ma, L. Chen, J. He, S. Liu, G. Jiang. *J. Environ. Sci.* **25** (2013) 873–881.
30. M. Lin, R. Zou, H. Shi, S. Yu, X. Li, R. Guo, L. Yan, G. Li, Y. Liu, L. Dai. *Mat. Sci. Eng. C* **50** (2015) 300–308.
31. D.R. Dreyer, S. Park, C.W. Bielawski, R.S. Ruoff. *Chem. Soc. Rev.* **39** (2010) 228–240.
32. L. Zhang, Z. Wang, Z. Lu, H. Shen, J. Huang, Q. Zhao, M. Liu, N. He, Z. Zhang. *J. Mater. Chem. B* **1** (2013) 749–755.
33. W. Miao, G. Shim, C.M. Kang, S. Lee, Y.S. Choe, H.G. Choi. *Biomaterials* **34** (2013) 9638–9647.
34. Y.K. Kim, M.H. Kim, D.H. Min. *Chem. Commun.* **47** (2011) 3195–3197.
35. K. Yang, J. Wan, S. Zhang, Y. Zhang, S.T. Lee, Z. Liu. *ACS Nano* **5** (2011) 516–522.
36. S.J. Zhu, H. Zhen, Y.J. Li, P. Wang, X.Y. Huang, P. Shi. *J. Nanopart. Res.* **16** (2014) 2530–2541.
37. H.L. Xiong, Z.Y. Guo, W. Zhang, H.Q. Zhong, S.H. Liu, Y.H. Ji. *J. Photochem. Photobiol. B: Biol.* **138** (2014) 191–201.
38. Z. Liu, J.T. Robinson, X. Sun, H. Dai. *J. Am. Chem. Soc.* **130** (2008) 10876–10877.
39. X. Sun, Z. Liu, K. Welscher, J.T. Robinson, A. Goodwin, S. Zaric, H. Dai. *Nano Res.* **1** (2008) 203–212.
40. Z. Xu, S. Zhu, M. Wang, Y. Li, P. Shi, X. Huang. *ACS Appl. Mater. Interfaces* **7** (2015) 1355–1363.
41. H. Bao, Y. Pan, Y. Ping, N.G. Sahoo, T. Wu, L. Li, J. Li, L.H. Gan. *Small* **7** (2011) 1569–1578.
42. S. Zhang, K. Yang, L. Feng, Z. Liu. *Carbon* **49** (2011) 4040–4049.
43. A.R. Maity, A. Chakraborty, A. Mondal, N.R. Jana. *Nanoscale* **6** (2014) 2752–2758.
44. L. Yan, Y.N. Chang, L.N. Zhao, Z.J. Gu, X.X. Liu, G. Tian, L.J. Zhou, W.L. Ren, S. Jin, W. Yin, H. Chang, G. Xing, X. Gao, Y. Zhao. *Carbon* **57** (2013) 120–129.
45. B.A. Chen, M. Liu, L.M. Zhang, J. Huang, J.L. Yao, Z.J. Zhang. *J. Mat. Chem.* **21** (2011) 7736–7741.
46. Y. Wang, P. Zhang, C. Fang, C. Liu, L. Zhan, Y.F. Li, C.Z. Huang. *RSC Adv.* **2** (2012) 2322–2328.
47. G. Shim, J.Y. Kim, J. Han, S.W. Chung, S. Lee, Y. Byun, Y.K. Oha. *J. Control. Release* **189** (2014) 80–89.
48. A. Karmakar, Y. Xu, T. Mustafa, G. Kannarpady, S.M. Bratton, A. Radominska-Pandya, A. Crooks, A.S. Biris. *RSC Adv.* **5** (2015) 2411–2420.
49. K. Liu, Y. Wang, H. Li, Y. Duan. *Colloids Surf B Biointerfaces* **128** (2015) 86–93.
50. M. Wojtnoszak, K. Urbas, M. Peruzynska, M. Kurzawski, M. Drożdżik, E. Mijowska. *Chem. Phys. Lett.* **568–569** (2013) 151–156.
51. Y.M. Zhang, Y. Cao, Y. Yang, J.T. Chen, Y. Liu. *Chem. Commun.* **50** (2014) 13066–13069.
52. V. Mailander, K. Landfester. *Biomacromolecules* **10** (2009) 2379–2400.
53. J.A. MacKay, M. Chen, J.R. McDaniel, W. Liu, A.J. Simnick, A. Chilkoti. *Nat. Mater.* **8** (2009) 993–999.
54. S.R. MacEwan, D.J. Callahan, A. Chilkoti. *Nanomedicine* **5** (2010) 793–806.

SAFETY ISSUE OF NANOPARTICLES WHICH ARE USED FOR STEM CELL LABELLING AND TRACKING

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24.1. INTRODUCTION

Various kinds of nanoparticles (NPs) have been used for the labelling and tracking of stem cells to determine their destinations and final differentiated fates after transplantation. Ideally, NPs should be biodegradable, non-toxic to stem cells, and should not disturb their multilineage differentiation potential. NPs should give a strong signal, allowing good visualisation of the stem cells. Moreover, the labelling of cells with NPs should be uncomplicated and imaging can be done *in vivo* in a non-invasive manner. The NPs should not induce adverse or immune reactions in the human body after the transplantation of labelled stem cells. The development of novel NPs is exciting; however, the safety issue should not be neglected. This review will therefore focus on this growing aspect of current NPs and the safety issue of stem cell labelling. Toxicity reports of common nanoparticles are selected and summarized in Table 1. The details of each report will be discussed in the following paragraphs of this review.

Table 1. Common nanoparticles and selected toxicity reports on various kinds of cell lines, stem cells, and animal models

Nanoparticles	Selected toxicity reports	Ref.
Superparamagnetic iron oxide nanoparticles	Aggravated clinical symptoms in animal model of labeled MSCs	[25]
	Impaired chondrogenesis on hMSCs	[23]
	Accelerated hMSC proliferation via diminishing intracellular H ₂ O ₂ and affected the expression of the protein regulators of cell cycle	[28]
	Inhibitory effect on osteogenic differentiation and Wnt signaling pathway in hMSCs	[29]
	Accelerated hMSC proliferation, altered gene expression and impaired chondrogenesis and osteogenesis	[30]
	Oxidative damage to lipids, proteins and DNA; lipid peroxidation in hMSCs	[31]
Gadolinium oxide nanoparticles	Cytotoxicity and genotoxicity via DNA damage on human skin fibroblast cell lines	[41]
	Necrosis of primary mouse bone marrow stromal cells via lysosomal rupture and ROS injury	[42]
Fluorescent polymer nanoparticles	Increase in the IL-8 release in hMSCs and altered gene expression	[46]

Nanoparticles	Selected toxicity reports	Ref.
Quantum dots	Apoptosis in mouse blastocysts, inhibited cell proliferation, early-stage blastocyst death in mouse	[60]
	Apoptosis in mouse myoblast cells line	[62]
	Impaired hematopoiesis in an invertebrate model organism	[63]
Fluorescent silica nanoparticles	Abnormal morphology and altered gene expression of human neural stem cells	[67]
	Cell death at high particle doses in neural stem cells	[68]
	Enhanced proliferation of human adipose tissue-derived stem cells through ERK1/2 activation	[69]
	Metabolic stress in hMSCs through EGR1, CCND, and E2F1 genes	[70]

MSC: mesenchymal stem cells; hMSC: human mesenchymal stem cells

24.2. MAGNETIC NANOPARTICLES

Magnetic resonance imaging (MRI) is a non-invasive, deep tissue penetrating imaging technique which provides excellent contrast between the different soft tissues of the body, and reconstructs 2D and 3D images of the tissues and organs easily. Magnetic NPs are categorised as T1 or T2 contrast agents for MRI depending on the relaxation processes. Among the magnetic NPs, superparamagnetic iron oxide NPs (SPIOs) and gadolinium oxide NPs are the most popular choices for T2 and T1 MRI-based stem cell labelling and tracking respectively. SPIOs have been widely used for stem cell labelling and tracking.

24.2.1. Superparamagnetic iron oxide nanoparticles

SPIOs have been shown to be biocompatible, biodegradable, and non-toxic to different kinds of stem cells as well as having no adverse effects on stem cell phenotypes or differentiation [1-12]. Additionally, several kinds of SPIOs have been used for stem cell labelling and tracking *in vivo* in MRI [13-16], as well as in clinical applications and animal models [17-22].

