Nano Based Drug Delivery

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Jitendra Naik**

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PREFACE

Successful treatment of various illnesses and disorders heavily relies on efficient drug delivery vehicles. In order enhance the therapeutic index of drugs, requires target of drugs to a particular tissue, cell or intracellular compartment. It can be achieved by controlling over release kinetics, protection of the active agent or combination of both. The drug delivery systems must provide controlled permeability and distribution of drug, targeting only those organs or biomolecules to be treated.

Recently, nano drug delivery systems have immersed as a powerful technique for the treatment of many illnesses such as infectious diseases, cancer and genetic disorders. They could be used in combination with different therapies including radio-therapy, gene-therapy etc. Controlled released nanoparticulate drug delivery systems can overcome the limitations of conventional/ traditional drug delivery systems such as low bioavailability as well as biodistribution and bring reduction in the daily doses, reduce the chance for both under and over dosing and number of repeated administration. Unlike conventional/traditional drug delivery systems, it provides more localized and better use of active agents by increasing bioavailability and biodistribution thus increase patient compliance. With nano drug delivery systems, it is possible to target those specific sites, where achievement of active molecules is difficult by other drug delivery systems. Thus, it facilitates cell-specific targeting minimising undesirable side effects on vital tissues.

The aim of this book is to overview recent advances and achievements in nano drug delivery systems and to provide wide coverage and possible future applications in the field.

LIST OF CONTRIBUTORS

Vojtech Adam, Department of Chemistry and Biochemistry, Laboratory of Metallomics and Nanotechnology, Mendel University in Brno, Zemedelska 1, CZ-613 00 Brno, Czech Republic, European Union; Central European Institute of Technology, Brno University of Technology, Technicka 3058/10, CZ-616 00 Brno, Czech Republic, European Union

Sanaalarab Al-Enazy, Department of Pharmacology & Toxicology, University of Texas Medical Branch, Galveston, Texas, USA

Norah Albekairi, Department of Pharmacology & Toxicology, University of Texas Medical Branch, Galveston, Texas, USA

Narsireddy Amreddy, Department of Pathology; Stephenson Cancer Center, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104, USA

Anne Caroline Andrade Cardoso, Department of Pharmaceutical Technology, Faculty of Pharmacy, Universidade Federal Fluminense, Rio de Janeiro, Brazil

Anish Babu, Department of Pathology; Stephenson Cancer Center, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104, USA

Sudipta Basu, Department of Chemistry, Indian Institute of Science Education and Research (IISER)-Pune, Pune, 411008, Maharashtra, India

Kessiane Belshoff de Almeida, Department of Pharmaceutical Technology, Faculty of Pharmacy, Universidade Federal Fluminense, Rio de Janeiro, Brazil

Eduardo André Bender, Federal University of Pampa, Uruguaiana, Brazil

Samanta Cardozo Mourão, Department of Pharmaceutical Technology, Faculty of Pharmacy, Universidade Federal Fluminense, Rio de Janeiro, Brazil

Harsiddhi Chaudhary, University of Mumbai, Bharati Vidyapeeth's College of Pharmacy, Department of Pharmaceutics, CBD Belapur, Sector-8, Navi-Mumbai-400614, India

Yong Chen, Department of Chemistry, State Key Laboratory of Elemento-Organic Chemistry, Nankai University, Tianjin 300071, P. R. China; Collaborative Innovation Center of Chemical Science and Engineering (Tianjin), Nankai University, Tianjin 300071, P. R. China

Anthony D.M. Curtis, School of Pharmacy, Institute for Science and Technology in Medicine, Keele University, Keele, ST5 5BG, UK

Gökhan Dikmenn, Central Research Laboratory, Eskisehir Osmangazi University, Turkey, 26480

Simona Dostalova, Department of Chemistry and Biochemistry, Laboratory of Metallomics and Nanotechnology, Mendel University in Brno, Zemedelska 1, CZ-613 00 Brno, Czech Republic, European Union; Central European Institute of Technology, Brno University of Technology, Technicka 3058/10, CZ-616 00 Brno, Czech Republic, European Union

Tomas Eckschlager, Department of Paediatric Haematology and Oncology, 2nd Faculty of Medicine and University Hospital Motol, Charles University, V Uvalu 84, Prague 5 CZ-150 06, Czech Republic, European Union

M. Sedef Erdal, Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Istanbul, 34116 Istanbul, Turkey

Junjun Fan, Department of Orthopaedic Surgery, Xi Jing Hospital, Fourth Military Medical University, Xi'an, China

Junbo Ge, Shanghai Institute of Cardiovascular Diseases, Zhongshan Hospital, Fudan University, 180 Feng Lin Road, Shanghai 200032, China

Lütfi Genç, Faculty of Pharmacy, Department of Pharmaceutical Technology, Anadolu University, Turkey, 26470

M. Clara Gonçalves, Departamento de Engenharia Química, Instituto Superior Técnico, Universidade de Lisboa, Av. Rovisco Pais, 1049-001 Lisboa, Portugal; Centro de Química Estrutural, Instituto Superior Técnico, Universidade de Lisboa, Av. Rovisco Pais, 1049-001 Lisboa, Portugal

Florin Graur, University of Medicine and Pharmacy "Iuliu Hatieganu" Cluj-Napoca Str. Victor Babeş Nr. 8, 400012 Cluj-Napoca, Romania; Regional Institute of Gastroenterology and Hepatology "Octavian Fodor" Cluj-Napoca Str. Croitorilor 19-21, Cluj-Napoca, Romania

Evren Gündoğdu, Department of Radiopharmacy, School of Pharmacy, University of Ege, 35100 Bornova, Izmir, Turkey

Gamze Güney Eskiler, Department of Medical Biology, Medical Faculty, Uludag University, Turkey, 16059

Sevgi Güngör, Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Istanbul, 34116 Istanbul, Turkey

Sinem Güngördük, Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Istanbul, 34116 Istanbul, Turkey

Clare Hoskins, School of Pharmacy, Institute for Science and Technology in Medicine, Keele University, Keele, ST5 5BG, UK

Dehui Hu, Department of Pharmacology, Institute of Medical Sciences, Shanghai Jiao Tong University School of Medicine, 280 South Chongqing Road, Shanghai, 200025, PR China; Translational Medicine Collaborative Innovation Center, Shanghai Jiao Tong University School of Medicine, 280 South Chongqing Road, Shanghai, 200025, PR China

Satoshi Gojo, Department of Regenerative Medicine, Kyoto Prefectural University of Medicine, Kyoto, Japan

Barbara Gomes Lima, Department of Pharmaceutical Technology, Faculty of Pharmacy, Universidade Federal Fluminense, Rio de Janeiro, Brazil

Sandra Elisa Haas, Federal University of Pampa, Uruguaiana, Brazil

Zbynek Heger, Department of Chemistry and Biochemistry, Laboratory of Metallomics and Nanotechnology, Mendel University in Brno, Zemedelska 1, CZ-613 00 Brno, Czech Republic, European Union; Central European Institute of Technology, Brno University of Technology, Technicka

Zheyong Huang, Shanghai Institute of Cardiovascular Diseases, Zhongshan Hospital, Fudan University, 180 Feng Lin Road, Shanghai 200032, China

Kisan Ramchandra Jadhav, University of Mumbai, Bharati Vidyapeeth's College of Pharmacy, Department of Pharmaceutics, CBD Belapur, Sector-8, Navi-Mumbai-400614, India

Vilasrao Kadam, University of Mumbai, Bharati Vidyapeeth's College of Pharmacy, Department of Pharmaceutics, CBD Belapur, Sector-8, Navi-Mumbai-400614, India

Emine Kahraman, Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Istanbul, 34116 Istanbul, Turkey

Irina Kalashnikova, Department of Obstetrics & Gynecology, University of Texas Medical Branch, Galveston, Texas, USA

Daisuke Kami, Department of Regenerative Medicine, Kyoto Prefectural University of Medicine, Kyoto, Japan

Rene Kizek, Department of Chemistry and Biochemistry, Laboratory of Metallomics and Nanotechnology, Mendel University in Brno, Zemedelska 1, CZ-613 00 Brno, Czech Republic, European Union; Central European

Institute of Technology, Brno University of Technology, Technicka 3058/10, CZ-616 00 Brno, Czech Republic, European Union

Patricia Alice Knupp Pereira, Department of Pharmaceutical Technology, Faculty of Pharmacy, Universidade Federal Fluminense, Rio de Janeiro, Brazil

Jiri Kudr, Department of Chemistry and Biochemistry, Laboratory of Metallomics and Nanotechnology, Mendel University in Brno, Zemedelska 1, CZ-613 00 Brno, Czech Republic, European Union; Central European Institute of Technology, Brno University of Technology, Technicka 3058/10, CZ-616 00 Brno, Czech Republic, European Union

Yu Liu, Department of Chemistry, State Key Laboratory of Elemento-Organic Chemistry, Nankai University, Tianjin 300071, P. R. China; Collaborative Innovation Center of Chemical Science and Engineering (Tianjin), Nankai University, Tianjin 300071, P. R. China

Daniela Lopes, UCIBIO/REQUIMTE, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal

Juliana Lopes de Araujo, Department of Pharmaceutical Technology, Faculty of Pharmacy, Universidade Federal Fluminense, Rio de Janeiro, Brazil

Lei Lu, Department of Pharmacology, Institute of Medical Sciences, Shanghai Jiao Tong University School of Medicine, 280 South Chongqing Road, Shanghai, 200025, PR China; Translational Medicine Collaborative Innovation Center, Shanghai Jiao Tong University School of Medicine, 280 South Chongqing Road, Shanghai, 200025, PR China

Letícia Marques Colomé, Federal University of Pampa, Uruguiana, Brazil

M. Cristina L. Martins, INEB – Instituto de Engenharia Biomédica, Universidade do Porto, Porto, Portugal; ICBAS – Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Porto, Portugal

Joana C. Matos, Departamento de Engenharia Química, Instituto Superior Técnico, Universidade de Lisboa, Av. Rovisco Pais, 1049-001 Lisboa, Portugal

Fiorella Mollo Zibetti, Department of Pharmaceutical Technology, Faculty of Pharmacy, Universidade Federal Fluminense, Rio de Janeiro, Brazil

Gabriel A. Monteiro, Departamento de Bioengenharia, Instituto Superior Técnico, Universidade de Lisboa, Av. Rovisco Pais, 1049-001 Lisboa, Portugal; IBB – Institute for Bioengineering and Biosciences, Instituto Superior Técnico

Anupama Munshi, Stephenson Cancer Center, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104, USA; Department of Radiation Oncology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104, USA

Ranganayaki Muralidharanm, Department of Pathology; Stephenson Cancer Center, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104, USA

Moritz Nazarenius, Fachbereich Physik, Philipps Universität Marburg, Germany

Cláudia Nunes, UCIBIO/REQUIMTE, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal

Mehmet Evren Okur, Department of Pharmacology, School of Pharmacy, University of Anadolu, 26470, Eskisehir, Turkey

Neslihan Üstündağ Okur, Department of Pharmaceutical Technology, School of Pharmacy, University of Istanbul Medipol, 34083 Istanbul, Turkey

Yıldız Özsoy, Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Istanbul, 34116 Istanbul, Turkey

Wolfgang J. Parak, Fachbereich Physik, Philipps Universität Marburg, Germany

Anuja Patil, University of Mumbai, Bharati Vidyapeeth's College of Pharmacy, Department of Pharmaceutics, CBD Belapur, Sector-8, Navi-Mumbai-400614, India

Sandeep Palvai, Department of Chemistry, Indian Institute of Science Education and Research (IISER)-Pune, Pune, 411008, Maharashtra, India

Guoxian Pei, Department of Orthopaedic Surgery, Xi Jing Hospital, Fourth Military Medical University, Xi'an, China

Ning Pei, College of Science, Shanghai University, 99 Shangda Road, Shanghai 200444, China

Frances M. Pietri-Vázquez, Medical School, University of Puerto Rico, Medical Sciences Campus, San Juan, Puerto Rico 00935, USA

Juying Qian, Shanghai Institute of Cardiovascular Diseases, Zhongshan Hospital, Fudan University, 180 Feng Lin Road, Shanghai 200032, China

Yuanyuan Qiu, Department of Pharmacology, Institute of Medical Sciences, Shanghai Jiao Tong University School of Medicine, 280 South Chongqing Road, Shanghai, 200025, PR China; Translational Medicine Collaborative Innovation Center, Shanghai Jiao Tong University School of Medicine, 280 South Chongqing Road, Shanghai, 200025, PR China

Deborah Quintanilha Falcão, Department of Pharmaceutical Technology, Faculty of Pharmacy, Universidade Federal Fluminense, Rio de Janeiro, Brazil

Rajagopal Ramesh, Department of Pathology; Stephenson Cancer Center, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104, USA; Graduate Program in Biomedical Sciences, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73104, USA

Salette Reis, UCIBIO/REQUIMTE, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal

Jeysika M. Reyes-González, Department of Biochemistry, University of Puerto Rico, Medical Sciences Campus, San Juan, Puerto Rico 00935, USA; Comprehensive Cancer Center, University of Puerto Rico, Medical Sciences Campus, San Juan, Puerto Rico 00935, USA

Pilar Rivera Gil, Fachbereich Physik, Philipps Universität Marburg, Germany

Erik Rytting, Department of Obstetrics & Gynecology, University of Texas Medical Branch, Galveston, Texas, USA; Department of Pharmacology & Toxicology, University of Texas Medical Branch, Galveston, Texas, USA; Center for Biomedical Engineering, University of Texas Medical Branch, Galveston, Texas, USA

Bruno Sarmento, INEB – Instituto de Engenharia Biomédica, Universidade do Porto, Porto, Portugal; IINFACTS – Instituto de Investigação e Formação Avançada em Ciências e Tecnologias da Saúde, Instituto Superior de Ciências da Saúde-Norte, Gandra-PRD, Portugal

Yunli Shen, Department of Cardiology, Shanghai East Hospital, Tongji University, 150 Jimo Road, Shanghai, 200120, China

Marie Stiborova, Department of Biochemistry, Faculty of Science, Charles University, Albertov 230, Prague 2 CZ-128 40, Czech Republic, European Union

Masashi Toyoda, Department of Vascular Medicine, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan

Marketa Vaculovicova, Department of Chemistry and Biochemistry, Laboratory of Metallomics and Nanotechnology, Mendel University in Brno, Zemedelska 1, CZ-613 00 Brno, Czech Republic, European Union; Central European Institute of Technology, Brno University of Technology, Technicka 3058/10, CZ-616 00 Brno, Czech Republic, European Union

Pablo E. Vivas-Mejía, Department of Biochemistry, University of Puerto Rico, Medical Sciences Campus, San Juan, Puerto Rico 00935, USA;

Comprehensive Cancer Center, University of Puerto Rico, Medical Sciences Campus, San Juan, Puerto Rico 00935, USA

Kun-Peng Wang, Department of Chemistry, State Key Laboratory of Elemento-Organic Chemistry, Nankai University, Tianjin 300071, P. R. China

Masatoshi Watanabe, Faculty of Engineering, Yokohama National University, Kanagawa, Japan

Zeyu Xiao, Department of Pharmacology, Institute of Medical Sciences, Shanghai Jiao Tong University School of Medicine, 280 South Chongqing Road, Shanghai, 200025, PR China; Translational Medicine Collaborative Innovation Center, Shanghai Jiao Tong University School of Medicine, 280 South Chongqing Road, Shanghai, 200025, PR China; Collaborative Innovation Center of Systems Biomedicine, Shanghai Jiao Tong University School of Medicine, 280 South Chongqing Road, Shanghai, 200025, PR China

Tetsuzo Yoshimura, Tokyo University of Technology, School of Computer Science 1404-1 Katakura, Hachioji, Tokyo 192-0982, Japan

Yufeng Zhou, School of Mechanical and Aerospace Engineering, Nanyang Technological University, Singapore

ABOUT THE EDITOR

Dr. J. B. Naik, Professor and Head, Department of Chemical Engineering at North Maharashtra University, received his Ph.D. in 1999 from the North Maharashtra University Jalgaon, India. From January 1998 to July 2008 he was a faculty member of University Department of Chemical Technology, Dr. Babasaheb Ambedkar Marathwada University, Aurangabad.