However, in 2004, Kostura *et al.* reported that Feridex (Ferumoxide) labelling of human mesenchymal stem cells (hMSCs) impairs chondrogenesis but does not affect cell viability, proliferation, adipogenesis, or osteogenesis [23]. Although the multilineage differentiation of SPIO-labelled mesenchymal stem cells (MSCs) is not altered in an *in vivo* rat model, subtle but significant phenotypic alterations are observed following the subcutaneous implantation of labelled MSCs grown on a collagen-GAG scaffold compared to those grown on implanted, unlabelled control scaffolds [24]. Schafer *et al.* noted that the use of SPIOs for the labelling of MSCs aggravates clinical symptoms in

experimentally-induced autoimmune encephalomyelitis, while treatment with unlabelled MSC leads to disease amelioration compared to controls [25]. The authors concluded that *in vivo* application of SPIO-labelled MSC needed to be performed with caution because the cell-derived exposure of iron can lead to disease aggravation. Furthermore, labelling of hMSCs with ferucarbotran had functional effects on hMSCs by decreasing the migration capacity and colony-forming ability of cells [26]. Later, in 2010, both SPIOs and the static magnetic field were identified as independent factors which affect the functional biology of hMSCs [27].

Huang *et al.* found that ferucarbotran stimulated hMSC proliferation in particle and ionic (free iron, Fe) forms *via* diminishing intracellular H₂O₂ and affected the expression of protein regulators of the cell cycle [28]. In a follow-up study, the inhibitory effect of ferucarbotran on osteogenic differentiation and the Wnt signalling pathway in hMSCs was reported [29]. Cell proliferation and viability of labelled hMSCs was found to be increased by amine (NH₃⁺)-surface-modified SPIOs, and the cell cycle of labelled cells was accelerated [30]. The osteogenic and chondrogenic differentiation potential of hMSCs was impaired or inhibited, while adipogenic potential was preserved. These findings have raised toxicity and other safety concerns in regard to using newly-developed SPIO NPs for stem cell labelling and tracking.

Novotna *et al.* reported oxidative damage to biological macromolecules in human bone marrow MSCs labelled with iron oxide NPs [31]. An increase of oxidative injury to lipids, proteins and DNA as a consequence of exposure to SPIOs was detected in labelled cells. Particularly, the levels of lipid peroxidation were high and increased further with time, regardless of the type of nanoparticle. Also, Diana *et al.* found that SPIO loading significantly reduced movement in fetal stem cell populations (human amniotic fluid and chorionic villi stem cells) without increasing the production of reactive oxygen species [32]. Moreover, motility impairment was directly proportional to the amount of loaded SPIOs, while chemoattractant-induced recovery was obtained by increasing serum levels.

On the other hand, long-lasting hypointensive signals in cardiac magnetic resonance were believed to originate from SPIO-engulfed macrophages during long-term stem cell tracking, rather than the transplanted stem cells. Recently, Huang *et al.* reported that the hypointensive signal of cardiac MRI was primarily caused by extracellular iron particles in the long-term tracking of transplanted swine MSCs after myocardial infarction [33]. Also, 6 months after transplantation, cardiac MRI identified 32 (64 %) of the 50 injection sites, where massive Prussian blue-positive iron deposits were detected by pathological examination. However, iron particles were predominantly distributed in the extracellular space, and a minority was distributed within Cluster of Differentiation 68 (CD68)-positive macrophages and other CD68-negative cells. No sex-determining region Y DNA of donor MSCs was detected in the myocardium. The authors suggested that consideration should

be given to both the false-positive signal and the potential cardiac toxicity of long-standing iron deposits in the heart.

The other limitations of SPIOs are well illustrated in their use of stem cell tracking [34]. For example, SPIO-labelled cells become diluted in a localised site due to cell division, and consequently, the generated image is inevitably weakened. Furthermore, due to the *in vivo* migration of SPIO-labelled stem cells, the density of cells is considerably reduced over time, again leading to weakening of the MRI signal [35]. On the other hand, SPIO labelling itself cannot determine whether or what the SPIO-labelled stem cell differentiates into, although through the use of combined imaging techniques such as immunohistochemistry and confocal microscopy, the function of the stem cell can be determined [36].

24.2.2. Gadolinium oxide nanoparticles

Contrary to T2 agents, which produce hypointensive signals (dark spots) on MRI images, gadolinium-based T1 agents produce bright hyperintensive signals. Gadolinium oxide NPs have been used to label human aortic endothelial cells and T1 enhancement of the internalised NPs was maintained for up to 7 days [37]. Currently, the use of gadolinium oxide NPs in cell tracking is still in the early stages, and they have been used for labelling brain cancer cells [38] and hematopoietic cells [39]. Surface modification of gadolinium oxide NPs with poly(ethylene glycol) (PEG) could increase their biocompatibility [40]. However, terbium-doped gadolinium oxide NPs induced cytotoxicity in a dose-dependent manner and promoted genotoxicity *via* DNA damage on human skin fibroblast cell lines [41]. Recently, Jin *et al.* reported that europium-doped Gd₂O₃ nanotubes caused the necrosis of primary mouse bone marrow stromal cells through lysosomal rupture and release of cathepsin B, and the overproduction of reactive oxygen species (ROS) injury to the mitochondria and DNA [42]. Prior to applying gadolinium oxide NPs in cell labelling and tracking, their effects on stem cell function and differentiation need to be further investigated.

24.3. FLUORESCENT NANOPARTICLES

Fluorescence imaging provides high sensitivity, high resolution, and feasibility for the real-time monitoring biological phenomena. Due to low cost and accessibility, fluorescence imaging is widely used in tracking stem cells. The main fluorescent nanoparticle products on the market are fluorescent polymer NPs, quantum dots (QDs) and fluorescent silica NPs.

24.3.1. Fluorescent polymer nanoparticles

Due to their biocompatibility and inertness, polystyrene (PS) and polycarbonate are widely used for the production of biomedical devices and

laboratory equipment. Currently, the most common fluorescent polymer NPs are PS NPs, which are mainly prepared through emulsion polymerisation [43]. Some of the PS NPs are frequently used in phagocytosis assessment of phagocytic cells. Phosphonate-functionalised PS NPs prepared by mini-emulsion polymerisation were incorporated by MSCs, and the cell viability was found to be unaffected [44]. Furthermore, the multi-lineage potential of MSCs was well preserved. PS does not degrade in the cellular environment, so PS NPs usually exhibit no short-term cytotoxicity [45].

Very few reports have addressed the safety issue of PS NPs on stem cell labelling [46,47]. Two sets of either bioinert (PS without carboxylic groups on the surface) or biodegradable (poly(L-lactic acid) (PLLA) without magnetite) particles were uptake by hMSCs and hematopoietic stem cells (hHSCs) [46]. Flow cytometry and microscopy analysis showed high uptake rates and no toxicity for all four tested particles in hMSCs and hHSCs. The PLLA-Fe particle showed a significant increase in the interleukin-8 (IL-8) release in hMSCs but not in hHSCs. For hHSCs and hMSCs, multi-lineage differentiation was not influenced by the particles when analysed with lineage-specific clusters of differentiation markers. On the other hand, quantitative polymerase-chain-reaction (qPCR) analysis showed significant changes in the expression of some (but not all) investigated lineage markers for both primary cell types. In another report, conjugated polymer-based water-dispersible NPs were used to label bone marrow-derived rat MSCs successfully and labelled MSCs migrated to the site of injury and retained their labels in an *in vivo* liver regeneration model [47].

24.3.2. Quantum dots

QDs are colloidal, crystalline semiconductor NPs made from II–VI or III–V elements (*e.g.* PdS, CdSe) [48]. QDs have wide absorption range from ultraviolet (UV) to visible, and are much more resistant (can be up to 100 times) to photochemical degradation than fluorescent dyes, which makes them useful for the long-term tracking of cells and monitoring of biological changes [49]. However, QDs have several inherent problems. First, QDs generally contain toxic heavy metal ions such as Pd²⁺, Se²⁺ and Cd²⁺. Second, the involvement of dark states and blinking phenomena in QDs requires higher doses to improve the brightness and accuracy of quantitative measurements in cell tracking and imaging [50]. Nonetheless, higher QD doses may raise the signal to noise *ratio* and result in non-specific binding.

Although QDs have been utilised for stem cell tracking and imaging for many years [51–58], most toxicity and pharmacokinetic studies have focused on the behaviours of II B–VI A and III A–V A QDs in rodents [59]. Investigations of the IV A QDs are still in the early stages. Initial safety and proof of concept studies of one- and two-cell QD-labelled mouse embryos reveal that fluorescent semiconductor nanocrystal QDs are compatible with early mammalian

embryonic development [51]. *In vivo* experiments further show that *in utero*-labelled neural stem and progenitor cells (NSPCs) continue to develop in an apparently normal manner [51]. However, it has been shown that CdSe-core QDs induce apoptosis in mouse blastocysts, inhibit cell proliferation, retard early post-implantation blastocyst development, and increase early-stage blastocyst death *in vitro* and *in vivo* [60]. The reproductive and developmental toxicity of manufactured nanomaterials including metallic and metal oxide-based particles, fullerenes (C(60)), carbon black, and luminescent particles has been reviewed in a previous paper [61].

Copper oxide QDs induce apoptosis in mouse myoblast cells line (C2C12) [62]. Further *in vivo* studies have shown that copper oxide QDs bind to genomic DNA, significantly decrease the viability of cells in culture in a concentration-dependent manner, and inhibit mitochondrial caspases 3 and 7. CdTe QDs, especially those that are smaller in size, resulted in impaired haematopoiesis in an invertebrate model organism, *Bombyx mori* [63]. QD exposure promoted the mitotic nucleus in prohaemocytes and haematocytes similar to peripheral blood stem cells in humans, but aggravated apoptosis. A decrease in haematopoiesis was accompanied by shrinkage and death of haematopoietic organs via an increase in reactive oxygen species.

According to our review, QDs still have some toxicity or harmful effects on several kinds of stem cells. The lack of studies on animal models using primates and human stem cells also limits our understanding of QDs. Further application of QDs in stem cell labelling and tracking merits further research.

24.3.3. Fluorescent silica nanoparticles

There are only a few reports applying fluorescent silica NPs for stem cell labelling and tracking. On the contrary, fluorescent silica NPs produced in research laboratories were widely referred to in publications of non-stem cell labelling and tracking. Cyanine dye-doped silica NPs were reported to directly discriminate live and early-stage apoptotic mesenchymal and embryonic stem cells through a distinct external cell surface distribution [64], which makes them ideal for stem cell labelling and tracking. On the other hand, mesoporous silica NPs are also used for the delivery of growth factors to induce advanced differentiation of transplanted stem cells [65,66].

However, hNSCs aggregated and exhibited abnormal morphology when exposed to silica and titanium oxide NPs [67]. Moreover, all of the particles affected the gene expression of nestin (stem cell marker) and neurofilament heavy polypeptide (NF-H, neuron marker). These results may indicate the potential toxicity of accumulated NPs for long-term usage or continuous exposure. Silica NPs with different [–NH₂, –SH and poly(vinylpyrrolidone) (PVP)] surface modifications cause cell death at high particle doses, except for PVP-coated SiO₂ NPs [68]. Among the tested neural tissue-type cells, neural stem cells and astrocytes internalise plain SiO₂, SiO₂–NH₂ and SiO₂–SH NPs,

while neurons do not take up any NPs at all. Their data indicate that the PVP coat, by lowering the particle-biomolecular component interactions, reduces the biological effects of SiO₂ NPs on the investigated neural cells.

It has been reported that scaffolds containing silica NPs may enhance the proliferation of human adipose tissue-derived stem cells through ERK1/2 activation [69], meaning that single component silica-derived NPs could be useful for bioscaffolds in stem cell therapy. Not surprisingly, silica NPs were found to induce significant metabolic stress in hMSCs in a recent report [70]. Alterations in the cytoplasmic organisation, nuclear morphology, cell cycle progression, and expression of genes linked to cell cycle-dependent metabolic stress through EGR1, CCND, and E2F1 genes, which are the primary indicators of metabolic stress, have been observed. The authors suggest that the acute and chronic toxicity mechanisms of silica NPs should be investigated in greater depth with special reference to food safety.

24.4. CONCLUSION

We summarise the toxicity reports of existing or commercial NPs which can act as contrast agents for MRI and fluorescence imaging for stem cell labelling and tracking. Prior to the extensive use of these NPs in stem cell therapy in humans, concerns regarding NPs, including the unclear safety profile, signal loss in long-term tracking, limitation of single modality NPs, viability and ultimate fate of labelled cells, and effects on multilineage differentiation potential will have to be clarified. Careful investigation of the effects of NPs on human stem cells and animal models using primates will facilitate our understanding and should be carried out ahead of the application of novel NPs in clinical trials.

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REFERENCES

1. A.S. Arbab, G.T. Yocum, A.M. Rad, A.Y. Khakoo, V. Fellowes, E.J. Read, J.A. Frank. *NMR Biomed.* **18**(2) (2005) 553–559.
2. D. Choi, J.H. Kim, M. Lim, K.W. Song, S.S. Paik, S.J. Kim, H.J. Cheong, J.S. Jeon, H.S. Park, Y.S. Song, H. Khang, J.H. Won. *Tissue Eng. Part C Methods* **14**(1) (2008) 15–23.
3. A. Focke, S. Schwarz, A. Foerschler, J. Scheibe, J. Milosevic, C. Zimmer, J. Schwarz. *Magn. Reson. Med.* **60**(6) (2008) 1321–1328.
4. A. Heymer, D. Haddad, M. Weber, U. Gbureck, P.M. Jakob, J. Eulert, U. Nöth. *Biomaterials* **29**(10) (2008) 1473–1483.
5. J. Krejci, J. Pacherník, A. Hampl, P. Dvorák. *Gen. Physiol. Biophys.* **27**(3) (2008) 164–173.
6. Z. Li, Y. Suzuki, M. Huang, F. Cao, X. Xie, A.J. Connolly, P.C. Yang, J.C. Wu. *Stem Cells* **26**(4) (2008) 864–873.
7. K.W. Au, S.Y. Liao, Y.K. Lee, W.H. Lai, K.M. Ng, Y.C. Chan, M.C. Yip, C.Y. Ho, E.X. Wu, R.A. Li, C.W. Siu, H.F. Tse. *Biochem. Biophys. Res. Commun.* **379**(4) (2009) 898–903.
8. L. Wang, J. Deng, J. Wang, B. Xiang, T. Yang, M. Gruwel, T. Kashour, B. Tomanek, R. Summer, D. Freed, D.S. Jassal, G. Dai, M. Glogowski, R. Deslauriers, R.C. Arora, G. Tian. *Magn. Reson. Med.* **27**(1) (2009) 108–119.
9. A. Crabbe, C. Vandeputte, T. Dresselaers, A.A. Sacido, J.M. Verdugo, J. Eyckmans, F.P. Luyten, K. Van Laere, C.M. Verfaillie, U. Himmelreich. *Cell Transplantation* **19** (2010) 919–936.
10. V.K. Verma, S.R. Kamaraju, R. Kancharla, L.K. Kona, S.S. Beevi, T. Debnath, S.P. Usha, R. Vadapalli, A.S. Arbab, L.K. Chelluri. *Int. J. Nanomed.* **10** (2015) 711–726.
11. X.X. Li, K.A. Li, J.B. Qin, K.C. Ye, X.R. Yang, W.M. Li, Q.S. Xie, M.E. Jiang, G.X. Zhang, X.W. Lu. *Int. J. Nanomed.* **8** (2013) 1063–1073.
12. M.R. Pickard, P. Barraud, D.M. Chari. *Biomaterials* **32**(9) (2011) 2274–2284.
13. P.W. So, T. Kalber, D. Hunt, M. Farquharson, A. Al-Ebraheem, H.G. Parkes, R. Simon, J.D. Bell. *Cell Transplantation* **19**(4) (2010) 419–429.
14. B. Zhou, H. Shan, D. Li, Z.B. Jiang, J.S. Qian, K.S. Zhu, M.S. Huang, X.C. Meng. *Magn. Reson. Med.* **28**(3) (2010) 394–399.
15. J.H. Lee, M.J. Jung, Y.H. Hwang, Y.J. Lee, S. Lee, D.Y. Lee, H. Shin. *Biomaterials* **33**(19) (2012) 4861–4871.
16. J. Han, B. Kim, J.Y. Shin, S. Ryu, M. Noh, J. Woo, J.S. Park, Y. Lee, N. Lee, T. Hyeon, D. Choi, and B.S. Kim. *ACS Nano* **9**(3) (2015) 2805–2819.
17. K. Hekimoglu, Y. Ustundag, A. Dusak, B. Kalaycioglu, H. Besir, H. Engin, O. Erdem. *Eur J. Radiol.* **77**(3) (2011) 468–472.
18. Y. Hirokawa, H. Isoda, Y.S. Maetani, S. Arizono, K. Shimada, T. Okada, T. Shibata, K. Togashi. *Radiology* **251**(2) (2009) 388–397.
19. M.R. Loebinger, P.G. Kyrtatos, M. Turmaine, A.N. Price, Q. Pankhurst, M.F. Lythgoe, S.M. Janes. *Cancer Res.* **69**(23) (2009) 8862–8867.
20. M. Rief, M. Wagner, T. Franiel, V. Bresan, M. Taupitz, C. Klessen, B. Hamm, P. Asbach. *Magn. Reson. Med.* **27**(9) (2009) 1223–1229.
21. L.P. Nucci, H.R. Silva, V. Giampaoli, J.B. Mamani, M.P. Nucci, L.F. Gamarra. *Stem Cell Res. Ther.* **6**(1) (2015) 27.