Dr. Naik is working on the development of controlled/sustained released micro/nano particles for anti-diabetic & antihypertensive as well as non-steroidal anti-inflammatory drugs, taste masking of bitter drugs, formulation and development of some herbal drugs, development of biocompatible polymers and polyelectrolyte (anti-scaling agents). He has published/ communicated more than seventy research papers in peer-reviewed scientific journals.

Dr. Naik has completed many government projects based on various drug delivery systems including nanoparticulate drug delivery system; projects sponsored by University Grants Commission, Council of Scientific and Industrial Research, Department of Science and Technology (Nano-mission) and Defense Research and Development Organization, New Delhi India. Additionally, he is a coordinator of Technical Education Quality Improvement Program (TEQIP-II) sponsored by World Bank and MHRD, New Delhi and Special Assistance Program sponsored by UGC, New Delhi. Moreover, he is a life member of Indian Institute of Chemical Engineers (IICChE); Indian Society for Technical Education (ISTE); Indian Desalination Association (InDA); Indian Pharmaceutical Association (IPA) and Fellow Member of the Council of Engineering and Technology (India).

NANOTECHNOLOGY AND ITS IMPLICATIONS IN THERAPEUTICS

**Anuja Patil, Harsiddhi Chaudhary,
Kisan Ramchandra Jadhav*, and Vilasrao Kadam**

University of Mumbai, Bharati Vidyapeeth's College of Pharmacy, Department
of Pharmaceutics, CBD Belapur, Sector-8, Navi-Mumbai-400614, India

*Corresponding author: drkrj24@gmail.com

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1.1. INTRODUCTION

Nanotechnology is the engineering and manufacturing of materials at the atomic and molecular scale resulting into the construction of structures in the nanometre scale size range (often 100 nm or smaller), without changing unique properties. Indeed, the physical and chemical properties of materials can considerably improve or radically change as their size is reduced to small clusters of atoms. Small size means different arrangements and spacings for surface atoms, and these govern the object's physics and chemistry [1]. The prefix of nanotechnology derives from 'nanos' – the Greek word for dwarf. Nanotechnology is becoming important in fields such as microelectronics, health care, engineering, construction, and agriculture. Recently, the application of nanotechnology in the field of health care is receiving considerable acknowledgement. Today, various treatments are available that are time consuming and are also very expensive, whereas the use of nanotechnology provides quicker and much cheaper treatments.

The field of nanotechnology was first introduced by Professor Richard P. Feynman in 1959 (Nobel laureate in physics, 1965). Nanotechnology has gained the status as one of the critical research endeavours of the early 21st century, as scientists harness the unique properties of atomic and molecular assemblages built at the nanometre scale. The ability to manipulate the physical, chemical, and biological properties of these particles provides researchers with the capability to rationally design and use nanoparticles for drug delivery, as image contrast agents, and for diagnostic purposes [2].

Nanotechnology [3] is a multidisciplinary field covering a large and diverse array of devices and materials in the nanometre scale, derived from engineering, physics, chemistry, and biology. The field of applied nanotechnology in medicine is rapidly growing and the application of nanotechnology to therapeutics has led to advances in drug delivery, biomaterials, biomedical devices, intelligent processes, and many other areas of medicine and applied biomedical sciences.

Nanotechnology is the construction of useful materials, devices and systems used to control matter at an incredibly small scale between 1–1000 nm. Such nanoscale objects can be useful by themselves, or as part of larger devices containing multiple nanoscale objects.

In biopharmaceutical delivery there are a number of features of nanotechnology which make it an appropriate tool to tackle major issues [3-5]:

- Reduction of particle size and increased surface area, enhancing solubility;
- Increasing oral bioavailability;
- Targeting of tissues, cells and cellular receptors;

- Vaccine and gene delivery;
- Delivery of large macromolecule drugs to intracellular sites of action;
- Co-delivery of two or more drugs or therapeutic moiety for combination therapy;
- Allows passage through biological membranes, especially the blood brain barrier;
- Design of new nanoporous membranes for controlled-release devices;
- Artificial surface engineering of implants to increase biocompatibility;
- Visualisation of sites of drug delivery by combining therapeutic agents with imaging modalities;
- Determining the *in vivo* efficacy of a therapeutic agent.

Materials used in nanotechnology could be subdivided into several categories:

1. *Nanoparticles* are solid colloidal particles ranging in size from 1–1000 nm which are comprised of macromolecular materials and used therapeutically; for example, as an adjuvant in vaccines or drug carriers, in which active ingredients are dissolved, entrapped or encapsulated and to which active principle is attached or adsorbed. The chief advantages of nanoparticles are (1) improved bioavailability by enhancing aqueous solubility, (2) increasing residence time in the body (increasing half-life for clearance/increasing specificity for its cognate receptors, and (3) targeting drug to specific location in the body (its site of action). This leads to concomitant reduction in the amount of the drug required and dosage toxicity, thus allowing the safe delivery of toxic therapeutic drugs and the protection of non-target tissues and cells from severe side effects. It is progressively used in different applications, including drug carrier systems and to cross organ barriers such as the blood-brain barrier, cell membrane, etc. The primary manufacturing methods of nanoparticles include the Emulsion-Solvent evaporation method, the Double emulsion and Evaporation method, the Salting out method, the Emulsion-Diffusion method, and the Solvent Displacement/Precipitation method, among others. They are made up of biocompatible lipids and provide sustained effects by either dissolution or diffusion. Nanoparticles can be used in biomedical applications, where they assist laboratory diagnostics, or in medical drug targeting. They are used for *in vivo* applications such as contrast agents for magnetic resonance imaging (MRI), for tumour therapy or cardiovascular disease [6-8].

2. *Nanocages* are unique classes of plasmonic nanoparticles with compact sizes varying from 10 to over 150 nm, porous walls, hollow interiors, and easily modified surface chemistry. They are prepared using a remarkably simple galvanic replacement reaction between solutions containing metal precursor salts, mainly chloroauric acid (HAuCl_4), and Ag nanostructures prepared

through polyol reduction. The electrochemical potential difference between the two species allows the reaction, with the reduced metal depositing on the surface of the Ag nanostructures. The resultant Au is deposited epitaxially on the surface of the Ag nanocubes, adopting their underlying cubic form. Concomitant with this deposition, the interior Ag is oxidised and removed, together with alloying and dealloying, to produce hollow and, ultimately, porous structures commonly known as Au nanocages. This approach is versatile; based upon the initial shape of an Ag template, a vast range of morphologies (*e.g.* nanorings, prism shaped nanoboxes, nanotubes, and multi walled nanoshells or nanotubes) are available. This novel class of hollow nanostructures is finding its use as both a contrast agent for optical imaging in the early stages of tumour detection, and as a therapeutic agent for photothermal cancer treatment. Gold nanocages can target tumour cells *in vitro* by complexing with antibodies through the gold thiolate-linkage. When coated with smart thermosensitive-polymer, gold nanocages can also serve as drug delivery vehicles, emptying their contents in response to near-infrared irradiation [6,9-11].

3. *Nanocomposites* are materials that are formed by introducing nanoparticulates (often referred to as filler) into a macroscopic sample material (often referred to as the matrix). This is part of the growing field of nanotechnology. After adding nanoparticulates to the matrix material, the resulting nanocomposite may manifest drastically enhanced properties. For example, adding carbon nanotubes tends to significantly add to the electrical and thermal conductivity. Depending upon the matrix materials, nanocomposites can be classified as ceramic matrix nanocomposites (CMNC), metal matrix nanocomposites (MMNC), polymer matrix nanocomposites (PMNC). CMNC matrix materials include Al_2O_3 , SiC, SiN, *etc.*, while metal matrices employed in MMNC are mainly Al, Mg, Pb, Sn, W and Fe, and a whole range of polymers, *e.g.* vinyl polymers, condensation polymers, polyolefins, speciality polymers (including a variety of biodegradable molecules) are used in PMNC. Processing methods for CMNC include the Powder Process, the Polymer Precursor process, and the sol-gel process, while processing methods for MMNC include Spray Pyrolysis, Liquid Infiltration, Rapid Solidification Process (RSP) with ultrasonication, High Energy Ball Milling, Chemical Processes (Sol-gel, Colloidal), *etc.* Important methods of synthesis for PMNC are intercalation of nanoparticles with the polymer or pre-polymer from solution, *in situ* intercalative polymerisation, melt intercalation, direct mixture of polymer and particulates, template synthesis, *in situ* polymerisation, sol-gel process, *etc.* These nanocomposites are useful in numerous areas ranging from packaging to biomedical applications [6,12,13].

4. *Nanofibres* are defined as fibres with a diameter of less than 100 nm. They can be prepared by interfacial polymerisation, electrospinning and phase separation techniques. Carbon nanofibres are graphitised fibres produced by catalytic synthesis. The development of nanofibres has improved the scope for

fabricating scaffolds that can potentially resemble the architecture of natural human tissue at the nanometre scale. The high surface area to volume ratio of the nanofibres coupled with their microporous structure promotes cell adhesion, proliferation, migration, and differentiation, all of which are highly desired properties for tissue engineering applications. Because of their potential, the nanofibre-based systems are also being sought for a variety of other biological and non-biological applications. Currently, nanofibres are finding use as scaffolds for musculoskeletal tissue engineering (including bone, cartilage, ligament, and skeletal muscle), skin tissue engineering, neural tissue engineering, vascular tissue engineering, and controlled delivery of drugs, proteins, and DNA [6,14].

5. *Nanorings* are small rings formed of crystal. A zinc-oxide nanoring was the first nanoring discovered by researchers. They are synthesised by a spontaneous self-coiling process of nanobelts. Many layers of nanobelts are rolled together as coils, layer-by-layer. The seamless nanorings, each made of a uniformly deformed single crystal of zinc oxide, could be used for nanoscale devices and serve as a model system for studying electrical and mechanical coupling at the nanoscale. These rings, which range in diameter from 1–4 microns and which are 10–30 nm thick, form in a horizontal tube furnace when a mixture of zinc oxide, indium oxide and lithium carbonate – at a ratio of 20 : 1 : 1 – is heated to 1,400 °C under a flow of argon gas [6].

6. *Nanorods* are one type of morphological nanoscale objects. Each of their dimensions range from 1–100 nm. They may be prepared from metals or semiconducting materials. Nanorods are produced by direct chemical synthesis. One potential application of nanorods is in display technologies, because the reflectivity of the rods can be changed by altering their orientation with an applied electric field. Another application is for microelectromechanical systems (MEMS). Nanorods, along with other noble metal nanoparticles, also serve as theragnostic agents. Nanorods absorb in the near infrared (IR), and generate heat when excited with IR light. This property makes nanorod a useful nanotool in cancer therapeutics. Nanorods can be conjugated with tumour-targeting motifs and ingested. When a patient is exposed to IR light (which passes through body tissue), nanorods selectively taken up by tumour cells are locally heated, destroying only the cancerous tissue, while protecting the healthy cells [6].

7. *Nanoshell* consists of a spherical core of a particular compound enclosed by a shell of a few nm in thickness. Currently, gold nanoshells (AuNSs) are being investigated as nanocarriers for drug delivery systems and have both diagnostic as well as therapeutic applications, together with photothermal ablation, hyperthermia, and drug delivery, and diagnostic imaging, particularly in oncology. Localised surface plasmon resonance, biocompatibility, low immunogenicity, and facile functionalisation make AuNSs valuable nanocarriers. AuNSs used for drug delivery can be spatially and temporally triggered to release controlled quantities of drugs inside the target cells when

illuminated with a near-infrared (NIR) laser. Recently, many research groups have confirmed that these AuNS complexes are able to carry anti-tumour drugs (e.g. doxorubicin, paclitaxel, small interfering RNA, and single-stranded DNA) into cancer cells, which augment the efficacy of the treatment. AuNSs can also be conjugated with active targeting ligands such as antibodies, aptamers, and peptides to increase the particles' specificity towards the desired targets [6,15].

8. *Quantum dots* (QDs) are tiny light-emitting particles on the nanometre scale, and are emerging as a new class of fluorescent labels for biology and medicine. QDs are novel type of semiconductor nanocrystals made up of an inorganic elemental core (e.g. cadmium, mercury) surrounded by a metal shell. Two universal approaches for the preparation of QDs have been reported over the last decade: (1) the formation of nanosized semiconductor particles through colloidal chemistry and (2) epitaxial growth and/or nanoscale patterning *i.e.* employing lithography-based technology. As compared to organic dyes and fluorescent proteins, they cover unique optical and electronic properties, with size-tunable light emission, superior signal brightness, resistance to photobleaching, and broad absorption spectra for the simultaneous excitation of multiple fluorescence colours. QDs also provide a versatile nanoscale scaffold for designing multifunctional nanoparticles with both imaging and therapeutic functions. When functionalised with targeting ligands such as antibodies, peptides or small molecules, QDs can be used to target tumour biomarkers as well as tumour vasculatures with high affinity and specificity. QDs can be used as imaging probes for both *in vitro* and *in vivo* cellular and molecular imaging [6,16,17].

9. *Fullerenes* are a class of allotropes of carbon which are graphene sheets rolled into tubes or spheres. The structures of fullerenes can be designated as symmetric cages of all sp^2 carbons, which belong to either 5- or 6-member rings on the cage surface. Fullerene cages of different sizes (C_{60} , C_{70} , and so on) consist of these rings in different numbers and ratios. It has been deduced that size, hydrophobicity, three-dimensionality and electronic configurations make them an attractive entity in medicinal chemistry. Their distinct carbon cage structure coupled with immense scope for derivatisation make them a potential therapeutic agent. The fullerene family, and especially C_{60} , has appealing photo, electrochemical and physical properties, which can be used in various medical fields. The geometry of fullerene matches with the shape of hydrophobic cavity of human immunodeficiency virus (HIV) proteases, thus making them able to fit inside the cavity and hence inhibiting the access of substrates to the catalytic site of the enzyme. It can be used as a radical scavenger and antioxidant. At the same time, when exposed to light, fullerene can produce singlet oxygen in high quantum yields. This action, and also the direct electron transfer from excited states of fullerene and DNA bases, altogether can be used to cleave DNA. Additionally, fullerenes have been used as a carrier for gene and drug delivery systems [6,18,19].