22. M. Ramos-Gomez, E.G. Seiz, A. Martinez-Serrano. *J. Nanobiotechnol.* **13**(1) (2015) 20.
23. L. Kostura, D.L. Kraitchman, A.M. Mackay, M.F. Pittenger, J.W. Bulte. *NMR Biomed.* **17**(7) (2004) 513–517.
24. E. Farrell, P. Wielopolski, P. Pavljasevic, S. van Tiel, H. Jahr, J. Verhaar, H. Weinans, G. Krestin, F.J. O'Brien, G. van Osch, M. Bernsen. *Biochem. Biophys. Res. Commun.* **369**(4) (2008) 1076–1081.
25. R. Schafer, M. Ayturan, R. Bantleon, R. Kehlbach, G. Siegel, J. Pintaske, S. Conrad, H. Wolburg, H. Northoff, J. Wiskirchen, R. Weissert. *Cell Transplant.* **17**(8) (2008) 923–941.
26. R. Schafer, R. Kehlbach, M. Müller, R. Bantleon, T. Kluba, M. Ayturan, G. Siegel, H. Wolburg, H. Northoff, K. Dietz, C.D. Claussen, J. Wiskirchen. *Cytotherapy* **11**(1) (2009) 68–78.
27. R. Schafer, R. Bantleon, R. Kehlbach, G. Siegel, J. Wiskirchen, H. Wolburg, T. Kluba, F. Eibofner, H. Northoff, C.D. Claussen, H.P. Schlemmer. *BMC Cell Biol.* **11** (2010) 22.
28. D.M. Huang, J.K. Hsiao, Y.C. Chen, L.Y. Chien, M. Yao, Y.K. Chen, B.S. Ko, S.C. Hsu, L.A. Tai, H.Y. Cheng, S.W. Wang, C.S. Yang, Y.C. Chen. *Biomaterials* **30**(22) (2009) 3645–3651.
29. Y.C. Chen, J.K. Hsiao, H.M. Liu, I.Y. Lai, M. Yao, S.C. Hsu, B.S. Ko, Y.C. Chen, C.S. Yang, D.M. Huang. *Toxicol. Appl. Pharmacol.* **245**(2) (2010) 272–279.
30. Y.K. Chang, Y.P. Liu, J.H. Ho, S.C. Hsu, O.K. Lee. *J. Orthop. Res.* **30**(9) (2012) 1499–1506.
31. B. Novotna, P. Jendelova, M.Kapcalova, P.Jr. Rossner, K. Turnovcova, Y. Bagryantseva, M. Babic, D. Horak, E. Sykova. *Toxicol. Lett.* **210**(1) (2012) 53–63.
32. V. Diana, P. Bossolasco, D. Moscatelli, V. Silani, L. Cova. *PLoS One* **8**(11) (2013) e78435.
33. Z. Huang, C. Li, S. Yang, J. Xu, Y. Shen, X. Xie, Y. Dai, H. Lu, H. Gong, A. Sun, J. Qian, J. Ge. *Int. J. Nanomed.* **10** (2015) 1679–1690.
34. E. Bull, S.Y. Madani, R. Sheth, A. Seifalian, M. Green, A.M. Seifalian. *Int. J. Nanomed.* **9** (2014) 1641–1653.
35. R. Guzman, N. Uchida, T.M. Bliss, D. He, K.K. Christopherson, D. Stellwagen, A. Capela, J. Greve, R.C. Malenka, M.E. Moseley, T.D. Palmer, G.K. Steinberg. *Proc. Natl. Acad. Sci. USA* **104**(24) (2007) 10211–10216.
36. A.S. Arbab, S.D. Pandit, S.A. Anderson, G.T. Yocum, M. Bur, V. Frenkel, H.M. Khuu, E.J. Read, J.A. Frank. *Stem Cells* **24**(3) (2006) 671–678.
37. Y. Loai, N. Sakib, R. Janik, W. Foltz, H.L. Cheng. *Mol. Imag.* **11**(2) (2012) 166–175.
38. L. Faucher, A.A. Guay-Bégin, J. Lagueux, M.F. Côté, E. Petitclerc, M.A. Fortin. *Contrast Media Mol. Imaging* **6**(4) (2011) 209–218.
39. A. Hedlund, M. Ahrén, H. Gustafsson, N. Abrikosova, M. Warntjes, J.I. Jönsson, K. Uvdal, M. Engström. *Int. J. Nanomed.* **6** (2011) 3233–3240.
40. N. Abrikosova, C. Skoglund, M. Ahrén, T. Bengtsson, K. Uvdal. *Nanotechnology* **23**(27) (2012) 275101.
41. M.I. Setyawati, P.K. Khoo, B.H. Eng, S. Xiong, X. Zhao, G.K. Das, T.T. Tan, J.S. Loo, D.T. Leong, K.W. Ng. *J. Biomed. Mater. Res. Part A* **101**(3) (2013) 633–640.
42. Y. Jin, S. Chen, J. Duan, G. Jia, J. Zhang. *J. Inorganic Biochem.* **146** (2015) 28–36.

43. S.B. Brijmohan, S. Swier, R.A. Weiss, M.T. Shaw. *Ind. Eng. Chem. Res.* **44** (2005) 8039–8045.
44. A. Tautzenberger, S. Lorenz, L. Kreja, A. Zeller, A. Musyanovych, H. Schrezenmeier, K. Landfester, V. Mailänder, A. Ignatius. *Biomaterials* **31**(8) (2010) 2064–2071.
45. C. Loos, T. Syrovets, A. Musyanovych, V. Mailänder, K. Landfester, G.U. Nienhaus, T. Simmet. *Beilstein J. Nanotechnol.* **5** (2014) 2403–2412.
46. I. Brustle, T. Simmet, G.U. Nienhaus, K. Landfester, V. Mailänder. *Beilstein J. Nanotechnol.* **6** (2015) 383–395.
47. E. Akhan, D. Tuncel, K.C. Akcali. *BioMed Res. Int.* **2015** (2015) 298430.
48. A.P. Alivisatos. *Science* **271** (1996) 933–937.
49. W.C. Chan, S. Nie. *Science* **281** (1998) 2016–2018.
50. J. Yao, D.R. Larson, H.D. Vishwasrao, W.R. Zipfel, W.W. Webb. *Proc. Natl. Acad. Sci. USA* **102**(40) (2005) 14284–14289.
51. J.R. Slotkin, L. Chakrabarti, H.N. Dai, R.S. Carney, T. Hirata, B.S. Bregman, G.I. Gallicano, J.G. Corbin, T.F. Haydar. *Dev. Dynam.* **236**(12) (2007) 3393–3401.
52. S. Danner, H. Benzin, T. Vollbrandt, J. Oder, A. Richter, C. Kruse. *Int. J. Cell Biol.* **2013** (2013) 918242.
53. S.H. Hsu, Y.Y. Lin, S. Huang, K.W. Lem, D.H. Nguyen, D.S. Lee. *Nanotechnology* **24**(47) (2013) 475102.
54. M.C. Collins, Gunst P.R., B.J. Muller-Borer. *Methods Mol. Biol.* **1199** (2014) 141–154.
55. Y.S. Kim, J.Y. Kim, D.M. Shin, J.W. Huh, S.W. Lee, Y.M. Oh. *Tuberc. Respir. Dis. (Seoul)* **77**(3) (2014) 116–123.
56. W. Shang, X. Zhang, M. Zhang, Z. Fan, Y. Sun, M. Han, L. Fan. *Nanoscale* **6**(11) (2014) 5799–5806.
57. X. Wen, Y. Wang, F. Zhang, X. Zhang, L. Lu, X. Shuai, J. Shen. *Biomaterials* **35**(16) (2014) 4627–4635.
58. Y.W. Wang, K. Yang, H. Tang, D. Chen, Y.L. Bai. *Int. J. Nanomed.* **9** (2014) 4809–4817.
59. X. Wu, F. Tian, J.X. Zhao, M. Wu. *Expert Opin. Drug Metab. Toxicol.* **9**(10) (2013) 1265–1277.
60. W.H. Chan, N.H. Shiao. *Acta pharmacologica Sinica* **29**(2) (2008) 259–266.
61. M. Ema, N. Kobayashi, M. Naya, S. Hanai, J. Nakanishi. *Reproduct. Toxicol.* **30**(3) (2010) 343–352.
62. T. Amna, H. Van Ba, M. Vaseem, M.S. Hassan, M.S. Khil, Y.B. Hahn, H.K. Lee, I.H. Hwang. *Appl. Microbiol. Biotechnol.* **97**(12) (2013) 5545–5553.
63. T. Liu, R. Xing, Y.F. Zhou, J. Zhang, Y.Y. Su, K.Q. Zhang, Y. He, Y.H. Sima, S.Q. Xu. *Biomaterials* **35**(9) (2014) 2942–2951.
64. L. Accomasso, E. Cibrario Rocchietti, S. Raimondo, F. Catalano, G. Alberto, A. Giannitti, V. Minieri, V. Turinetti, L. Orlando, S. Saviozzi, G. Caputo, S. Geuna, G. Martra, C. Giachino. *Small* **8**(20) (2012) 3192–3200.
65. A.E. Garcia-Bennett, M. Kozhevnikova, N. König, C. Zhou, R. Leao, T. Knöpfel, S. Pankratova, C. Trolle, V. Berezin, E. Bock, H. Aldskogius, E.N. Kozlova. *Stem Cells Transl. Med.* **2**(11) (2013) 906–915.
66. D. Bocking, O. Wiltshcka, J. Niinimäki, H. Shokry, R. Brenner, M. Lindén, C. Sahlgren. *Nanoscale* **6**(3) (2014) 1490–1498.

67. K. Fujioka, S. Hanada, Y. Inoue, K. Sato, K. Hirakuri, K. Shiraishi, F. Kanaya, K. Ikeda, R. Usui, K. Yamamoto, S.U. Kim, Y. Manome. *Int. J. Mol. Sci.* **15**(7) (2014) 11742–11759.
68. E. Izak-Nau, K. Kenesei, K. Murali, M. Voetz, S. Eiden, V.F. Puentes, A. Duschl, E. Madarász. *Nanotoxicology* **8**(1) (2014) 138–148.
69. K.J. Kim, Y.A. Joe, M.K. Kim, S.J. Lee, Y.H. Ryu, D.W. Cho, J.W. Rhie. *Int. J. Nanomed.* **10** (2015) 2261–2272.
70. V.S. Periasamy, J. Athinarayanan, A.A. Akbarsha, A.A. Alshatwi. *Appl. Biochem. Biotechnol.* **175**(2) (2015) 1181–1192.

Chapter

25

ACOUSTIC WAVE RESONATORS FOR BIOMEDICAL APPLICATIONS

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25.1. AN ELECTROACTIVE SOLUTION

Materials used as scaffolds for tissue engineering applications are designed to match the structural, morphological, mechanical, and chemical properties of the tissue or organ that will be replaced. Human cells in human tissues are anchorage dependent, the microstructure and surface properties of the scaffolds therefore being a critical issue. In this way, the behaviour of cells cultured on substrates is highly dependent on these characteristics.

Surfaces can influence cell behaviour in different aspects such as the growth, adhesion, or morphology of cells. Beyond this, an electrically charged base for tissue engineering applications can be an interesting and promising approach. This fact is particularly important as many body tissues are subjected to varying electro-mechanical solicitation.

Conductive materials have thus been used for tissue engineering applications, but some drawbacks exist concerning these materials, such as the need for an external power source to promote electrical stimuli in the cells. Piezoelectric materials are materials that generate varying surface charges under mechanical solicitation and that do not require additional energy sources or electrodes for the generation of the electrical signal [1]. These materials are thus suitable for active tissue engineering strategies in which electroactive response and scaffold microstructure play an essential role.

Therefore, the piezoelectric polymer poly(vinylidene fluoride) (PVDF) has attracted interest for its biomedical applications in the fabrication of sensors and actuators and supports for cell cultures. Similarly, aluminium nitride (AlN) has become a very attractive biocompatible piezoelectric material as it is also compatible with complementary metal-oxide-semiconductor (CMOS) technology.

This chapter deals with the design and development of piezoelectric biocompatible materials for potential use in tissue engineering applications as a piezoelectric-based scaffold; further, the latest advances in these developments are shown.

25.2. PIEZOELECTRIC PVDF

Piezoelectric polymers are a good option for developing piezoelectric-based acoustic wave devices and sensors as well as actuators. In particular, PVDF shows good biocompatibility, chemical resistance, and, in particular, excellent electroactive properties such as piezo-, pyro-, and ferroelectricity. PVDF and its copolymers are the polymers with the highest piezoelectric effect. It has a high dynamic range and wideband and belongs to a new class of piezoelectric plastics characterised by a relatively low dielectric constant, moderate piezoelectric coupling, low acoustic velocities, and low density. One of the mayor advantages of these piezoelectric films is their low acoustic impedance close to water, human tissue, and other organic materials, allowing for a more efficient transduction of acoustic signals due to close impedance matching; this makes them suitable for biological applications. PVDF has three main actuation/sensing modes depending of the fabrication process: inplane mode, thickness mode, and thickness shear mode [2].

A PVDF film under an electric field with a controlled temperature after a mechanical stretch shows very strong piezoelectric and pyroelectric properties. Thin piezoelectric films can also be obtained using soft lithographic techniques by casting and poling, allowing compatibility with microelectromechanical systems (MEMS) technology [3].

PVDF can be made piezoelectric because fluorine is much more electronegative than carbon. The fluorine atoms will attract electrons from the carbon atoms to which they are attached. The $-CF_2-$ groups in the chain will be very polar, so when they are placed in an electrical field they will align. Conversely, when the piezopolymer deforms, a macroscopic dipole appears. This can be obtained by submitting the film of polymer to a sufficiently high electric field after mechanical stretching. The obtained polarisation is mainly due to the spatial rearrangement of the polar segments of the macromolecular chains. By machining the material in one or two perpendicular directions prior to the polarisation process, different piezoelectric behaviours can be obtained.

This section deals with the design, fabrication, and characterisation of a piezoelectric PVDF for potential use in tissue engineering applications.

25.2.1. Numerical model for the PVDF polymer

Previously, the importance of applying electrical signals to body tissues by using piezoelectric materials has been demonstrated. In addition, there are studies showing the feasibility of carrying electrical/mechanical signals on demand by applying an appropriate (wireless) stimuli in order to deform the piezoelectric scaffold microstructure [4,5]. In this sense, the resonance frequency of the piezoelectric implant has to be well known in order to apply the correct excitation.

The basic principle of operation for a generic acoustic-wave resonator is a travelling wave combined with a confinement structure to produce a standing wave whose frequency is determined jointly by the velocity of the travelling wave and the dimensions of the confinement structure.

A complete model for acoustic systems can be built using the three-port one-dimensional multilayer Mason model [6]. In order to get a more realistic model as a reference, the finite element method (FEM) model fits very well. FEM is a flexible method capable of modelling complicated device geometries, nonuniform material properties, and quite general boundary conditions.

PVDF has an orthorhombic symmetry (mm^2 class) and is usually operated in thickness mode when the frequency of operation is more than 500 kHz. It has three main actuation/sensing modes depending on the fabrication process: inplane mode (d_{31} , d_{32}), thickness mode (d_{33}), and shear mode (d_{15} , d_{24}).

25.2.1.1. FEM model

For a proposed prototype, a disc-shaped PVDF polymer, a three-dimensional (3D) piezoelectric elastic linear solid can be used; the electric potential is applied on both principal surfaces of the disc to avoid electrode modelling. This simplifies the model and reduces simulation times without losing accuracy. The piezoelectric polymer is in thickness excitation mode where the electric field is in the direction of acoustic wave propagation.

The elastic matrix $\mathbf{c}$, the piezoelectric coupling matrix $\mathbf{e}$, and the dielectric coefficients matrix at constant strain $\boldsymbol{\varepsilon}$ are expressed as follows:

$$\mathbf{c} = \begin{bmatrix} c_{11} & c_{12} & c_{13} & 0 & 0 & 0 \\ c_{12} & c_{22} & c_{23} & 0 & 0 & 0 \\ c_{13} & c_{23} & c_{33} & 0 & 0 & 0 \\ 0 & 0 & 0 & c_{44} & 0 & 0 \\ 0 & 0 & 0 & 0 & c_{55} & 0 \\ 0 & 0 & 0 & 0 & 0 & c_{66} \end{bmatrix} \text{ Pa}$$

$$\mathbf{e} = \begin{bmatrix} 0 & 0 & 0 & 0 & e_{15} & 0 \\ 0 & 0 & 0 & e_{24} & 0 & 0 \\ e_{31} & e_{32} & e_{33} & 0 & 0 & 0 \end{bmatrix} \frac{\text{C}}{\text{m}^2}$$

$$\boldsymbol{\varepsilon} = \begin{bmatrix} \varepsilon_{11} & 0 & 0 \\ 0 & \varepsilon_{22} & 0 \\ 0 & 0 & \varepsilon_{33} \end{bmatrix}$$

The three major axes are unique in the orthorhombic symmetry, where the presence of three separate longitudinal diagonal terms and three separate shear diagonal terms are shown in the elastic matrix $\mathbf{c}$.

A modelling of this system is shown in Figure 1, where the meshing and the vibrational response is depicted.

Figure 1. Meshing aspect of 10 μm -thick PVDF (left) and its vibrational response (right)

25.2.2. Resonator fabrication

Rolls of piezo film are produced in a clean room environment. The process begins with the melt extrusion of PVDF resin pellets into sheet form, followed by a stretching step that reduces the sheet. Stretching at temperatures well below the melting point of the polymer causes chain packing of the molecules into parallel crystal planes, called β -phase. To obtain high levels of piezoelectric activity, the β -phase polymer is then exposed to very high electric fields to align the crystallites relative to the poling field.

A disc-shaped piezoelectric can be obtained from commercial metalised PVDF piezoelectric film sheet, as shown in Figure 2. The piezopolymer is commercialised in metalised sheets of copper or silver [7].