10. *Carbon Nanotubes* (CNTs) are allotropes of carbon with a nanostructure that can have a length-to-diameter ratio greater than 1,000,000. They display extraordinary strength and unique electrical properties, and are efficient conductors of heat. Inorganic nanotubes have also been synthesised. Nanotubes are classified as single-walled nanotubes (SWNTs) and multi-walled nanotubes (MWNTs). Single-walled boron-nitride (BN) nanostructures are hypothetically stronger and lighter than steel. Once BN nanostructures are surrounded by polymers, they can serve to ruggedise the surface of metal parts, as well as forming the basis for oxidation-proof coating. The three most commonly used methods for the synthesis of carbon nanotubes includes the arc-discharge method, the laser ablation method and the chemical vapour deposition techniques. Their remarkable structural, mechanical, and electronic properties are because of their small size and mass, their strong mechanical potency, and their high electrical and thermal conductivity. CNTs have been successfully used in pharmacy and medicine due to their high surface area, which is able to adsorb or conjugate with a wide variety of therapeutic and diagnostic agents (drugs, genes, vaccines, antibodies, biosensors, *etc.*). For the first time, they have been demonstrated as an excellent vehicle for drug delivery directly into cells without metabolism by the body. Other applications of CNTs include tissue regeneration, biosensor diagnosis, enantiomer separation of chiral drugs, extraction and analysis of drugs and pollutants. Recently, CNTs have been revealed to be promising antioxidants [6,20].

Application of nanotechnology in areas of drug delivery and therapy has the potential to revolutionize the treatment of many diseases. In the past two decades, several nanotherapeutics were approved by Food and Drug Administration (FDA) for the treatment of cancer, pain, and infectious diseases (Table 1). Advanced therapy can only be achieved through the rational design of nanotherapeutics that leads to the development of nanoplateforms of particular size, shape, and surface properties that are crucial for biological interactions and consequent therapeutic effects. In the market, nanotherapeutic products include nanocrystals, liposomes, nanoemulsions, nanocomplexes, and nanoparticles, as listed in Table 1. The majority of nanotherapeutics on the market is intended for parenteral administration. These are followed by nanotherapeutics designed for oral administration. The choice of the delivery route, and consequently the barriers to be crossed, are of particular importance for drug-delivery systems [21].

Table 1. Marketed nanotechnology based formulations

Nanotechnology based approach	Drug	Major indication	Dosage form	Brand name
Nanocrystals	Sirolimus	Graft rejection, Kidney transplantation	Tablet	Rapamune®
	Fenofibrate	Hyper-cholesterolemia	Tablet	Tricor® / Lipanthyl® / Lipidil®
	Aprepitant	Postoperative nausea and vomiting, Cancer	Capsule	Emend®
	Olanzapine	Schizophrenia	Powder and solvent for prolonged release suspension for injection	Zypadhera®
Nanoemulsion	Cyclosporine	Prophylaxis of organ rejection following organ transplant	Soft capsule	Neoral®
	Ritonavir	HIV Infection	Soft capsule	Norvir®
Liposome	Amphotericin B	Fungal infection	Suspension (iv)	AmBisome®
	Daunorubicin	Cancer advanced HIV-associated Kaposi's sarcoma	Suspension (iv)	DuanoXome®
	Doxorubicin	Breast Neoplasms Kaposi's sarcoma	Suspension (iv)	Myocet®
	Cytarabine	Meningeal neoplasms	Suspension (intrathecal)	DepoCyt®
	Propofol	Anaesthetic	Emulsion (iv)	Diprivan® Propofol-Lipuro®
	Paclitaxel	Breast neoplasms	Powder for suspension for infusion	Abraxane®
Nanocomplex	Sodium ferric gluconate	Iron deficiency anaemia	Solution (iv)	Ferrlecit®

1.2. CURRENT TECHNOLOGIES AND APPLICATIONS OF NANOTECHNOLOGY

1.2.1. Polymeric nanomedicine for cancer therapy

As the world population ages, the incidence of cancer is increasing continuously; regardless of the tremendous efforts to treat cancer, there has been very little actual improvement in cancer therapeutics over the past few years. Nanomedicine, a branch of nanotechnology, refers to highly specific, molecular-scale medical intervention for treating disease or repairing damaged tissues [22,23]. The nanomedicine-based diagnostics such as gold nanoshells, iron oxide nanocrystals, and quantum dots have gained tremendous impetus in the diagnosis of cancer, although their practical application has been limited by problems such as toxicity, instability, and lack of selectivity for the disease site. Recently, researchers have provided technical solutions to overcome these limitations by physically or chemically attaching biocompatible polymers on the surfaces of diagnostic nanomedicines. Most of the polymers used for these systems are biocompatible and/or biodegradable, and are thus approved by the FDA. The drug is usually either dispersed within the polymeric nanoparticle or conjugated with the polymeric backbone. In the former case, the encapsulated drugs are slowly released from the polymer matrix by diffusion. In the latter case, surface erosion or bulk degradation of the polymer matrix can play a primary role in drug release, and various techniques may be used to adjust the release rate. Several natural and synthetic water soluble polymers and their derivatives have been investigated for their potential use in polymer therapeutics, including poly(ethylene glycol) (PEG), *N*-(2-hydroxypropyl) methacrylamide copolymers, poly(vinyl pyrrolidone), chitosan, hyaluronic acid, poly(ethyleneimine) (PEI), dextran and poly(aspartic acid) [24,25]. Polymer-based nanomedicine (Figure 1), mainly includes the use of polymer-DNA complexes (polyplexes), polymer-drug conjugates [26] and polymer micelles [27] bearing hydrophobic drugs, and has gained increasing consideration due to its ability to improve the efficacy of cancer therapeutics [28]. Due to their small size and excellent biocompatibility, nanosized polymer therapeutic agents can remain in the bloodstream for longer duration, hence allowing them to reach the target site. Additionally, chemical modification of polymer therapeutic agents by the use of ligands which are capable of specifically binding receptors that are over-expressed in cancer cells can markedly enhance therapeutic efficiency.

Figure 1. Schematic illustration of representative polymeric nanomedicines: (a) polymer-drug conjugates; (b) polymer-protein conjugates; (c) polymer-DNA complexes; (d) polymeric micelles; (e) dendrimers

1.2.1.1. Nanotechnology in cancer therapy

Current nanotechnologies in cancer chemotherapy include the following issues [29]:

- ✓ Conventional anticancer drugs, when administered intravenously, can lead to the distribution of drugs throughout the entire body *via* the bloodstream, and also affect both malignant as well as normal cells, thus producing adverse effects.
- ✓ The interstitium of a tumour is characterised by high hydrostatic pressure which is opposite to that of most normal tissues, leading to an outward convective interstitial flow that can wash the drug away from the tumour.
- ✓ Although the drug is successfully delivered to the tumour interstitium, its efficacy may be hampered by the cancer cells that have acquired multidrug resistance (MDR) [30]. MDR is mainly characterised by over-expression of the plasma membrane P-glycoprotein (P-gp), which is able to drive the drugs away from the cell. Several strategies have been used to circumvent P-gp-mediated MDR, which includes the co-administration of P-gp inhibitors and anchoring the anticancer drugs within the nanoparticles. The latter strategy should allow the drug to avoid recognition by P-gp at the plasma membrane, enabling its delivery to the cell cytoplasm or nucleus [31].

1.2.1.2. Opportunities and challenges for cancer therapeutics

Angiogenesis in cancer [32-35]

In the case of cancer, solid tumours [36] smaller than 1–2 mm³ are not vascularised because oxygen and nutrients can reach the centre of the tumour by simple diffusion. Moreover, solid tumours larger than the critical volume of 2 mm³ enter a state of cellular hypoxia that marks the onset of tumoral angiogenesis, *i.e.* the developing of new blood vessels from existing vessels.

A fine balance between factors capable of stimulating and inhibiting blood vessel formation regulates angiogenesis. This balance tips in favour of angiogenesis when hypoxia induces the cancer cell to release pro-angiogenic molecules such as growth factors.

Various strategies for interfering in the angiogenic process have been investigated, including the inhibition of endogenous angiogenic factors (*e.g.* growth factors), degradative enzymes [*e.g.* matrix metalloproteases (MMPs)] which digest the extracellular matrix and allow the blood vessels to form towards the tumour tissue and endothelial cell processes (*e.g.* differentiation, activation, migration, and proliferation), which are necessary for angiogenesis. Moreover, the tumour vasculature caused by angiogenesis in cancer is morphologically abnormal, and various cell-surface proteins have been associated with promoting angiogenesis. Thus, it should be possible to selectively destroy tumour neovasculature devoid of significantly affecting normal vessels.

In terms of nanomedicine, recently prepared nanocells are polymer-based nuclear nanoparticle embedded within an extranuclear PEGylated lipid envelope. This nanocell bears temporal release of two therapeutic agents wherein an anti-angiogenic agent is released from the outer envelope, blocking vascularisation, after which a chemotherapeutic agent is released from the inner nanoparticle to kill the cancer cells. This study confirmed that the combination of traditional chemotherapy with anti-angiogenic agents in a polymeric nanoparticulate system improved the therapeutic index while reducing toxicity.

1.2.1.3. Passive tumour targeting

Most anticancer drugs used in conventional chemotherapy have no tumour selectivity and are randomly distributed within the body, resulting in a reasonably low therapeutic index. Furthermore, recent studies have shown that polymer-conjugated drugs and nanoparticulates show prolonged circulation in the blood and accumulate passively in tumours, even in the absence of targeting ligands, suggesting the existence of a passive retention mechanism. Tumour blood vessels are generally denoted by abnormalities such as a relatively high proportion of proliferating endothelial cells, increased tortuosity, pericyte deficiency and aberrant basement membrane formation.

This defective vascular structure, which is probably the result of the rapid vascularisation necessary to provide oxygen and nutrients for fast-growing cancers, reduces lymphatic drainage and renders the vessels permeable to macromolecules. Due to the reduction in the lymphatic drainage, the permeant macromolecules are not removed efficiently, and are thus maintained in the tumour. This passive targeting phenomenon (Figure 2) is known as the enhanced permeability and retention (EPR) effect [37,38].

Figure 2. Passive drug targeting through the enhanced permeability and retention (EPR) effect. The polymeric nanoparticles preferentially accumulate in solid tumors, owing at least in part to leaky tumor vessels and an ineffective lymphatic drainage system.

1.2.1.4. Active tumour targeting

Active drug targeting is typically achieved by chemical attachment to a targeting component that strongly interacts with antigens (or receptors) present on the target tissue, resulting into the preferential accumulation of the drug in the targeted organ, tissue, or cells. The use of a targeting moiety not only reduces adverse side effects by allowing the drug to be delivered to the specific site of action, but also facilitates cellular uptake of the drug by receptor-mediated endocytosis, which is an active process requiring a significantly lower concentration gradient across the plasma membrane than

simple endocytosis. Active targeting frequently makes use of monoclonal antibodies. However, nowadays folate targeting is preferred.

Folates [39-41] are low molecular weight vitamins that are mandatory for eukaryotic cells and their conjugates have the ability to deliver a variety of drugs or imaging agents to pathological cells without causing harm to normal tissues. (Figure 3).

Figure 3. Receptor-mediated endocytosis of folate-conjugated drugs. The folate receptors recognize the conjugates, which are subsequently subjected to membrane invagination. As the endosomal compartment acidifies, the conjugate and the drugs are released from the receptor into the cytosol.

Advantages of folate targeting:

1. Folate is known to be non-immunogenic.
2. Folate-conjugated drugs or nanoparticles are very quickly internalised *via* receptor-mediated endocytosis.
3. In addition, the use of folate as a targeting moiety is believed to evade cancer cell multidrug-efflux pumps [41].

1.2.1.5. Polymer-based nanomedicine for treating cancer

Effective gene therapy requires two vital components [42], namely:

1. An efficacious therapeutic gene that can be expressed at a target cell; and
2. A safe and efficient delivery system that can transport the therapeutic gene to the specific tissue or organ.

Two different types of carriers have been examined for use in delivering genes to the target site: “viral and non-viral vectors”.

Viral vectors, which cover retroviruses, adenoviruses, and adeno-associated viruses [43], are capable of introducing their genetic materials into the host cells, which results in high gene transfection efficiency.

Limitations of viral vectors:

1. The large genetic materials required for gene therapy are difficult to encapsulate;
2. Risks based on their immunogenicity and oncogenic potential [44, 45].

Thus, non-viral vectors, especially polymers, have recently turned out to be a promising alternative that exhibits considerably lower safety risks and can be tailored to specific therapeutic needs through relatively simple changes in the preparation, purification and chemical modification steps [46,47]. Genetic material of various sizes can be delivered by non-viral vectors, and can be prepared easily and inexpensively. However, when compared to viral vectors, they show relatively low transfection efficiency and a short duration of gene expression.

The most commonly used non-viral vectors include polymeric gene carriers [42,48] and polymer micelles.

Polymeric gene carriers

Poly(ethyleneimine) (PEI)

Most of the polymer-based non-viral vectors are cationic in nature and can interact electrostatically with negatively charged DNA to produce nanosized ionic complexes (polyplexes) [49-51]. In general, polyplexes reveal optimal transfection efficiency when they have a positive net charge generated by the presence of more a cationic polymer than DNA [51]. This allows the polyplexes to interact efficiently with the negatively charged cell surface proteoglycans that mediate subsequent endocytosis [52]. Erythrocyte aggregation and/or interaction with plasma components such as albumin and fibrinogen can occur upon the intravenous administration of positively charged polyplexes. Polyplexes, when chemically conjugated to PEG or various Targeting moieties, can minimise such problems.

There are several factors affecting transfection efficiency and cytotoxicity of PEI-based polyplexes:

1. The molecular weight of the PEI [53],
2. The ionic strength of the solution,
3. Its degree of branching,
4. The zeta potential of the polyplexes and
5. Their particle size, confirming that low molecular weight PEI (LMW-PEI, 11.9 kDa) produces a higher transfection efficiency and lower cytotoxicity than high molecular weight PEI (HMW-PEI, 1616 kDa) [53].

Researchers have chemically conjugated PEG to the PEI backbone. It is usually accepted that the surface modification of biomolecules with PEG leads to increased blood circulation time [54], because PEG appreciably decreases the uptake of the polyplexes by macrophages in the liver and spleen through a phenomenon called the “stealth effect”. Other *advantages of PEG* are:

1. PEG is a biocompatible polymer;
2. Cytotoxicity is decreased when it is conjugated with polyplexes;
3. Greater transfection efficiency.

In recent years, PEGylated polyplexes bearing ligands capable of targeting specific cells are prepared, as this leads to an increase in both the blood circulation time and the transfection efficiencies of the polyplexes. These efforts may be broadly divided into three different approaches [55]:

- (1) PEG is chemically grafted to PEI, such that the PEG functional groups are located at the end of the chain, thus enabling chemical conjugation of the ligands (Figure 4a).
- (2) polyplexes prepared by mixing PEI and DNA are modified with heterobifunctional PEG, followed by the chemical attachment of the ligands (Figure 4b).
- (3) PEI has been ligand-modified and used to form polyplexes, which are then surface-decorated with PEG (Figure 4c).

Out of the three approaches, the first two have shown better transfection efficiencies, most probably because the ligand is conjugated with the distal end of the PEG, which has more access to the target cells.