Figure 2. Disk-shaped piezoelectric PVDF with silver ink electrodes

The circular disk has a diameter of 20 mm and a thickness of 110 μm . Silver ink printed electrodes and copper electrodes patterned with standard lithography on both sides of the disk were used. Printed inks have low sheet resistivity, high current density capability, and are robust mechanically. The overlap between the top and bottom electrode defines an active circular area where the electrical signal for impedance measurements is applied through metal patterned PVDF arms.

25.2.3. System characterisation

The frequency response of the PVDF-based piezoelectric implant will show the main characteristic of this polymer. To achieve this, it is necessary to conduct a frequency response analysis of the piezopolymer to measure impedance and phase values, and finally obtain the resonance frequency of the system.

A piezoelectric material behaves like a series RLC resonator circuit. At the resonance frequency the impedance changes its magnitude; therefore, measuring the electric potential will result in the desired response. A faster and easier way to achieve this is by using an impedance gain-phase analyser device.

One-port electrical characterisation is performed with a precision impedance analyser by measuring the absolute impedance value as a function of frequency. Custom polyamide fixation is used to make proper contact with the surface of the electrode arms and to provide the clamped mechanical boundary conditions as depicted in Figure 3 (left). The admittance measurement is obtained in the frequency range of 2–10 MHz in order to excite the thickness mode resonance frequency. Thickness modes are so-called high frequency modes whose frequencies are determined by the plate thickness, which is the smallest dimension. In this case, the electric field is in the direction of the acoustic wave propagation. The connection of the system can be seen in Figure 3 (right).

Figure 3. Custom polyamide setup (left) and system connection (right)

25.2.3.1. Cu electrode vs Ag electrode

The frequency responses (admittance vs frequency) for two piezoelectric disks are obtained by using copper and silver electrodes. A frequency sweep between 7 and 12 MHz is applied at room temperature. The results are shown in Figure 4.

Figure 4. Cu-electrode PVDF vs Ag-electrode PVDF

It is clear that the PVDF piezoelectric disc with silver electrodes has a lower resonance frequency than the one with copper electrodes. As predicted by the Sauerbrey equation [8], this lower frequency is due to the greater thickness of the silver electrode which thus produces a shift in the frequency response of the resonator compared with the disk with copper electrodes.

25.2.4. Influence of PVDF microstructures on cell morphology

Piezoelectric polymers demonstrate a large potential for tissue engineering applications once electrical signals can be detected in the human body and can be referred to as a universal property of living tissue. Thus, piezoelectric PVDF samples can be tailored both with respect to morphology and electroactive response depending on the intended applications, including bone, muscle, and neuronal tissue engineering applications, as shown in Table 1 [9].

Table 1. Contact angle values (mean $\pm$ SD) of the different PVDF samples depending on microstructure and poling state and their potential applications in tissue engineering. “Poled +” and “Poled –” means poled samples with a positive and negative surface charge, respectively.

PVDF samples	Contact angle values	Applications in tissue engineering
non-poled β -PVDF films	$83.1^\circ \pm 2.2^\circ$	bone
“poled +” β -PVDF films	$51.3^\circ \pm 3.1^\circ$	muscle
“poled –” β -PVDF films	$45.0^\circ \pm 1.6^\circ$	cartilage
β -PVDF porous membranes	$94.3^\circ \pm 2.6^\circ$	neuronal
aligned β -PVDF fibres	$135.1^\circ \pm 3.0^\circ$	spinal cord injury
random β -PVDF fibres	$115.6^\circ \pm 3.3^\circ$	wound healing

It has been shown that piezoelectric polymers allow appropriate design to develop novel and efficient tissue engineering strategies for cells that need electromechanical stimuli for their suitable development. In particular, suitable piezoelectric scaffolds can take advantage of the mechanical activity of tissues to generate the necessary varying electrical signals for proper tissue regeneration, as is the case for bone and muscle, which include electromechanical activity in their formation and normal activity.

25.2.5. More applications...

Reis *et al.* presented preliminary results on the use of a piezoelectric actuator in an orthopaedic application [10]. The use of the converse piezoelectric effect to mechanically stimulate bone was achieved with PVDF actuators implanted in osteotomy cuts in a sheep *femur* and *tibia* (Figure 5).

Figure 5. Actuator position inside the *tibia* obtained *post-mortem*—scale bar 1 cm

The bone deposition rate was significantly higher in the mechanically stimulated areas. In these areas, increased osteopontin expression was observed. The present *in vivo* study suggests that piezoelectric materials and the converse piezoelectric effect may be used to effectively stimulate bone growth.

Pärssinen *et al.* worked with nonelectroactive α -PVDF and electroactive β -PVDF to investigate the substrate polarisation and polarity influence on focal adhesion size and number as well as on human adipose stem cell differentiation [11]. The surface charge of the poled PVDF films (positive or negative) influenced the hydrophobicity of the samples, leading to variations in the conformation of adsorbed extracellular matrix proteins, which ultimately modulated the stem cell adhesion on the films, thus inducing their osteogenic differentiation.

25.3. BIOCOMPATIBLE ALN MEMBRANE

Nowadays, the development of the thin-film bulk acoustic wave resonator (FBAR) is constantly growing. This technology was born as a direct extension of quartz crystal resonators. These are bulk acoustic wave (BAW)-based thickness mode wave resonators, permitting a reduction in the piezoelectric thickness of the implant and thus conducting an increased resonance frequency. Therefore, high sensibility in gravimetric applications was reached in addition to their compatibility with integrated circuit (IC) technology.

AlN is a piezoelectric material, previously primarily used in the electronics industry as a circuit substrate due to its relatively high thermal conductivity in combination with being an electrical insulator. It is a very attractive piezoelectric material for use in biomedical MEMS (BioMEMS) because it is biocompatible, exhibits high resistivity, high breakdown voltage, and high acoustic velocity, and it can be grown with the reactive sputtering technique at relatively low temperatures, thus making it compatible with integrated circuit technology. Material compatibility with integrated circuit fabrication opens the way for monolithic integration of the traditionally incompatible IC and electro-acoustic technologies.

The most common materials for thin-film electro-acoustic devices today include AlN, ZnO, and lead zirconium titanate (PZT). Extensive research on and development of AlN thin-film synthesis for high frequency surface acoustic wave (SAW) and BAW applications has resulted in AlN so far appearing to be the best compromise between performance and manufacturability and the prime candidate for the mass production of FBARs and filters. Material compatibility with IC fabrication opens the way for monolithic integration of the traditionally incompatible IC and electro-acoustic technologies.

AlN is most often in the wurtzite hexagonal crystallographic system around the c-axis, which is also the direction with the highest piezoelectric constant in addition to a high acoustic wave velocity.

Regarding the crystal structure of AlN, there are two main crystallographic orientations that provide piezoelectric behaviour. The crystallographic orientation (002) has the highest piezoelectric constant and has a diffraction peak at 36° , while the crystallographic orientation (100) has a diffraction peak at 33° .

This section deals with the deposition of AlN on a silicon substrate, where the feasibility of the final goal of fabricating unconventional piezoelectric materials for biomedical applications is shown.

25.3.1. Deposition process

A Pt layer is grown with magnetron sputter deposition on top of a Ti film deposited on a SiO_2 surface as an adhesion layer. The Pt bottom electrode is grown with sputter deposition. A Ti film provides an adhesion layer for growing highly c-axis (002) oriented AlN films. The thicknesses of the Pt and Ti is 150 nm and 10 nm, respectively. In addition, this coating provides seeding capability and serves as a buffer layer, lowering the discrepancy in the lattice parameter between substrate and film.

The Pt layer will serve as the bottom and top electrode to apply voltage to excite the piezoelectric if that is required. A scheme of the used piezoelectric heterostructure is shown in Figure 6.

Figure 6. Scheme of the heterostructure of AlN/Pt/Ti/SiO₂/Si

AlN (002)-oriented films are grown on the Pt layers using reactive sputter deposition, *via* sputtering material from an Al metallic target, using an Ar-plasma to produce Ar ions to sputter the Al material in a N_2 atmosphere to provide the nitrogen needed for the AlN films.

25.3.2. Characterisation

A straightforward way to discover the crystalline structure of a material and its preferential orientation is by using X-ray diffraction (XRD). It is a nondestructive technique that reveals detailed information about the chemical composition and the crystalline structure of a material. Atoms in a material are organised in a 3D arrangement forming a series of parallel planes separated by a distance that depends on the nature of the material. When a monochromatic X-ray beam is projected on the material, a diffraction pattern according to Bragg's Law is obtained:

$$n \lambda = 2 d \sin(\theta) \quad (1)$$

where λ is the wavelength of the X-ray beam, d is the distance between atomic crystal layers, θ is the incidence angle, and n is an integer.