Figure 4. Schematic representation of three strategies used for the formation of PEGylated ligand-containing PEI/DNA complexes

Poly(L-lysine) (PLL)

PLL, which forms polyelectrolyte complexes with DNA, is another polymer that can be used as a non-viral gene carrier. PLL is a linear polypeptide comprising of repeated lysine residues, which have primary ϵ -amino groups and are protonated in the physiological environment. The cationic nature of PLL enables it to interact electrostatically with negatively charged DNA, forming nanoparticulate polyplexes that exhibit different properties depending on the molecular weight of the utilised PLL. The LMW PLLs (< 3 kDa) do not form many stable complexes with DNA. The HMW versions of PLL can form nanosized complexes with DNA, but show relatively high toxicities, and the complexes tend to aggregate in aqueous solution. To address such problems, researchers have modified PLL with PEG and various targeting moieties [56]. PEGylation is expected to improve the stability in addition to pharmacokinetic properties of polyplexes.

Synthetic biodegradable polycations

Ideally, a gene carrier should not only carry the gene to specific cells with high efficacy, it should also degrade and be excreted from the body after a given time period. Since the non-degradable cationic polymers (e.g. PEI) are not easily removed from the body, they may accumulate within cells or tissues, leading to serious side effects. In an effort to overcome these issues, researchers have assessed the use of biodegradable polycations, such as poly(α -(4-aminobutyl)-L-glycolic acid) (PAGA) [57], poly(β -amino ester) [58], poly(4-hydroxy-L-proline ester), poly(phosphoester) [59], polyphosphazene and degradable PEI, as gene carriers.

PAGA, which showed 2-fold higher transfection efficiency than PLL without any measurable cytotoxicity, showed rapid initial degradation within 100 min and complete degradation within 6 months in physiological buffer (pH 7.3) at 37.1 °C. In contrast to PAGA, after 3 months under the same conditions, PLL showed negligible degradation. Poly(amino ester), prepared by the addition of primary amines to diacrylate esters, showed lower cytotoxicity and transfection efficiency and was found to be similar to unmodified PEI.

Polymer micelles

Polymeric micelles [60], which were introduced by Ringsdorf in 1984, are formed by amphiphilic block copolymers in aqueous solution [61]. Polymeric micelles are capable of enhancing the solubility of hydrophobic drugs stems from their unique structural composition, which is characterised by a hydrophobic inner core sterically stabilised by a hydrophilic shell. A polymeric micelle can serve as a nanosized container into which drugs can be incorporated by chemical, physical, or electrostatic interactions [62].

The use of polymeric micelles as drug carriers offers several advantages over conventional dosage forms:

1. The protection of drugs from harsh biological environments (*e.g.* low pH and hydrolytic enzymes);
2. The agents can be imbibed into the hydrophobic inner core, appreciably improving the aqueous solubility of hydrophobic drugs;
3. The small size of polymeric micelles (10–100 nm in diameter) should facilitate drug targeting and reduce the side effects of chemotherapy;
4. They have prolonged retention time in circulation. The presence of hydrophilic polymers on the surfaces of nanoparticulate systems is known to hamper protein adsorption and opsonisation of the particles by the reticulo-endocytic system (RES).

PEG-poly(amino acid) [63-65]

PEG has been widely used as the hydrophilic segment of polymeric micelles. Due to their biocompatibility and hydrophilic nature, PEG-based polymeric micelles have shown no significant cytotoxicity and are not often recognised by the RES system, allowing prolonged circulation in the bloodstream. The PEG chains of polymeric micelles possess high chain mobility in an aqueous environment and have a large excluded volume, potentially decreasing the interactions of the polymeric micelles with constituents of biological fluids. In addition, the PEG molecules in the outer layer of the polymeric micelles can inhibit hydrophobic interactions between the inner cores of different micelles, thus blocking inter-particle aggregation. When amphiphilic block copolymers are prepared with heterobifunctional PEG with different functional groups, the polymeric micelles can be modified with targeting moieties for drug delivery to specific cells and/or tissues.

Poly(ethylene oxide)-block-poly(L-amino acid) [PEO-b-p(L-AA)s] is the modified version of the PEG-poly(amino acids). In addition to loading of therapeutic substances by both chemical and physical means, the use of PEO-b-p(L-AA) also facilitates chemical modification of the core-forming blocks. The p(L-AA)s are biodegradable, biocompatible and relatively nontoxic. They undergo hydrolysis and/or enzymatic degradation in biological fluids to produce biocompatible L-amino acid materials.

PEG-polyester

Biocompatible polyesters have been widely used for drug delivery, because they are slowly degraded in the body and thus an additional removal procedure after implantation is not necessary. They are also useful for the preparation of amphiphilic block copolymers that are capable of forming micelles in aqueous solutions. The representative polyesters that can be used as the hydrophobic segments of the copolymers include poly(glycolic acid), poly(D-lactic acid), poly(ϵ -caprolactone) (PCL), and poly(D, L-lactic acid), as well copolymers of lactide/glycolide.

A model for the cellular internalisation of the drug incorporated in PCL-b-PEO micelles [66-67] has been proposed by the researchers. They proposed that:

- (i) by means of endocytosis, the micelle bearing the drugs enters the cytoplasmic compartment;
- (ii) from micelle-incorporated drugs, the drug molecules eventually diffuse out of the micelle and distribute through the cytoplasm; and
- (iii) Some of the micelles inside the cell may disassemble into single chains and act locally to penetrate the membranes of cellular organelles (Figure 5).

Figure 5. Cellular internalization of free drug and drug incorporated in PCL-b-PEO micelles: (a) free drug diffuses through the cell membrane; (b) micelle bearing the drug enters the cytoplasmic compartment by endocytosis; (c) eventually diffuses out of the micelle and distributes through the cytoplasm; and (d) some of the micelles inside the cell may disassemble into single chains and act locally to permeabilize the membranes of the cellular organelles (dotted arrows)

1.2.2. Ligand based dendritic systems for tumour targeting

Medications that can selectively target tumours whilst at the same time avoiding access of the drug to non-target areas employ the use of homing devices termed ligands that can bind to specific epitopes expressed on the surface of the necrotic mass of cells. Dendrimers [68] are nanosized, non-immunogenic, and hyper-branched vehicles that can be efficiently tailored for the spatial distribution of bioactives, thereby reducing untoward cytotoxicity on normal cells. These nanoparticulate drug delivery vehicles provide a unique platform that has exactly placed functional groups so that multiple copies of ligands can be attached to them and facilitate targeting to the tumour surface or neo-vascularising vessels proliferating around these cells.

1.2.2.1. Dendrimers

Dendrimers are uni-molecular polymeric systems synthesised in a re-iterative manner. At the same time, their synthesis can be optimised to control their size, shape, molecular mass, composition and reactivity. Dendrimers have hyper-branched structure with precisely placed functional groups that bear an important role in controlling the properties of therapeutic moieties that are encapsulated or complexed with it.

Types of dendrimers

1) Poly(amidoamine) (PAMAM) dendrimer

These were the first dendritic structures to have been exhaustively investigated. Tomalia's PAMAM dendrimer received widespread attention. PAMAM dendrimers are synthesised by the divergent method starting from ammonia or ethylenediamine initiator core reagents. Products up to generation 10 (a molecular weight of over 930,000 g mol⁻¹) have been obtained. The polydispersity index of 5.0–10.0 G PAMAM dendrimers is less than 1.08, indicating that the particle size distribution is very uniform for each generation. PAMAM dendrimers have the ability for condensation of DNA followed by transfection due to the presence of positive charge on the surface [69].

2) Poly(propylenimine) (PPI) dendrimer

PPI dendrimers are hyper-branched macromolecules that are amine terminated. The divergent method is basically used to synthesise the PPI dendrimers [70]. Nitrogen of primary amine and nitrogen of tertiary amine are two types of nitrogen atoms in a PPI dendrimer. Tertiary nitrogen atoms are more acidic, with a pKa of around 6–9, whereas primary nitrogen atoms are more basic, with a pKa of around 10. PPI dendrimers are synthesised by a divergent approach, in a sequence of repetition of double Michael addition of acrylonitrile to primary amines followed by heterogeneously catalysed the hydrogenation of nitriles. This repeated reaction results in a doubling of the number of primary amines. 1,4-Diaminobutane is utilised as a dendrimer core during the synthesis of PPI dendrimers. A variety of molecules with primary or secondary amine groups can also be used as the core in dendrimer synthesis [71].

3) Liquid crystalline (LC) dendrimers

Mesogenic LC monomers *e.g.* mesogen functionalised carbosilane dendrimers are found in LC dendrimers. Rod-like (calamitic) or disk-like (discotic) molecules form thermotropic LC phases or mesophases [72].

Frey and coworkers attached several mesogenic units to carbosilane dendrimers, such as cyanobiphenyl [73] and cholesteryl [74]. Mesogenic 3,4-bis-(decyloxy)benzoyl groups functionalised PPI dendrimers of different generations (1.0–5.0 G) were investigated for mesogenic activity as *per* the reported study. Apart from the fifth generation dendrimer, all other lower generation dendrimers displayed a hexagonal columnar mesophase in which the dendrimers had a cylindrical conformation [75,76]. The fifth generation dendrimer lacks mesomorphism. This lack of mesomorphism for the fifth generation dendrimer was due to its inability to reorganise into a cylindrical shape. Boiko *et al.* reported the debut synthesis of photosensitive LC dendrimer with terminal cinnamoyl groups in 2001 [77]. These LC dendrimers are being investigated by scientists for biomedical applications. Recently, Pedziwiatr-Werbicka and coworkers suggested that amino terminated carbosilane dendrimers have potential to deliver short-chain siRNA and anti-HIV oligodeoxynucleotide to HIV-infected blood cells. Although these dendrimers had limited application in the delivery of long-chain double stranded nucleic acids, the dendriplexes of carbosilane dendrimers and anti-HIV nucleic acid were stable and less cytotoxic to blood cells compared to the plain dendrimers, suggesting their utility in the delivery of bioactives.

4) Core Shell (tecto)-dendrimers

Core-shell or tecto-dendrimers represent a polymeric architecture with highly ordered structure. This highly ordered structure was obtained as a result of the controlled covalent attachment of dendrimer building blocks [78]. Tecto-dendrimers are composed of a core dendrimer that may or may not contain the therapeutic agent, surrounded by dendrimers. The synthesis of tecto-dendrimers has been reported with fluorescein as the core reagent for detection and folate as the targeting moiety [79]. The solubility problems encountered in previous studies with aromatic fluorescein isothiocyanate (FITC) moieties on dendrimeric surfaces were solved by such conjugates. This conjugate was found to be overwhelmingly superior to those dendrimeric conjugates containing both FITC and folic acid attached to the surface [80]. In contrast to simple dendrimers, the synthesis procedure for tecto-dendrimers is comparatively simple and thus later inflating the application of dendrimers. Schilrreff *et al.* investigated the cytotoxicity of tecto-dendrimers in order to point out their application in biomedical fields. In this study, tecto-dendrimers with amine-terminated 5.0 G PAMAM dendrimers as a core, surrounded by a shell composed of 2.5 G PAMAM dendrimers with surface carboxyl groups, were investigated for cytotoxicity towards SK-Mel-28 human melanoma cells. The inhibition of growth of melanoma cells occurred at a concentration which is safe to healthy keratinocytes epithelial cells [78] with these tecto-dendrimers. Hence, tecto-dendrimers could be explored for application in the field of nanomedicine, including drug delivery.

5) Chiral dendrimers

Dendrimers based upon the construction of constitutionally different but chemically similar branches to the chiral core are referred to as chiral dendrimers. Chiral, non-racemic dendrimers with well-defined stereochemistry are a particularly interesting subclass with potential applications. These have applications in asymmetric catalysis and chiral molecular recognition. Ghorai *et al.* described the first molecules of anthracene-capped chiral dendrimers which were derived from a 1,3,5-trisubstituted aromatic core and carbohydrate units in the interior and periphery. These were claimed to be suitable for anchoring other useful functionalities aimed at applications as drug delivery system and light harvesting materials [81]. Evidence supporting the above claim is keenly awaited, particularly in the field of drug delivery application of chiral dendrimers.

6) Peptide dendrimers

Peptide dendrimers are radically branched macromolecules that contain a peptidyl branching core and/or peripheral peptide chains. Peptide dendrimers can be divided into three categories. The first category with peptides only as surface functionalities is referred to as grafted peptide dendrimers. The second category composed entirely of amino acids is known as peptide dendrimers, while the third one utilises amino acids in the branching core and surface functional groups but having non-peptide branching units. The synthesis of peptide dendrimers is the best and frequently performed by divergent and convergent methods. The availability of solid-phase combinatorial methods facilitates large libraries of peptide dendrimers to be produced and screened for desired properties. Peptide dendrimers have been used in industry as surfactants and in biomedical field as multiple antigen peptides (MAP), protein mimics and vehicles for drug and gene delivery [82,83]. In addition, Darbre and Reymond have utilised peptide dendrimers as esterase catalysts [84].

7) Glycodendrimers

Glycodendrimers encompass sugar moieties such as glucose, mannose, galactose and/or disaccharide into their structure. The vast majority of glycodendrimers have saccharide residues on their outer surface. Glycodendrimers containing a sugar unit as the central core, from which all branches emanate, have also been described. Glycodendrimers are generally divided into three categories: i) carbohydrate-centred, ii) carbohydrate-based, and iii) carbohydrate-coated dendrimers [85]. One anticipated application of these dendrimers is site-specific drug delivery to the lectin-rich organs. These dendrimers were anticipated to display better association with lectin-supported systems compared to mono-carbohydrate-anchored systems [86].

8) Hybrid dendrimers

Hybrid dendrimers are a combination of dendritic and linear polymers in hybrid block or graft copolymer forms. The formation of dendritic hybrids is due to the spherical shape and a large number of surface functional groups of dendrimers. The small dendrimer segment coupled to multiple reactive chain ends provides an opportunity to use them as surface active agents, also as compatibilisers or adhesives, or hybrid dendritic linear polymers. The dendritic hybrids obtained from various polymers with dendrimers generated the compact, rigid, uniformly shaped globular dendritic hybrids that have been explored for various aspects in the field of drug delivery [87,88].

9) PAMAM-organosilicon (PAMAMOS) dendrimers

Inverted unimolecular micelles that consist of hydrophilic, nucleophilic PAMAM interiors and hydrophobic organosilicon (OS) exteriors are known as radially layered PAMAMOS dendrimers (PAMAMOS). PAMAMOS dendrimers offer unique potential for novel application in electronics, chemical catalysis, nano-lithography, photonics, *etc.* It is possible due to its unique properties such as constancy of structure and ability to form complex and encapsulate various guest species with nanoscopic topological precision [89].

Synthesis of dendrimers

Dendrimers are symmetrical, highly branched polymers possessing a compact spherical structure (diameter ranging from 1.1 nm for 1.0 G PAMAM to 9 nm for 8.0 G PAMAM dendrimer). They are normally synthesised from a central polyfunctional core which is achieved by the repetitive addition of monomers. The core is characterised by a number of functional groups. The addition of monomers to each functional group results in next dendrimer generation, as well as the expression of end groups for further reaction [90]. The size of the dendrimer increases as the generation number increases. A stage will soon be reached when the dendrimer attains its maximum size and becomes tightly packed, giving the appearance of a ball. Divergent and convergent methods are most frequently used for dendrimer synthesis. Additionally, other approaches like hypercores and branched monomers growth, double exponential growth, lego chemistry and click chemistry are also used.

1) Divergent approach

The divergent approach is comprised of two steps. First, is the activation of functional surface groups, and second is the addition of branching monomer units. The approach includes reacting the core with two or more moles of reagent containing at least two protecting/branching sites, followed by the removal of protecting groups. This will lead to the formation of first generation dendrimers. The process is then repeated several times until the dendrimer of

the desired size is formed. This method gives PAMAM starburst dendrimers. Compared to other methods, the divergent approach has some overriding advantages such as the ability to modify the surface of dendrimer molecules by changing the end groups at the outermost layer. The fact that the overall chemical and physical properties of dendrimer can be configured to specific needs is the other major advantage.[91,92].

2) Convergent approach

The convergent approach is an alternative method of dendrimer synthesis that was first proposed by Hawker and Frechet in 1990. Only one kind of functional group on the outermost generation is the main constraint of divergent growth method. Convergent growth would overcome such a weakness. Convergent method involves two stages, firstly a reiterative coupling of protected/deprotected branch to produce a focal point functionalised dendron; and secondly, divergent core anchoring steps to produce various multidendron dendrimers. Some outstanding dividends of this method are the precise control over molecular weight and the production of dendrimers with functionalities in precise positions and number are some of the outstanding dividends of this method [93]. A significant limitation of this method is the difficulty in synthesising dendrimers in large quantities, because of repeated, reactions occurring during the convergent approach that necessitates the protection of active sites.

3) Hypercores and branched monomers

The pre-assembly of oligomeric species to hasten up the rate of dendrimer synthesis is the requirement of this method. Oligomeric species are linked together to yield dendrimers in fewer steps and/or higher yields in this method. Essentially, a hypercore with multiple attaching groups is grown from a core molecule and the surface units are linked to a branched monomer with focal point activation, leading to the synthesis of blocks that are then attached to the hypercore to generate higher generation dendrimers.

4) Double Exponential

This approach allows the preparation of monomers for both divergent and convergent growth from a single starting material. The starting material is similar to a rapid growth technique for linear polymer. The two resultant products are then reacted to give an orthogonally protected trimer that can be used to repeat the growth again. The advantage of the double exponential growth approach is rapid synthesis and also the applicability to either the divergent or convergent method [94].

5) Lego chemistry

Various approaches have been explored by scientists in order to simplify the synthetic procedure for dendrimers, in terms of cost as well as duration of synthesis. Lego chemistry is one of the outcomes of these explorations. Lego chemistry is based on the application of highly functionalised cores and branched monomers. It has been utilised in the synthesis of phosphorus dendrimers. The basic synthetic scheme has undergone several modifications and has resulted in a refined scheme wherein a single step can amplify the number of terminal surface groups from 48–250. This method also encompasses the advantage of utilising the minimum volume of solvent, allowing a simplified purification procedure with eco-friendly by-products like water and nitrogen, apart from higher growth in the number of terminal surface groups in fewer reactions [95].

6) Click chemistry

Another approach for the fast and reliable synthesis of dendrimers is based on click chemistry. In click chemistry, small units are joined together. High chemical yield with innocuous by-products is the main characteristic of the click chemistry reaction. The use of simple reaction conditions, easily available reagents, and benign solvent are the additional profits of click chemistry. Following the click chemistry strategy, dendrimers with various surface groups can be obtained in high purity and excellent yield. 2.0 G and 3.0 G triazole dendrimers were synthesised using Cu(I)-catalysed click chemistry reactions. The obtained dendrimers were isolated as a pure, solid sample with only sodium chloride as the major by-product using chromatographic procedure [95].

Dendrimer Generations

Dendrimer generation is hyperbranching when going from the centre of the dendrimer towards the periphery, which results in homostructural layers between the focal points (branching points). The number of focal points when going from the core towards the dendrimer surface is the generation number, *i.e.* a dendrimer with five focal points when going from the centre to the periphery is denoted a 5th generation dendrimer. This term, here abbreviated to simply a G5-dendrimer, *e.g.* a 5th generation PPI, is abbreviated to a “G5-PPI”-dendrimer. The core part of the dendrimer is sometimes denoted generation “zero”, or presented as “G0”, in the terminology. As hydrogen substituents are not considered focal points, the core structure thus presents no focal points. Intermediates during the dendrimer synthesis are sometimes denoted half-generations [96,97].

Properties of dendrimers [98]:

- (1) Monodispersive nature;
- (2) Globular shape;
- (3) Efficient carrier system for drugs due to their highly controlled architecture;
- (4) Highly stable carriers and can be stored for longer periods;

Dendrimers consist of three characteristic scaffolds:

- (1) Multifunctional initiator core;
- (2) Inner generations, which are composed of repeating branched units; and
- (3) The outermost generation with attached exterior surface groups (Figure 6)

Figure 6. Dendrimer: Unique architecture facilitates (a) encapsulation, (b) conjugation of drugs, (c) prevent their opsonisation by mononuclear phagocytic system (MPS), and (d) provides numerous sites of attachment

The term “exo-receptors” means the terminal functionalities of the dendrimers, which are involved in complexation of therapeutic moieties, whereas the term “endo-receptors” means groups present in the interior responsible for drug entrapment. Dendrimers may have the same functional group at the terminal junctions, which can be an important feature for substrate binding to these functionalities. Multivalency provided by the

dendrimer can play a superior role in the increased affinity of substrates to its complementary receptors, and is purely by co-operation or on a statistical basis. This multiple binding mimics nature (*e.g.* protein–protein & protein–membrane binding), and results in significantly increased activity. Thus, attaching multiple copies of the ligand to dendritic surface promotes increased access to the target area, where the movement of the carrier system/ligand by simple diffusion is a problem. Nanoparticulate architecture of dendrimers favours its access in the highly permeable tumour vasculature and its high molecular weight causes its localisation and prevents its escape [99]. The process is termed the EPR effect.

- Dendrimers (MW > 40 kDa) were found to remain in the blood for longer periods of time when compared with lower molecular weight polymers.
- The host-guest chemistry facilitates the encapsulation of hydrophobic drugs at the same time also stimulates the attachment of hydrophilic moieties.
- The hydrophilic exterior of these robust nanostructures prevents their recognition by mononuclear phagocytic system (MPS) and hence prevents their subsequent removal by opsonisation.

At the physiological pH (~ 7.4) the tertiary amine groups of these dendrimers remain deprotonated and the branches converge to the central core. This prevents the release of drugs in the environment. However, once the dendrimers enter the tumour vasculature, which has a somewhat more acidic micro-environment, the amine groups protonate, and they repel to undergo a conformational change, thus promoting the release of drug.

1.2.2.2. Receptor specific dendritic nanoconstructs

The exo-groups that are at the surface of dendrimers can be designed so that few of the branches are conjugated to the drug, and the remaining ones are tailored to targeting moieties or ligands. Ligands attaching to the dendrimer confirm its destination to the target site and thus later prevent the delivery of the drug to non-target areas. Thus, polyvalency, *i.e.* the presence of multiple functional groups, aids with targeting the drug at its desired location. Spatial accessibility for targeting is provided by the presence of multiple branching sites in a dendrimer that provides enhanced interaction to the receptor. Targeted delivery offers increased therapeutic index, reduction in the required dose as well as toxicity. Dendrimers provide a unique platform that can couple the targeting moiety, drug, imaging agent and fluorescent probe simultaneously, without affecting the integrity of individual components. Dendrimers are different from various other carriers, which is better

understood by the ability to attach any or all of these molecules in a well-defined and controllable manner onto a robust dendritic surface. This ability clearly differentiates dendrimers from other carriers such as micelles, liposomes, emulsion droplets, and engineered particles. Numerous tailorable surfaces on the dendrimer make it possible to attach various ligands and thus delivery to their specific receptors on the tumour cell surface or on the angiogenic microcapillaries growing around these cells. Specific cell surface receptors recognise structural analogues possessed by the ligands. Once the complex reaches the target site, it is internalised and subsequently releases the therapeutic moiety. The ligands thus play a prominent role in inhibiting or stimulating a patho-physiological response. Thus, conjugation of these ligands to dendrimers provides enhanced intracellular trafficking of these macromolecules in the necrotic tumour cells.

1.2.3. Nanomedicine in the diagnosis and therapy of neurodegenerative disorders

Alzheimer's and Parkinson's diseases *i.e.* neurodegenerative and infectious disorders, amyotrophic lateral sclerosis, and stroke are rapidly increasing as population's age. Early diagnosis would enable improved disease outcomes. Drugs, vaccines or regenerative proteins present "real" possibilities for positively affecting disease outcomes, but are limited in that their entry into the brain is commonly restricted across the blood–brain barrier (BBB). Such obstacles can be overcome by polymer science and nanotechnology which may improve diagnostic and therapeutic outcomes [100,101].

1.2.3.1. Barriers to central nervous system (CNS) drug delivery [102]

Formidable barriers that hinder delivery of diagnostic and therapeutic agents to CNS separates the brain from the rest of the body. Comprehending physiological features of these barriers is necessary for discovery of the means toward the effective delivery of drugs and imaging agents. Walls of capillaries in BBB separate the brain from circulating blood (Figure 7).

Figure 7. The BBB is formed by brain microvessel endothelial cells (BMVEC) that form tight junctions and express different transport systems such as Pgp, glucose transporter (GLUT1), large amino acid transporter (LAT1), excitatory amino acid transporters (EAAT1-3), transferrin receptor and others.

1.2.3.2. Nanocarriers for CNS drug delivery [103]

Nanocarriers are mainly low molecular weight drugs or therapeutic proteins that are chemically linked to water-soluble polymers. Linking to water soluble polymers increase drug solubility and drug stability, or enable the site-specific transport of drugs to target tissues affected by disease. Also, PEG-coated liposomes carrying chemotherapeutic drugs have been tested for clinical use.

Liposomes

Liposomes [104] are vesicular structures composed of unilamellar or multi-lamellar lipid bilayers surrounded by internal aqueous compartments.

Liposomes possess following advantages:

1. Varying sizes from several nanometres to several microns;
2. Large amounts of drug molecules can be incorporated into liposome aqueous compartments (water soluble compounds) or within lipid bilayers (lipophilic compounds) comparatively;
3. Reticuloendothelial system (RES) rapidly clears conventional liposomes from circulation;

4. Extended circulation time can be accomplished with small sized liposomes (~ 10 nm) composed of neutral, saturated phospholipids and cholesterol;
5. Liposomes with a surface modified with PEG ("PEGylation") reduces opsonisation of liposomes in plasma. Also it decreases its recognition and removal by the mononuclear phagocyte system (MPS) in liver and spleen;
6. PEGylated (or "stealth") liposomes have circulation half-life of around 50 h in humans. One such instance is that of doxorubicin encapsulated in PEGylated liposome, Doxil, which was approved for treatment of ovarian cancer, AIDS related Kaposi's sarcoma SS and metastatic breast cancer;

Encapsulation of a drug into liposomes prolongs drug circulation time in the blood stream, reduces drug side effects, and enhances drug therapeutic effects in CNS.

PEGylated liposomes [105,106] coupled with monoclonal antibodies to glial fibrillary acidic protein (GFAP), an antigen expressed in astrocytes, show altered brain penetrance. Immunoliposomes are incapable of penetrating a normal BBB, while immunoliposomes used to treat glial brain tumours that express GFAP can reach their disease site when the BBB is partially permeabilised. Thus, the mechanism(s) of brain accumulation in disease may involve EPR of circulating liposomes at sites of disease-induced BBB compromise.

Alternatively, MP captures liposomes, which then cross the BBB. Liposomes have also been conjugated with mannose, transferrin and insulin receptors at the surface of brain capillaries. In particular, the transferrin receptor is necessary as it delivers iron across the BBB. The expression of this receptor in BBB increases during certain pathologies, for instance after stroke. Thus, transferrin-conjugated liposomes successfully targeted the post-ischemic brain endothelium in rats. Another example of a brain-targeting vector is a genetically engineered monoclonal antibody to human insulin receptor, 83-14 Mab. This vector was also used for targeting liposomes with the aid of a reporter gene to the brain.

Nanoparticles

Nanoparticles, used for drug and gene delivery are often composed of insoluble polymer(s). During their formulation, the drug is captured within the precipitating polymer, forming nanoparticles, and is then released upon the degradation of a polymer in the biological environment. The methods for the preparation of nanoparticles commonly employ the use of organic solvents, which may result in the degradation of immobilized drug agents, especially

biomacromolecules. Efficient cell uptake is achieved when the nanoparticle size does not exceed 100–200 nm.

Also, the nanoparticle surface is often modified by PEG that increases its dispersion stability and extends its circulation times in the body. For example, poly(butylcyanoacrylate) nanoparticles [107] were evaluated for the CNS delivery of many drugs. These nanoparticles were coated with PEG-containing surfactants, such as Tween 80. After injection, they localised in the choroid plexus, *via* mater and ventricles, and, to a lower extent, in the capillary endothelial cells. Also, some evidences suggest that increased brain delivery with surfactant-coated poly(butylcyanoacrylate) nanoparticles may be associated with non-specific permeabilisation of BBB and toxicity. Drugs delivered to CNS in these constructs included analgesics (Dalargin, Loperamide), anti-cancer agents (Doxorubicin), anti-convulsants (NMDA receptor antagonist, MRZ 2/576) and peptides.

More recently nanoparticles conjugated with metal chelators, Desferioxamine or D-Penicillamine, were shown to cross the BBB, chelate metals, and exit through the BBB with their complexed metal ions. This method may prove to be useful for reducing the metal load in neural tissue, thus later mitigating the harmful effects of oxidative damage during Alzheimer disease and other CNS diseases.

Nanospheres

Nanospheres are a subset of nanoparticles. Nanospheres [108] are hollow species prepared by microemulsion polymerisation or covering colloidal templates with a thin layer of polymer material followed by template removal. Such nanospheres remained carboxylated; polystyrene nanospheres (20 nm) were evaluated for CNS drug delivery in the vasculature under normal conditions after intravenous injection. However, they extravasated into the brain during cerebral ischemia-induced stress that partially opened the BBB. Such nanospheres may have the potential for imaging agents during ischemia, stroke and other conditions that disrupt the BBB and for the CNS delivery of drugs.

Nanosuspensions

Drug nanosuspensions [109] represent crystalline drug particles often stabilised by non-ionic PEG-containing surfactants or mixtures of lipids. They are manufactured by a variety of techniques such as media milling, high-pressure homogenisation or using emulsions and microemulsions as templates. These procedures often result in irregular shaped, rather polydisperse materials of near micron or sub-micron particle size range. Major advantages of this technology include its simplicity, high drug loading capacity and applicability to many drugs including very hydrophobic compounds.

Nanosuspension surfaces can be modified to increase its delivery to the brain after systemic administration similar to that of regular nanoparticles.

Polymeric micelles

Polymeric micelles [110] (“micellar nanocontainers”) have also been developed as carriers of drugs and diagnostic imaging agents. They form spontaneously in aqueous solutions of amphiphilic block copolymers [111]. They also have a core-shell architecture with a core of hydrophobic polymer blocks (*e.g.*, poly(propylene glycol) (PPG), poly(D,L-lactide), poly(caprolactone), *etc.*) and a shell of hydrophilic polymer blocks (often PEG). The size of polymeric micelles usually varies from 10–100 nm. Their core can incorporate considerable amounts (up to 20–30 % wt) of water-insoluble drugs that prevent premature drug release and degradation. The shell stabilises micelles in dispersion and masks the drug from interactions with serum proteins and untargeted cells. Diffusion helps in the release of the drug from the micelle after reaching the target cells (Figure 8).