The silicon wafers are N-type mirror polished Si (100); the X-ray powder diffraction (XRD) spectra is shown in Figure 7.

Figure 7. XRD spectrum of an AlN/Pt/Ti/SiO₂/Si heterostructure showing he characteristic peaks of the Pt and Ti layers and the Si substrate, in addition to the critical AlN (002) peak that reveals the high orientation of the AlN necessary to yield the high piezoelectric coefficient measured for these films

The XRD $2\theta/\omega$ diffraction pattern taken from the XRD analysis of the AlN/Pt/Ti/SiO₂/Si multilayer is scanned between 20° and 80°. Figure 6 shows an XRD $2\theta/\omega$ scan of the AlN/Pt/Ti/SiO₂/Si layered film exhibiting high c-axis orientation for the AlN film. The presence of a hexagonal AlN (002) diffraction peak at 36.1° (note the logarithmic scale) in addition to the spurious Si diffraction peak at 33.1° (not to be confused with the AlN (100) diffraction

peak at 33.1°) can be appreciated. These results reveal the presence of a mainly (002) textured AlN film. The diffraction peaks of Pt (111) at 40° and Ti (002) at 38.5° of the electrode and a peak for Al (331) at 44.8° are also shown. The latter indicates the presence of an amount of Al that has not been nitrided.

A direct method of deposition of AlN on a Si substrate that avoids the polishing steps and thus reduces fabrication times has been demonstrated. Thin AlN films with high (002) orientation can be produced on surfaces with an root-mean-square (rms) roughness of ≤ 1 nm; this statement is supported by the XRD spectra shown in Figure 6, which indicates that AlN films exhibit high intensity for the (002) peak when grown on an atomically flat SiO_2 surface.

The columnar structure of the obtained AlN showed a highly textured film, thus allowing the fabrication of SAW resonators and piezoelectric actuators. The Pt film has proven to be a good buffer layer serving also as the bottom and top electrodes. Regarding these experiments, a huge field for biomedical devices based on AlN is awaiting exploration.

Several applications regarding the use of piezoelectric AlN exist. Olivares *et al.* have shown the piezoelectric actuation of a microbridge based on thin-film AlN [12]. Figure 8 displays two images obtained from a scanning electron microscope (SEM) video showing a typical microactuator in two different states. Figure 8a shows the microactuator bended upwards after a voltage of 16 V has been applied; Figure 8b shows its equilibrium position. As can be seen in Figure 8b, the bridge is convex in its equilibrium state due to the combined residual stresses of the different layers, adding an extra height at the centre of the bridge of about $10\text{ }\mu\text{m}$ for this particular device.

Figure 8. Microactuator bended after a voltage has been applied (a), and in its equilibrium state (b)

The authors have demonstrated the viability of using piezoelectric AlN for the actuation of suspended microbridges. The microbridges achieved a deflection of up to $0.22 \mu\text{m V}^{-1}$; this actuation response is sufficiently good for Radio Frequency (RF) microswitching applications.

Furthermore, Sinha *et al.* reported AlN films with nanoscale thickness for nanoelectromechanical systems (NEMS) switches [13]. The nanoactuator shown in Figure 9 was fabricated as a beam clamped at both ends and successively cut by a focused ion beam to free one end and test different length structures as bending cantilevers. The actuation voltage can be applied to each of the two piezoelectric layers separately and consequently permits the nanomechanical actuator to operate as a unimorph or as a bimorph structure.

Results lead to the demonstration of bimorph actuation at the nanoscale using AlN piezoelectric films that have preserved the same stress-free state and high piezoelectric coefficients as their macroscopic counterparts.

Figure 9. The sample was tilted to provide a 3D view of the nanoactuator and to show its constituent layers: a 350 nm-thick stack formed by two 100 nm-thick AlN layers and three 50 nm-thick Pt layers. The released cantilevered beam also shows very small out-of-plane deflections. This is indicative of low levels of stress gradients in the nano-AlN films. The inset schematic illustrates a cross-sectional view of the stack of layers used to make the device and the operating principle of the bimorph nanoactuator.

Furthermore, Iborra *et al.* demonstrated the fabrication of micromachined suspended bridge structures formed by AlN/polysilicon bimorphs [14].

25.4. AlN AND UNCD... A PROMISE

BioMEMS technology has been inherited from the IC Industry and has resulted from the integration of structural and functional microparts such as microchannels, microreservoirs, microsensors, and microactuators. It has recently been developed to overcome several limitations imposed by the current state of the art in medical technology.

In spite of the remarkable advantages that BioMEMS technology brings, the need still exists for a biocompatible–bioinert material capable of persisting in the harsh environment of the human body without suffering from biofouling and that is capable of sustaining microfabrication processes while keeping its properties intact. In this sense, AlN and diamond have been demonstrated to accomplish these aspects.

The fabrication of diamond-based devices requires the growth of diamond films on appropriate substrates followed by photolithography and etching to release moving structures, for example cantilevers and beams. Ultrananocrystalline diamond (UNCD) exhibits superior mechanical/tribological properties combined with smoother surface morphology ($\sim 4\text{--}7$ nm rms roughness) and the lowest diamond deposition temperature demonstrated today (400°C) compared with single crystal diamond, microcrystalline diamond, nanocrystalline diamond (NCD), and diamond-like films [15,16].

UNCD films are grown on substrates exposed to Ar-rich CH_4 microwave plasma (MPCVD) which yields 2–5 nm diamond grains and 0.4 nm-wide grain boundaries with sp^3 and sp^2 bondings. This nanostructure provides the name UNCD for distinction from NCD films grown with H-rich/ CH_4 plasmas that yield films with 30–100 nm grain sizes. UNCD exhibits a unique combination of high fracture strength (~ 5.4 GPa) and Young's modulus (~ 990 GPa), low stress ($\sim 50\text{--}80$ MPa), negligible stiction, exceptional chemical inertness, high electric field-induced electron emission, and surface functionalisation that makes it bioinert/biocompatible for applications in biosensors and biomedical devices.

For example, UNCD has been developed as a hermetic bioinert/biocompatible encapsulating coating for a Si microchip [17]. In that work, *in vivo* passive evaluation of UNCD-coated samples implanted in the eyes of rabbits were reported; six-months tests involving the implantation of UNCD-coated Si chips in the eyes of a pigmented rabbit demonstrated that UNCD is bioinert and biostable, thus evidencing the potential of low-temperature UNCD as a hermetic coating on implantable retinal microchips.

Following the implantation of the samples in the eyes of rabbits, no evidence of inflammation above and beyond was noted. As shown in Figure 10, there was no evidence of acute damage of the surface.

Figure 10. Comparison of surface morphology (SEM) of the UNCD coating before and after implantation in rabbit eyes: (a) before implantation; (b) after implantation. UNCD films were deposited at 400 °C using a CH₄ (1 %)/Ar (99 %) plasma.

In addition, Benedic *et al.* have demonstrated the fabrication of very high-frequency SAW devices based on a AlN/diamond layered structure. The experimental results showed that the Rayleigh wave and the higher mode were generated; a high frequency around 4 GHz was obtained [18].

As diamond-based substrates have the highest sound velocity among all materials, **Error! Bookmark not defined.** an AlN/diamond structure is a very promising device for thin-film bulk acoustic resonator (FBAR) applications. In this sense, AlN–UNCD integration becomes an important option for implantable resonators.

This section deals with the development of an AlN/UNCD-based FBAR for biomedical applications. In the following paragraphs, the fabrication and characterisation of a FBAR piezoelectric resonator is shown.

25.4.1. Geometry design

The simplest configuration of a membrane FBAR involves the acoustic resonance cavity formed by the creation of an air cavity underneath the bottom electrode by completely etching the Si substrate.

The typical thickness of the piezoelectric film is a few microns, while that of the metal electrodes is an order of magnitude less. To acoustically isolate the resonance cavity, parts of the substrate are removed to create a freestanding membrane.

A scheme of a FBAR device can be observed in Figure 11. In Figure 11a a freestanding heterostructure membrane can be seen. It is composed of a Pt/Ti/AlN/Pt/Ti/UNCD multilayer on a Si substrate, as shown in Figure 11b.

Figure 11. AlN FBAR on UNCD. a) 3D longitudinal cut image of the freestanding FBAR heterostructure, and b) lateral view scheme of the device describing the deposited layers.

For the cavity design, the substrate material as well as the used etching method has to be considered. In the case of Si, a common etching method is wet anisotropic etching; potassium hydroxide (KOH) is a strong base capable of attacking the Si substrate in an anisotropic way. Here, N-type mirror-polished Si (100) wafers are used as substrates, so the KOH will preferably etch the Si in the (100) plane. This produces the characteristic V anisotropic etch of the walls, forming an angle of 54.7° with respect to the main surface (Figure 12).