Figure 8. A scheme illustrating mechanism of Pluronics action in BBB: (A) inhibiting PgpATPase function in cell plasma membrane; (B) inhibiting respiration in mitochondria resulting in ATP depletion. Both effects combined result in (C) inhibition of the Pgp drug efflux system and (D) transport of the drug to the brain.

For CNS drug delivery, an early study used micelles of Pluronic block copolymers [112] PEG-block-poly(propyleneglycol)-block-PEG (PEG-b-PPG-b-PEG) as carriers. These micelles were conjugated with either insulin as targeting moieties or polyclonal antibodies against brain α_2 -glycoprotein. Both antibody and insulin-vectorised micelles were shown to deliver a drug or fluorescent probe to the brain *in vivo*. Moreover, there was an increase in the neuroleptic activity of a drug (haloperidol) solubilised in the targeted micelles in comparison to a free drug.

Charged molecules can be incorporated by Polyion complex micelles [113] (also referred as “block ionomer complexes”), which are novel nanosystems. Formation is a result of the reaction of double hydrophilic block copolymers, which contains ionic and non-ionic blocks along with macromolecules of opposite charge that includes oligonucleotides, plasmid DNA and proteins or surfactants of the opposite charge. For instance, by reacting trypsin or lysozyme (positively charged under physiological conditions) with an anionic block copolymer, block ionomer was made – PEG-poly(a,b-aspartic acid). These complexes spontaneously assemble into nanosized particles with core-shell architecture. The core has polyion complexes of a biomacromolecule and ionic block of the copolymer. Non-ionic block forms the shell. In the case of surfactant-based complexes, the core is composed of mutually neutralised surfactant ions and polyion chains. It contains hydrophobic domains of surfactant tail groups and has the ability to incorporate water-insoluble drugs. The complexes assume different morphologies that include vesicles and micelles of different shapes based on surfactant and block copolymer architectures.

These versatile nanomaterials can incorporate solutes of different structure with high loading capacity. Furthermore, they can release solutes upon changes in environmental conditions such as pH (acidification), concentration and chemical structure of elementary salt. DNA molecules *in vitro* and *in vivo* were efficiently delivered by nanomaterials.

Advances were made to develop stable polymeric micelles that do not dissociate during circulation in the body. Examples include amphiphilic scorpionlike block copolymers that have low critical micelle concentration (CMC) as well as various types of unimolecular micelles based on amphiphilic star-like macromolecules with covalently bound hydrophobic cores. Core-polymerisation was employed to stabilise micelles of heterotelechelic amphiphilic block copolymers which contained polymerisable groups at the ends of hydrophobic blocks. Wooley *et al.* have developed cage-like nanostructures on the base of polymeric micelles with hydrophobic core and cross-linked anionic shell. Additionally micelles with cross-linked ionic cores were prepared by self-assembly of ionic blocks of double hydrophilic block copolymers with a condensing agent, followed by the chemical cross-linking of ionic blocks [114]. The resulting micelles contain a hydrophilic PEO shell and a

cross-linked hydrophilic ionic core, which is swollen in water and may incorporate hydrophilic drugs as well as imaging agents.

Protein and block ionomers were additionally cross linked with each other to improve the stability of polyion complex micelles with immobilised proteins according to another study. Similarly, in surfactant-based block ionomer complexes, surfactant molecules were chemically linked to each other ("dimerised"), which formed stable vesicles. Altogether, polymeric micelles of various types comprise a versatile platform for the delivery of imaging and therapeutic agents. One should expect further development of these systems for CNS drug delivery.

Nanogels

Nanogels are nanosized networks of cross-linked polymers that often combine ionic and non-ionic chains, such as PEI and PEG or poly(acrylic acid) and Pluronic [115]. Such networks swell in water and can incorporate through ionic interactions of oppositely charged molecules such as oligonucleotides, siRNA, DNA, proteins and low molecular mass drugs. Their loading proceeds with very high capacities (up to 40–60 % wt) which are not achieved with conventional nanoparticles. Individual collapsed nanogel particles do not phase separate and form stable dispersions because of the solubility of PEG chains. Transport of oligonucleotides incorporated in nanogel particles across an *in vitro* model of the BBB was found satisfactory. It was because of the nanogels that the degradation of oligonucleotides decreased during their transport in brain microvessel endothelial cells (BMVEC). The surface of nanogels was modified by either transferrin or insulin, to further enhance delivery across the BBB. *In vivo* studies suggested that nanogel increased brain uptake of oligonucleotides while decreasing its uptake in the liver and spleen. To summarise, nanogels are promising carriers for CNS drug delivery, although they are in relatively early stages of development.

Nanofibres and nanotubes

Nanofibres and nanotubes [116] are carbon vapour grown, self-assembled from peptide amphiphiles or electrospun from most polymer materials. Carbon nanotubes have attracted attention in nanomedicine, even though there are also serious concerns regarding their safety. Electrospun continuous nanofibres are unique. It is because they represent nanostructures in two dimensions and macroscopic structures in another dimension. They are safer to manufacture than carbon nanotubes. They pose less of a risk of air pollution. Electrospun nanofibres of a degradable polymer, poly(lactic-co-glycolic acid) (PLGA) loaded with dexamethasone, have been used for neural prosthetic applications. A conducting polymer, poly(3,4-ethylenedioxythiophene), was deposited onto the nanofibre surface and the coated nanofibres were then mounted on the microfabricated neural microelectrodes, which were

implanted into brain. Electrical stimulation released the drug that induced a local dilation of the coat and increased permeability. Nanotubes and nanofibres can be administered systemically in future, if the toxicity issues are addressed, for example, by appropriate polymer coating. Continuous nanofibres are more likely to be used in implants and also in tissue engineering applications.

1.2.4. Nanoparticle applications in ocular gene therapy

Nanoparticles can serve as carriers for drugs, peptides, vaccines and oligonucleotides and have been successfully delivered to multiple targets including cancerous cells, as well as other diseased tissues. Nanoparticles also have great potential as a strategy for gene therapy. They can be used to treat genetic defects *in vitro* and *in vivo*. Viral vectors have been the preferred mechanism for transfer of nucleic acids into tissues of interest historically, and they have dominated the field for some time. In a phase I/II clinical trial using a recombinant adeno-associated viral vector (rAAV) containing the herpes-simplex-virus thymidine kinase gene to treat hormone-refractory prostate cancer, two patients (out of six) responded positively to therapy [117].

Yet another instance of a successful viral gene therapy treatment comes from a phase I clinical trial using adeno-associated viral vector (AAV) to deliver pigment epithelium-derived factor (PEDF) to the eyes of patients diagnosed with age-related macular degeneration (AMD) and showed a significant level of reduction in neoangiogenesis associated with disease progression. Modified HIV vectors have been used to preserve some retinal function.

Limitations of viral vectors [118]

1. Physical limitations include random integration into the host's genome, immunogenicity of the vector, and limitations in the insert size;
2. Significant toxic side effects can be observed such as stimulation of an immune response, inflammation and neutralising antibodies, which are associated with repeat treatment, and other potentially serious toxic outcomes including death;
3. In addition of literature concerning the use of the most common AAV vectors for direct gene delivery is contradictory on both issues of transduction efficiency and inflammatory response and on the duration and reproducibility of transgene expression;

The lack of a clearly superior viral candidate for future clinical application of gene therapy in the eye combined with the limitations of viral gene therapy mentioned above make the development of an efficacious non-viral vector for the eye of supreme importance.

Liposomes, DNA nanoparticles or combination of both are included into non-viral vectors. Although liposomes are promising, they have shown low transfection efficiency. This can cause significant inflammatory toxicity.

Alternatively, compacted DNA nanoparticles have proven to be a very useful vehicle for gene therapy and meet the majority of the requirements discussed above for a successful vector [119]. There are many different formulations of nanoparticles. They typically contain a segment of DNA or RNA (circular or linear) which is compacted with a polycationic polymer. Their size is quite small, typically ranging from 10–100 nm in diameter. These small particles are taken up at the cell surface and trafficked to the nucleus within a short period of time. The delivery of compacted DNA nanoparticles to the target yields medium to high transfection efficiency; in many cases, expression levels are several-fold greater than those observed after treatment with naked plasmid DNA. These results are dependent on specifics of the nanoparticle formulation, size, or electric charge. Excellent preliminary studies have been undertaken with poly(lactic acid) and poly(lactide co-glycolide) nanoparticles in the retina, and until now far they have not been used for gene transfer to the mammalian retina. Compacted PEG nanoparticles have been used to efficiently transfect post-mitotic cells *in vitro* and *in vivo*.

Advantages of non-viral vectors

1. These nanoparticles can be stably stored under a variety of conditions and concentrations (up to 12 mg ml⁻¹ of DNA);
2. They are tolerant of a wide range of temperatures, salt concentrations and pH;
3. DNA or RNA is protected from DNase or RNase degradation that is a tendency of non-viral vectors;
4. One of the most exciting features of compacted DNA/RNA nanoparticles is their insert capacity. Some DNA-compacted nanoparticles can contain plasmids up to 20 kb and retain full functional competence following *in vivo* administration;
5. Studies in humans and mice showed little to no toxicity in the targeted tissues. Also, they showed modest immune response when a high concentration of the nanoparticles was used;
6. No serious side effects were observed even after repetitive administration of nanoparticles is possible.

Limitations of non-viral vectors

1. Low transfection efficiency;
2. Short duration of action and expression;
3. One of the traditional limitations of non-viral vectors has been passage of the vector across two physiological barriers: the cell membrane and the nuclear membrane. Endocytosis is the most commonly accepted pathway for nanoparticle internalisation into the cytosol. Endocytosis

can be divided into two categories, phagocytosis (which requires specialised cells) or pinocytosis. The latter pathway can be subdivided into macropinocytosis (molecules > 120 nm), caveolin-mediated endocytosis (molecules 60 nm) and clathrin-mediated endocytosis (120 nm). Positively charged nanoparticles use clathrin-mediated endocytosis.

To summarise, compacted-DNA nanoparticle-mediated gene therapy provides a safe, effective and promising system for the delivery of therapeutic genes to target tissues in the eye. They drive very specific and high levels of gene expression and expression that can be sustained for several months. The safe use of compacted DNA nanoparticles in the clinical setting speaks to their viability as a potential treatment strategy for human conditions. The use of this system in the treatment of genetic diseases of the eye is a strong alternative to the existing collection of viral vectors.

1.2.5. Nanoparticles for the treatment of osteoporosis

There are no current effective prevention and treatment methods for this disease; even though the studies have been taking place since years. There are several major barriers that exist for the use of any pharmaceutical agents to stimulate new bone formation. First, the agents can cause non-specific bone formation in undesirable areas. This is because these agents are often delivered in non-specific ways (such as through the mouth, directly into the blood stream, *etc.*). Second, if delivered locally to the tissue around the area of low bone density, they rapidly diffuse to adjacent tissues. This limits their potential to promote prolonged bone formation in targeted areas of weak osteoporotic bone. Even the best strategies to sufficiently increase bone mass (although, to date, still unproven) require at least one year to see any change; for these reasons, this is a time period that is not acceptable, especially for the elderly. Thus for these reasons, nanotechnology will be used (or the design of materials with 10^{-9} m dimensions) to develop novel drug-carrying systems that will specifically attach to osteoporotic (not healthy) bones. Moreover, some of these novel drug carrying systems will then distribute pharmaceutical agents locally in order to quickly increase bone mass.

Nanoparticles offer a high potential for several biomedical applications, including bioanalysis and bioseparation, tissue-specific drug therapeutic applications, gene and radionuclide delivery are based on their unique mesoscopic physical, chemical, thermal, and mechanical properties. To be used effectively in fighting diseases, specific surface chemistry of the nanoparticles need to be tailored in order to have desired biomedical applications. Magnetic nanoparticles are also of interest [120]. Specifically, the main interest in the use of magnetic nanoparticles in biomedical applications is that a homogeneous external magnetic field exerts a force on them, and this is how they can be manipulated or transported to a specific diseased tissue by a

magnetic field gradient. They also have controllable sizes to match their dimensions either that of a virus (20–500 nm), of a protein (5–50 nm) or of a gene (2 nm wide and 10–100 nm long). After removal of the magnetic field, magnetic particles are of interest because they do not retain any magnetism.

Nanotechnology is specifically used here to prolong the release of bioactive agents that efficiently regenerate enough bone for a patient to return to a normal active lifestyle. To be specific, inorganic biodegradable nanoparticles (including ceramics like hydroxyapatite) will be functionalised with bioactive chemicals such as bone morphogenetic protein-2 (BMP-2) that bond to bone of low mass. Bioactive groups like these will be placed on the outer surface of the magnetic nanoparticle systems using various techniques (such as covalent chemical attachment). Once bonded specifically to osteoporotic bone and not healthy bone, magnetic nanoparticle systems will deliver bioactive compounds to locally increase bone mass. Lastly, the outer coating of the embedded nanoparticle systems will be created to have different biodegradation rates for the release of bone-building agents over various time spans. This allows for not only quick bone formation, but also long-term sustained bone regeneration. One potential advantage of formulating hyaluronan (HA) magnetic nanoparticles is that, as the magnetic particles accumulate, *e.g.*, in bone tissue, they can play an important role in detection through MRI to locate, monitor and control drug activities.

1.2.6. Applications of nanotechnology in diabetes

Diabetes mellitus, often referred to simply as diabetes, is a chronic metabolic disorder due to the relative deficiency of insulin secretion and varying degrees of insulin resistance. It is characterised by high circulating glucose. Excessive levels of either molecular oxygen or Reactive Oxygen Species (ROS) lead to an imbalance in the body's normal oxidative metabolism that leads to high glucose levels in the blood (hyperglycemia), resulting in metabolic disturbances (oxidative stress) and chronic complications in diabetes. The management of diabetic conditions by insulin therapy has several drawbacks like insulin resistance. Also, chronic treatment causes anorexia nervosa, brain atrophy and fatty liver. Several research studies are currently ongoing with the aid of nanosize particles to overcome such limitations in diabetes management [121,122].

1.2.6.1. Nanomedicine application in glucose and insulin monitoring

The major problems with conventional finger-prick capillary blood glucose self-monitoring are widely accepted [123]. It is painful (leading to non-compliance) and cannot be performed when the patient is sleeping or driving a motor vehicle (times when the patient is especially vulnerable to hypoglycemia). Since it is intermittent, it can miss dangerous fluctuations in blood glucose concentrations between tests. A new method that uses

nanotechnology to rapidly measure minute amounts of insulin and blood sugar level is a major step toward developing the ability to assess the health of the body's insulin-producing cells.

Microphysiometer

The microphysiometer is built from multiwalled carbon nanotubes, which are like several flat sheets of carbon atoms stacked rolled into very small tubes. It can be used to detect and monitor the response of cells to a variety of chemical substances, especially ligands for specific plasma membrane receptors. The nanotubes are electrically conductive. The concentration of insulin in the chamber can be directly related to the current at the electrode. The nanotubes operate reliably at pH levels characteristic of living cells. Current detection methods measure insulin production at intervals by periodically collecting small samples and then later measuring their insulin levels. The new sensor detects insulin levels continuously. This is possible by measuring the transfer of electrons produced when insulin molecules oxidise in the presence of glucose. When the cells produce more insulin molecules, the current in the sensor increases or *vice versa*, allowing insulin concentrations to be monitored in real-time [124].

Implantable sensor

An implantable sensor capable of long-term monitoring of tissue glucose concentrations by wireless telemetry has been developed for eventual application in people with diabetes. The implantable sensor is designed to give diabetes patients an alternative to finger-sticking or short-term glucose sensors. It also limit dangerous glucose level fluctuations known as glucose excursions. The use of PEG beads coated with fluorescent molecules to monitor diabetes blood sugar levels is very effective. In this method, the beads are injected under the skin and stay in the interstitial fluid. Glucose displaces the fluorescent molecules and creates a glow when glucose in the interstitial fluid drops to dangerous levels. This glow is seen on a tattoo placed on the arm. Sensor microchips are also being developed to continuously monitor key body parameters including pulse, temperature and blood glucose. A chip implanted under the skin would transmit a signal that could be monitored continuously [125].

1.2.6.2. Nanoparticles in the treatment of diabetes

Polymeric nanoparticles

Polymeric nanoparticles have been used as carriers of insulin and the use of biodegradable polymeric nanoparticles for controlled drug delivery has shown significant therapeutic potential. These are biodegradable polymers that are with the polymer-insulin matrix surrounded by the nanoporous membrane

containing grafted glucose oxidase. A rise in blood glucose level triggers a change in the surrounding nanoporous membrane that results in biodegradation and subsequent insulin delivery. Lowering of the pH in the delivery system's microenvironment is due to the glucose/glucose oxidase reaction. This may cause an increase in the swelling of the polymer system, leading to an increased release of insulin. *N,N*-dimethylaminoethyl methacrylate and poly(acrylamide) are the polymers investigated for such applications. This "molecular gate" system is composed of an insulin reservoir and a delivery ratecontrolling membrane which is made of poly[methacrylic acid-g-PEG] copolymer. The polymer swells in size at normal body pH (pH = 7.4) and closes the gates. It shrinks at low pH (pH = 4) when the blood glucose level increases, thus opening the gates and releasing the insulin from the nanoparticles. These systems release insulin by swelling which is caused due to changes in blood pH. The control of the insulin delivery depends on the size of the gates, the concentration of insulin, and the rate of gates' opening or closing (response rate). These self-contained polymeric delivery systems are still under research. Now, the delivery of oral insulin with polymeric nanoparticles has progressed to a greater extent in the recent year [126].

Oral insulin administration by using polysaccharides and polymeric nanoparticles

Polysaccharides are natural biodegradable hydrophilic polymers. These exhibit enzymatic degradation behaviour and good biocompatibility. The development of improved oral insulin administration is essential for the treatment of diabetes mellitus in order to overcome the problem of daily subcutaneous injections. Insulin undergoes degradation in the stomach due to gastric enzymes when administered orally; therefore, insulin should be enveloped in a matrix-like system to protect it from gastric enzymes. This can be achieved by encapsulating the insulin molecules in polymeric nanoparticles. Calcium phosphate-PEG-insulin combination was combined with casein (a milk protein) in one such study. The insulin is protected from the gastric enzymes by the casein coating. Due to casein's mucoadhesive property, the formulation remained concentrated in the small intestine for a longer period, resulting in slower absorption and longer availability in the bloodstream [127].

Insulin delivery through inhalable nanoparticles

Inhalable, polymeric nanoparticle-based drug delivery systems have been tried previously for the treatment of tuberculosis. Such approaches can be directed toward insulin delivery through inhalable nanoparticles. Insulin molecules can be encapsulated within the nanoparticles and can be administered into the lungs by inhaling the dry powder formulation of insulin. The nanoparticles should be small enough to avoid clogging up the lungs but large enough to avoid being exhaled. Such a method of administration allows the direct delivery of insulin molecules to the bloodstream without undergoing

degradation. A few studies have been performed to test the potential use of ceramic nanoparticles (calcium phosphate) as drug delivery agents. Porous hydroxyapatite nanoparticles have also been tested for the intestinal delivery of insulin. Preclinical studies in guinea pig lungs with insulin-loaded poly(lactide-co-glycolide) nanospheres demonstrated a significant reduction in blood glucose level with a prolonged effect over 48 h when compared with insulin solution. Insulin-loaded poly(butyl cyanoacrylate) nanoparticles when delivered to the lungs of rats were shown to extend the duration of hypoglycaemic effect over 20 h when compared with the pulmonary administration of insulin solution. The major factors limiting the bioavailability of nasally administered insulin include poor permeability across the mucosal membrane and rapid mucociliary clearance mechanism which removes the non-mucoadhesive formulations from the absorption site. Mucoadhesive nanoparticles made of chitosan/tripolyphosphate and starches have been evaluated to overcome these limitations. These nanoparticles showed good insulin-loading capacity. It provided the release of 75 % to 80 % insulin within 15 min after administration [123].

1.2.6.3. Applications of nanotechnology in diabetes

Diabetes is one of the major afflictions of modern western society. To date, diabetic patients control their blood-sugar levels *via* insulin introduced directly into the blood stream using injections. This unpleasant method is required since stomach acid destroys protein-based substances including insulin, making oral insulin consumption useless. The new system is based on inhaling the insulin (instead of injecting it) and on the controlled release of insulin into the bloodstream (instead of manually controlling the amount of insulin injected). The treatment of diabetes includes the proper delivery of insulin into the blood stream, which can be achieved by nanotechnology in the following ways:

Development of oral insulin

The production of pharmaceutically active peptides and proteins, such as insulin, in large quantities has become feasible. The oral route is considered to be the most convenient and comfortable means for the administration of insulin for less invasive and painless diabetes management. This leads to higher patient compliance. As hydrophilic drugs cannot diffuse across epithelial cells through the lipid bilayer cell membranes into the bloodstream, the intestinal epithelium is a major barrier to the absorption. Therefore, attention has been given to improving the paracellular transport of hydrophilic drugs. A variety of intestinal permeation enhancers including chitosan have been used for the assistance of the absorption of hydrophilic macromolecules. Hence, a carrier system is needed to protect protein drugs from the harsh environment in the stomach and small intestine, if given orally. Additionally, chitosan nanoparticles enhanced the intestinal absorption of protein molecules

to a greater extent than aqueous solutions of chitosan *in vivo*. The insulin loaded nanoparticles coated with mucoadhesive chitosan may prolong their residence in the small intestine, infiltrate into the mucus layer and subsequently mediate transiently opening the tight junctions between epithelial cells while becoming unstable and broken apart due to their pH sensitivity and/or degradability. The insulin released from the broken-apart nanoparticles could then permeate through the paracellular pathway to the bloodstream, which is its ultimate destination [128].

Microsphere for oral insulin production

The most promising strategy to achieve oral insulin is the use of a microsphere system. It is inherently a combination strategy. The oral drug delivery device for insulin is used to protect the sensitive drug from digestive enzymes and proteolytic degradation in stomach and upper part of gastrointestinal tract. Microspheres act as protease inhibitors by protecting the encapsulated insulin from enzymatic degradation within its matrix as well as permeation enhancers by effectively crossing the epithelial layer after oral administration [129].

Artificial pancreas

An artificial pancreas system is an automated, closed-loop system that combines a continuous glucose monitor, an insulin infusion pump, and a glucose meter for calibrating the monitor. The devices are designed to work together, monitor the body's glucose levels and automatically pump appropriate doses of insulin as determined by a computer algorithm [89]. The development of an artificial pancreas could be the permanent solution for diabetic patients. The concept of its work is simple: a sensor electrode repeatedly measures the level of blood glucose; this information feeds into a small computer that energises an infusion pump, and the needed units of insulin enter the blood stream from a small reservoir. Another way to restore body glucose is the use of a tiny silicon box containing pancreatic beta cells taken from animals. The box is surrounded by a material with a very specific nanopore size (about 20 nm in diameter). These pores are big enough to allow for glucose and insulin to pass through them, but small enough to impede the passage of much larger immune system molecules. These boxes can be implanted in diabetes patients under their skin. This could temporarily restore the body's delicate glucose control feedback loop without the need for a powerful immunosuppressant which may leave the patient at a serious risk of infection. Scientists are also trying to create a nanorobot which would have insulin departed in inner chambers, and glucose level sensors on the surface. When blood glucose levels increase, the sensors on the surface would record it and insulin would be released. Yet, this kind of nano-artificial pancreas is still only a theory. In the artificial pancreas, biosensors are also useful [130].

The nanopump

The nanopump is a powerful device with many possible applications in the medical field. The first major application of the pump, introduced by Debiotech, was insulin delivery. The pump injects insulin to the patient's body at a constant rate, balancing the amount of sugars in his or her blood. The pump can also administer small drug doses over a long period of time.

1.2.7. Nanosystems in inflammation

1.2.7.1. Targeting macrophages to control inflammation

The potential of macrophages for rapid recognition and clearance of foreign particles has provided a rational approach to macrophage-specific targeting with nanoparticles. Macrophages' have the ability to secrete a multitude of inflammatory mediators that allows them to regulate inflammation in many diseases. Therefore, macrophages are potential pharmaceutical targets in various human and animal diseases. Although macrophages are capable of killing most of the microbes, many microorganisms (*Toxoplasma gondii*, *Leishmania sp*, *Mycobacterium tuberculosis*, and *Listeria monocytogenes*) have developed the potential ability to resist the phagocytosis activity of macrophages. These pathogens subvert a macrophage's molecular machinery designed to kill them and come to reside in modified lysosomes. Therefore, nanoparticle-mediated delivery of antimicrobial agent(s) into pathogen-containing intracellular vacuoles in macrophages could be useful and help to eliminate cellular reservoirs [131]. This system can be used to achieve therapeutic drug concentrations in the vacuoles of infected macrophages. It is also used in the reduction of side effects associated with the drug administration and the release of pro-inflammatory cytokines. Poly(alkyl cyanoacrylates) (PACAs) nanoparticles have been used as a carrier for targeting anti-leishmanial drugs into macrophages. This nanomaterial did not induce interleukin-1 release by macrophages [132]. Therefore, in chronic diseases, similarly designed nanosystems could be very useful in targeting macrophage infections.

The antifungal and anti-leishmanial agent amphotericin B (AmB) has been complexed with lipids-based nanotubes in order to develop a less toxic formulation of AmB. Gupta and Vyas [133] formulated AmB in trilaurin based nanosize lipid particles (emulsomes) stabilised by soya phosphatidylcholine as a new intravenous drug delivery system for macrophage targeting. Nanocarrier-mediated delivery of macrophage toxins has proved to be a powerful approach in getting rid of unwanted macrophages in gene therapy. Also nanocarrier-mediated delivery of macrophage toxins is useful in other clinically relevant situations such as autoimmune blood disorders, T cell-mediated autoimmune diabetes, rheumatoid arthritis, spinal cord injury, sciatic nerve injury, and restenosis after angioplasty. Alternatively,

nanoparticles with macrophage-lethal properties can also be exploited. Exploiting a variety of macrophage cell receptors as therapeutic targets may prove a better strategy for antigen delivery as well as targeting with particulate nanocarriers.

1.2.7.2. Targeting inflammatory molecules

Many cell adhesion molecules have been discovered in the past two decades. Cell adhesion molecules are glycoproteins found on the cell surface which act as receptors for cell-to-cell and cell-to-extracellular matrix adhesion [134]. These cell adhesion molecules are divided into four classes called integrins, cadherins, selectins, and the immunoglobulin superfamily. These molecules are required for the efficient migration of inflammatory cells such as neutrophils and monocytes into inflamed organs and the generation of host response to infections. However, there is considerable evidence that excessive migration of neutrophils in inflamed lungs leads to exaggerated tissue damage and mortality. Therefore, a major effort is underway to fine tune the migration of neutrophils into inflamed organs. Recent advancements of the understanding of the cell adhesion molecules has impacted the design and development of drugs (*i.e.* peptide, proteins) basically for the potential treatment of cancer, heart and autoimmune diseases [135,136]. These molecules have important roles in diseases such as cancer [137], thrombosis and autoimmune diseases like type-1 diabetes. The arginylglycylaspartic acid (RGD) peptides have been used to target integrins $\alpha\beta3$ and $\alpha\beta5$, and peptides derived from the intercellular adhesion molecule-1 (ICAM-1) have been used to target the $\alpha\beta2$ integrin. Peptides derived from $\alpha\beta2$ can target ICAM-1 expressing cells. Cyclic RGD peptides have been conjugated to paclitaxel (PTX-RGD) and doxorubicin (Dox-RGD4C) for improving the specific delivery of these drugs to tumour cells. Mice bearing human breast carcinoma cells (*i.e.*, MDA-MB-435) survived the disease when treated with Dox-RGD4C, while all of the untreated control mice died because of the disease [138]. $\alpha\beta3$ and $\alpha\beta5$ integrins on the tumour vasculature during angiogenesis are targeted by this conjugate.

Extracellular regulated kinases (ERK) may regulate apoptosis and cell survival at multiple points that include increasing p53 and BAX action, increasing caspase-3 and caspase-8 activities, decreasing Akt activity, and increasing the expression of tumor necrosis factor alpha (TNF- α) [139]. Our research group is investigating the interaction of RGD-Rosette nanotubes (RGD-RNT) to $\alpha\beta3$ integrins, following cell signalling through P38 kinases and its function in human lung epithelial cells, and bovine and equine neutrophil migration. Cyclo(1,12)PenITDGEATDSGC peptide (cLABL peptide), derived from the I-domain of the α subunit of leukocyte function-associated factor-1 (LFA-1), is known to bind ICAM-1. The cLABL peptide has been conjugated with methotrexate (MTX) to give the MTX-cLABL conjugate. Because ICAM-1 is upregulated during tissue inflammation and several different cancers, this conjugate may be useful for directing drugs to inflammatory and tumour cells.

The anti-inflammatory activity of MTX is due to the suppression of the production of anti-inflammatory cytokines such as (interleukin-6) IL-6 and (interleukin-8) IL-8. Thus, the activity of MTX-cLABL conjugate was compared to MTX in suppressing the production of these cytokines in human coronary artery endothelial cells stimulated with TNF- α . MTX-cLABL is more selective in suppressing the production of IL-6 than IL-8, which is opposite to MTX. PLGA nanoparticles coated with cLABL peptides have also been shown to upregulate ICAM-1 [140]. More detailed information on the mechanism(s) of internal isolation and intracellular trafficking of cell adhesion molecules is required to be exploited for delivering drug molecules to a specific cell type or for the diagnosis of cancer and other diseases (heart and autoimmune diseases).

1.2.8. Nanotechnology in hypertension [141]

For the flow of blood through arteries, it requires some force, which is measured in terms of blood pressure. When the flow of blood changes, blood pressure may elevate or lower according to the flow. When blood pressure is elevated, the heart has to work harder than normal so that the proper flow of blood through blood vessels is possible. The blood pressure in the arteries is elevated; this phenomenon is known as hypertension, high blood pressure or arterial hypertension. It is a chronic disease. Blood pressure at rest is within the range of 100–140 mm Hg systolic (top reading) and 60–90 mm Hg diastolic (bottom reading) in normal conditions. If the blood pressure is at or above 140/90 mm Hg, it is termed high blood pressure. A blood pressure greater than 180/110 mm Hg is termed a 'hypertensive crisis.' Headaches, light-headedness, vertigo, tinnitus, and altered vision are the symptoms of hypertension. Physical examination can be performed by examining optic fundus in the back of the eye for the presence of hypertensive retinopathy.