Figure 12. Si (100) showing the characteristic V anisotropic etch of the walls forming an angle of 54.7° with respect to the main surface

25.4.2. Membrane fabrication

A membrane fabrication on the Si substrate involves several steps:

UNCD growth: UNCD is deposited on the front of a silicon wafer (N-type mirror polished Si (100)). The UNCD films are grown on the front of the silicon wafer (N-type mirror-polished Si (100)) using a microwave plasma chemical vapor deposition (MPCVD) system. This layer is used as a substrate for the AlN and also serves as a stop layer for the anisotropic wet etching of the Si when using KOH.

Si₃N₄ deposition: Silicon Nitride (Si₃N₄) is used as a mask for the wet etching of the Si when using KOH.

Pt/Ti deposition: A Pt electrode is grown with sputter deposition using a Ti adhesion layer. In addition, this coating provides seeding capability for the AlN and serves as a buffer layer. It also helps to improve the adhesion of the AlN to the UNCD.

AlN deposition: On the Pt/Ti electrode, AlN reactive sputter deposition is performed. AlN (002) is grown in a gas mixture of Ar and N with an Al target.

AlN pattern: The pattern is transferred to the AlN film by performing photoresist spin coating, UV exposure, resist development, and dry etching.

Electrode pattern: Patterning of the top Pt/Ti electrode is done by applying lift off techniques. First, a spin coating on the front side of the wafer, UV exposure, and resist development are conducted, followed by Pt/Ti sputter deposition and posterior removal of the photoresist.

Cavity: The cavity etching is done on the backside by using KOH.

In Figure 13, a cavity prototype using only UNCD as a membrane on the Si wafer substrate can be seen.

Figure 13. Cavity prototype. a) Front side of the UNCD film, and b) backside showing the different sizes of the cavities within dices

25.4.3. Device characterisation

25.4.3.1. X-ray diffraction (XRD)

Figure 14 shows an XRD θ - 2θ scan of the AlN/Pt/Ti/UNCD/SiO₂/Si layered film exhibiting high c-axis orientation for the AlN film. The presence of a hexagonal AlN (002) diffraction peak at 36.05° can be noted, revealing the presence of a mainly (002)-textured AlN film. Further, two Pt peaks, (111) at 40.05° and (002) at 46.5°, are present. The weak peaks at 44.7° and at 65.1° correspond to Al (002) and Al (202), respectively. The strong narrow peak at 33.2° comes from the Si wafer substrate and is the result of Si crystalline imperfections.

Figure 14. XRD spectrum of an AlN/Pt/Ti/UNCD/SiO₂/Si heterostructure showing the characteristic peaks of the Pt layers and Si substrate, in addition to the critical AlN (002) peak that reveals the high orientation of the AlN necessary to yield the high piezoelectric coefficient

The X-ray wavelength was $\lambda = 1.540598 \text{ \AA}$ and by using Bragg's law the c-lattice constant for the AlN layer could be calculated. The obtained value was $4.9681 \text{ \AA}$ which is in good agreement with the value of the AlN found elsewhere.

25.4.3.2. Scanning Electron Microscope (SEM)

The SEM is today a routinely used instrument for the examination of fine detail for a variety of samples. The instrument is, in simple terms, analogous to an optical microscope: the electron source (gun) is equivalent to the light source and the glass lens is replaced by electromagnetic lens. Information from the sample is collected and displayed on a viewing screen for visual interpretation.

In Figure 15a, a cross section of the AlN/Pt/Ti/UNCD membrane (cantilevered) is depicted. It exhibits a columnar microstructure; these columnar crystals are perpendicular to the substrate surface, in agreement with the XRD results where the AlN film is highly textured. In this case, the piezoelectric film reaches a thickness of 417 nm after five hours of growth (Figure 15b).

Figure 15. SEM cross section of the film for the AlN/Pt/Ti/UNCD membrane.
a) cantilevered membrane, and b) measurements on the SEM image

A clarified view of the generated Si cavity can be appreciated in the SEM image of the freestanding AlN/Pt/Ti/UNCD membrane on the Si substrate (Figure 16). An in-focus image of the tilted Si walls (54.7°) can be seen where the big square has the dimensions of the mask and the smaller square is the AlN/Pt/Ti/UNCD membrane. The white frame surrounding the big square is produced by the generated undercuts under the UNCD.

Figure 16. SEM image of the freestanding AlN/Pt/Ti/UNCD membrane on the Si substrate

The low temperature of the UNCD growth in addition to the AlN deposition makes this innovative system compatible with CMOS chips and CMOS-driven MEMS and NEMS systems. Successful integration of piezoelectric AlN and UNCD films may enable the development of a new generation of biocompatible piezoelectric-based drug delivery MEMS devices that will be implantable in the human body.

The feasibility of the fabrication of a FBAR AlN/diamond structure is a very promising device for FBAR applications, opening a huge field of biomedical applications.

25.5. COMPARISON TABLE

Table 2 summarises the different developed biomedical systems.

Table 2. Summary of the biomedical systems analysed

System	Piezo material	Fabrication	Biocompatible?	IC Compatible?	Application
polymer-based electroactive device	PVDF	melt extrusion followed by stretching step and exposed to very high electric fields	Yes	No	<ul style="list-style-type: none"> – tissue engineering – orthopaedics – osteogenic differentiation
thin-film electroactive device	AlN	magnetron sputter deposition	Yes	Yes	<ul style="list-style-type: none"> – AlN thin-film-based microbridge – NEMS switches. – micromachined suspended bridge structures
AlN/UNCD-based BioMEMS	AlN	magnetron sputter deposition + MPCVD	Yes	Yes	<ul style="list-style-type: none"> – hermetic biocompatible encapsulating coating for Si-microchips – AlN/diamond-based SAW devices. – FBAR piezoelectric resonators

REFERENCES

1. A. Arnau, *Piezoelectric transducers and applications*, Springer Verlag, 2008.
2. J. Rosenbaum, *Bulk Acoustic Wave Theory and Devices*, Artech House, Inc., 2000.
3. M. Koucky, *Piezoelectric Polymer Microstructures For Biomedical Applications*, MS Thesis, The Ohio State University, 2009.
4. G. Ciofani, S. Danti, L. Ricotti, D.D. Alessandro, S. Moscato, V. Mattoli, in *Piezoelectric Nanomaterials for Biomedical Applications*, G. Ciofani, A. Menciassi (Eds.), Springer, Germany, 2012, p.p. 213–238.
5. G.V. Cochran, M.P. Kadaba, V.R. Palmieri. *J. Orthop. Res.* **6** (1988) 145–147.
6. J. Rosenbaum, *Bulk Acoustic Wave Theory and Devices*, Artech House, Inc., 2000.
7. Measurement Specialities, <http://www.meas-spec.com/> (2015).
8. G. Sauerbrey. *Z. Phys.* **155** (1959) 206–222.
9. C. Ribeiro, D.M. Correia, S. Ribeiro, V. Sencadas, G. Botelho, S. Lanceros-Méndez. *Eng. Life Sci.* **15**(4) (2015) 351–356.
10. J. Reis, C. Frias, C.C. e Castro, M.L. Botelho, A.T. Marques, J.A. Oliveira Simões, F.C. e Silva, J. Potes. *J. Biomed. Biotechnol.* **2012** (2012) 613403.
11. J. Pärssinen, H. Hammarén, R. Rahikainen, V. Sencadas, C. Ribeiro, S. Vanhatupa, S. Miettinen, S. Lanceros-Méndez, P. Hytönen Vesa. *J. Biomed. Mater. Res. Part A* **103**(3) (2015) 919–928.
12. J. Olivares, E. Iborra, M. Clement, L. Vergara, J. Sangrador, A. Sanz-Hervás. *Sensor. Actuat. A: Phys.* **123-124** (2005) 590–595.
13. N. Sinha, G.E. Wabiszewski, R. Mahameed, V.V. Felmetsger, S.M. Tanner, R.W. Carpick. *Departmental Papers (MEAM)* (2009) 165.
14. E. Iborra, J. Olivares, M. Clement, L. Vergara, A. Sanz-Hervas, J. Sangrador. *Sensor. Actuat. A: Phys.* **115**(2-3) (2004) 501–507.
15. O. Auciello, J. Birrell, J.A. Carlisle, J.E Gerbi, X. Xiao, B. Peng, H.D. Espinosa. *J. Phys.: Condens. Matter* **16**(16) (2004) R539.
16. O. Auciello, A.V. Sumant. *Diam. Relat. Mater.* **19**(7) (2010) 699–718.
17. X. Xiao, J. Wang, C. Liu, J.A. Carlisle, B. Mech, R. Greenberg, D. Guven, R. Freda, M.S. Humayun, J. Weiland, O. Auciello. *J. Biomed. Mater. Res. Part B: Appl. Biomater.* **77**(2) (2006) 273–281.
18. F. Benedic, M.B. Assouar, P. Kirsch, D. Moneger, O. Brinza, O. Elmazria, P. Alnot, A. Gicquel. *Diam. Relat. Mater.* **17**(4) (2008) 804–808.