1.2.8.1. Cause

Hypertension is classified as either primary hypertension or secondary hypertension. Primary hypertension is caused by high salt intake, high alcohol consumption, and the use of high fat products. Also, stress plays a minor role. For adult primary hypertension, early life events like low birth weight, maternal smoking, lack of breast feeding, *etc.* can be implicated as risk factors. Some risk factors for secondary hypertension are endocrine conditions, sleep apnoea, obesity, excessive liquorice, illegal drugs, and herbal medicines. Hypertension may result from a complex interaction of genes and environmental factors.

1.2.8.2. Diagnosis and drugs

The diagnosis of hypertension is made on the basis of a persistently high blood pressure. Some typical tests performed in hypertension are given in Table 2.

Table 2. The tests used in the diagnosis of hypertension

System	Tests
Renal	Microscopic urinalysis, Proteinuria, BUN, Creatinine
Endocrine	Serum sodium, calcium, potassium, TSH
Metabolic	Fasting blood glucose, HDL, LDL, Total cholesterol, triglycerides
Others	Haematocrit, Chest radiograph, Electrocardiogram

Source: Harrison's principles of internal medicine

For the treatment of hypertension, a health care provider (Doctor) will advise to lose weight, stop smoking and start exercising. Some medicines for high blood pressure are:

- Diuretics – These pills help the kidneys to remove salt from blood, *e.g.* Chlorthalidone, Hydrochlorothiazide.
- Beta blockers – These pills will help heart beat slow and lower the pressure.
- Angiotensin-converting enzyme inhibitors (ACE Inhibitors) – These inhibitors make the blood vessels relax, so that the blood pressure lowers.
- Angiotensin II receptor blockers- These blockers work the same as ACE Inhibitors, *e.g.* Olmesartan, Medoxomil.
- Calcium channel blockers – These blockers stop calcium from entering the cells that will relax blood vessels.
- Renin Inhibitors – These newer types of medicines act by relaxing blood vessels to control blood pressure.
- Sometimes, patients have to take more than one drug/medicine to control blood pressure. These medicines may have several side effects such as:
 - Cough, Diarrhoea
 - Erection problems
 - Feeling tired, weak, nervous
 - Skin rash
 - Weight loss or gain without trying

1.2.8.3. Nanoparticle based hypertension treatment

In the pulmonary artery, when pulmonary vascular resistance increases and the right ventricle fails, this is known as pulmonary arterial hypertension (PAH). In the treatment of PAH, currently the most effective drugs have a very short life-cycle, difficult rendering systemic administration. As a future prospective, these difficulties can be overcome by using nanotechnology.

PAH, asthma and chronic obstructive pulmonary disease (COPD) share pathological features, such as inflammation and smooth muscle contraction. No existing drugs have the potential to deal with these pathomechanisms. Using a combination of long active vasoactive intestinal peptide (VIP) analogues with drug delivery systems can provide clinically useful agents for the treatment of pulmonary hypertension in asthma and COPD. Nanoparticles used as drug delivery systems:

Liposomes – Liposomes are concentric nanosized artificial vesicles with a spherical shape that can be produced from natural phospholipids and cholesterol. Liposomes size should be big enough to carry a sufficient amount of drugs. After the encapsulation of drugs, liposomes should protect their payload from degradation in the microenvironment of the pulmonary artery and a controlled, retarded release of the drugs.

Micelles – Self-assembled supermolecular structures consisting of amphiphilic macromolecules are micelles. When coming into contact with water, these amphiphilic molecules assemble to form nanoscopic core-shell structure that can be used as a reservoir for hydrophobic drugs. PEG-based cationic micelles are used for intratracheal gene transfer to the lung of rats with monocrotaline-induced PAH. A remarkable therapeutic efficiency was achieved without compromising biocompatibility using these nanoparticles.

Polymeric nanoparticles – From previous studies of microspheres and submicron particles, the use of polymeric nanoparticles was derived. Generating aqueous droplets in the range of 1–3 μm provides the chance to carry numerous nanoparticles in one droplet. The feasibility of polymeric microspheres as an inhalable carrier for prostaglandin E1 (PGE1) for the treatment of PAH was studied using PLGA microspheres. Another approach for the treatment of PAH is using combination of PEO-*w*-lactic acid (PELA) and hydrophilic prodrug (PROLI/NO) has not yet been successful, even using nanoparticles. Further studies are continuing for the success of this method.

In another approach, bioabsorbable polymeric nanoparticles formulated from a PEG-PLGA enabled the delivery of the nuclear factor κB (NF- κB) decoy oligodeoxynucleotide, which is directed against NF- κB binding site in the promoter region. This study in a rat model of monocrotaline-induced PAH showed that these nanoparticles prevent monocrotaline-induced NF- κB activation.

Nanocrystals and nanoprecipitates – Nifedipine (hypertension drug) nanoparticles are co-precipitated with steric acid to form a negative surface charged colloid. Destabilisation of the colloid was performed by using NaCl to disrupt the electrostatic repulsion between the particles in order to achieve agglomerated nanoparticles of a controlled size. Such nanoparticles are well suited for pulmonary delivery. Tranilast, an anti-allergic agent that has a potential for the co-treatment of pulmonary inflammation during PAH, can be processed as wet-milled crystalline particle that possess a mean diameter of 122 nm.

1.2.9. Biomedical nanotechnology

Three applications of nanotechnology are particularly suited to biomedicine, namely diagnostic techniques, drugs and prostheses and implants [142]. Interest is booming in biomedical applications for use outside the body, like diagnostic sensors and “lab-on-a-chip” techniques that are suitable for analysing blood and other samples, and for inclusion in analytical instruments for research and development (R&D) on new drugs. Many companies are developing nanotechnology applications for anticancer drugs, implanted insulin pumps, and gene therapy for inside the body. Many other researchers are working on prostheses and implants which include nanostructured materials.

The applications include:

1. Sensors needed for medical and environmental monitoring and for preparing pure chemicals and pharmaceuticals (Figure 9).
2. Light and strong materials for defence, aerospace, automotive and medical applications.
3. Lab-on-a-chip diagnostic techniques.
4. Sunscreens with ultraviolet-light absorbing nanoparticles.
5. The report also said that the following applications are expected in the next decade:
6. Longer-lasting medical implants.
7. The capability to map an individual’s entire genetic code almost instantaneously.
8. The ability to extend life by 50 % from present expectations.

Figure 9. A bioengineered cell rover swims through the human body in this artist's impression, performing drug delivery, waste removal, and cellular repair. It has an internal frame, skin panels, internal organelles, a propulsion scheme, and a control system.

Nanodrugs

Nanostructured materials becoming new drug compounds are not expected by the pharmaceutical companies. However, carbon buckyballs and nanotubes might be useful as drug delivery vehicles because their nanometre size enables them to move easily inside the body. The active compound might be inserted in a nanotube or bonded to a particle's surface. Also, other types of nanopowders or biomolecules are also useful. For example, albumin-bound paclitaxel (ABI-007) is a new nanoparticle delivery system for an established anticancer drug. ABI-007 is 130 nm long and consists of an engineered protein-stabilised nanoparticle that contains paclitaxel, which is used to treat breast, bladder, and more than a dozen other cancers. New delivery systems like these combine a drug with an artificial vector that can enter the body and move in it like a virus.

Cosmetics based on QDs are already sold in large quantities. Nanophase Technologies Corp. (Romeoville, IL) produces nanocrystalline materials like zinc oxide for use in sunscreens and other products. Particles between 3–200 nm are used for different purposes. These particles are protective and cause minimal damage to DNA in sunlight. QDs manufactured between 3–5 nm are suitable for binding specific biomolecules.

QDs are luminescent particles, more stable than the organic dyes used now and are non-toxic.

Prostheses and implants

Nanotechnology has many applications in tissue engineering as well. Researchers put a biological material in a mould, a straitjacket as it were, which forces it to assume the shape of a body part, such as a hipbone to replace damaged or missing tissue by a similar equivalent material. Biomimetic nanostructures start with a predefined nanochemical or physical structure. A nanochemical structure may be an array of large reactive molecules attached to a surface, while a nanophysical structure may be a small crystal. Using these nanostructures as seed molecules or crystals, a material will keep growing by itself according to the researchers. Other groups want to apply nanostructured materials in artificial sensory organs such as an electronic eye, ear, or nerve.

1.3. CONCLUSION

Particulate colloidal carriers (*i.e.* liposomes or nanospheres or nanocapsules) were developed. They are now proposed as a new approach for drug administration and vaccines. Tumour blood vessels present, indeed, several abnormalities in comparison with normal physiological vessels, often including a relatively high proportion of proliferating endothelial cells, an increased tortuosity, a deficiency in pericytes and an aberrant basement membrane formation. Long circulating carriers to diffuse into the tumoural tissue because of the resulting enhanced permeability of the tumour vasculature.

During neurological diseases, BBB permeability is increased dramatically, and it has been hypothesised that drug carrier systems such as polymeric nanoparticles could cross the BBB and penetrate into the central nervous system.

PEGylated PACA nanoparticles are one such system and have been shown to dramatically penetrate the brain during experimental allergic encephalomyelitis (EAE).

In a similar manner, tamoxifen-loaded onto nanoparticles was found to be able to reduce experimental autoimmune uveitis (EAU) by entering into the ocular tissues and delivering the drug specifically to the inflammatory cells. It is also possible using those systems to improve the intracellular penetration of non-intracellularly diffusible and/or unstable compounds if the physicochemical characteristics of colloidal carriers allow certain tissues or cells to be targeted. This is illustrated by the delivery, with the aid of nanotechnologies, of antisense oligonucleotides against junction oncogenes which are found in cancers such as certain leukaemias, Ewing sarcoma and thyroid papillary carcinomas. Tumours originate from a chromosomal translocation and are therefore only found in the tumour cells, making them interesting targets. Probably because of their short biological life and limited cellular uptake, successful results have never been obtained with antisense

oligonucleotides directed against junction genes on solid tumours *in vivo*. Because they are delivered to the right place in the body, nanotechnologies open new and exciting perspectives for the discovery of medicines and are more efficient. However, the preparation methods are often complex and need the use of organic solvents and surfactants that clearly represent a limitation for further medical applications.

We recently developed a new concept to obtain self-assembling nanoparticles in water without requiring any organic solvent or surfactant to overcome these inconveniences. These nanoparticles are made in a simple way, by mixing two aqueous solutions, a polymer of poly- β -cyclodextrins (p β CD) and a dextran bearing alkyl side chains (DM). When these solutions are mixed, the hydrophobic alkyl chains made up of dextran spontaneously form inclusion complexes with CDs, thus forming a molecular superstructure. The structure of these nanoparticles is a core rich in CD and a corona essentially made of dextran, which sterically stabilises them. Free CD entraps drugs or other molecules inside the cores.

With nanotechnology, it is possible to achieve efficient targeting and movement of drugs across barriers. Few challenges which can be encountered will be characterisation of molecular targets and the expression of molecules specifically in the targeted tissues. Understanding the fate of the drug after being delivered to the targeted organ will further improve the efficiency of nanosystems to be used in inflammation.

In diabetes management, the use of a microphysiometer or implantable sensors allows the monitoring of insulin concentrations in real time. For the treatment of diabetes, polymeric, polysaccharide and inhalable nanoparticles can be used as carriers for the administration of insulin. Applications of nanotechnology in developments of oral insulin, the use of microspheres for oral insulin production and in development of artificial pancreas will be effective in the treatment of diabetes. With the help of nanopumps, it will be possible to administer small drug doses over a long period of time at a constant rate, which will also help in balancing the amount of sugars in blood.

Polymeric nanoparticles provide clinically useful agents for the treatment of PAH, in which currently used potential drugs have a limitation of significantly short half-life, rendering systemic administration difficult. The use of nanoparticles in the form of liposomes, micelles, nanocrystals and nanoprecipitates can help to overcome these difficulties and may prove to be an efficient treatment for pulmonary hypertension in asthma and COPD.

All in all, the application of original physicochemical concepts to the formulation of particulate colloidal carriers may lead to efficient systems' development for the controlled administration of drugs to specific tissues, cells or even intracellular compartments.

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Chapter

2

NANODRUG ADMINISTRATION ROUTES

**Letícia Marques Colomé*, Eduardo André Bender,
and Sandra Elisa Haas**

Federal University of Pampa, Uruguaiana, Brazil

*Corresponding author: leticiacolome@unipampa.edu.br

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2.1. INTRODUCTION

Nanoparticles are colloidal structures less than 1 μm in size that have received considerable attention as drug delivery systems. Many drugs and other molecules can be carried in nanoparticles, and these systems can improve pharmacological effectiveness. Obstacles to achieving successful drug therapy are often linked to problems with drug bioavailability. Bioavailability depends on the administration route employed and the absorption and metabolism of the drug. In general, the choice of the delivery route includes aspects such as patient acceptability, the characteristics of the drug, and the accessibility and/or effectiveness of the drug regarding the site of application. Among the different drug delivery routes, the oral route is the most frequently used. However, nasal, ophthalmic, parenteral, dermal, transdermal, pulmonary, and others have also been considered for drug-loaded nanoparticle administration.

According to the administration route used, variations in the pharmacological effects of drug-loaded nanoparticles can occur. Many of these effects are related to physiological and physicochemical situations such as drug transport and metabolism, receptor affinity, membrane permeability, protein binding, gene regulation, and protein expression. The type of colloidal carrier system must also be taken into consideration, since many systems are used for drug transport, *e.g.* polymeric nanoparticles, liposome vesicles, cyclodextrins, and dendrimers. This review focuses on the different administration routes used for nanosystems in drug therapy, especially considering polymeric nanoparticles.

2.2. ORAL DRUG DELIVERY

The oral route is the most widely used pathway for drug delivery. It offers innumerable advantages in comparison to other routes for the patient, such as convenience, painless administration, and self-application, resulting in high compliance. The gastrointestinal tract provides a large surface area for absorption (300–400 m^2), an excellent blood supply, and extensive residence time, which are advantageous for drug absorption, especially for those with good solubility and permeability [1,2]. However, most drugs have problems of stability, permeability or solubility in the gastrointestinal tract, which results in low bioavailability, erratic absorption, large variations in *intra*- and *inter*-subject pharmacokinetics, and a lack of dose proportionality. Therefore, many new therapeutic drugs cannot be developed for use as conventional oral formulations, due to the inhospitable environment found in the gastrointestinal tract, such as high metabolic activity, pH variations, and the presence of a mucus layer.

In order to circumvent the limitations associated with the absorption process, attention has shifted to nanotechnology approaches. The encapsulation of drugs (or candidates), peptides, and proteins in polymeric nanoparticles have been used to improve the apparent water solubility, to enhance the intestinal permeability (mainly by the gut uptake), to control drug delivery, and to protect the drugs from gastrointestinal enzymes and local pH.

2.2.1. Improving drug solubility

Recent reports estimate that at least 40 % of new drug candidates are poorly soluble in water, resulting in low bioavailability. A great number of delivery systems have been developed to increase the oral bioavailability of these compounds, by increasing the dissolution rate and/or by increasing the dissolved drug levels [3]. Spironolactone is a specific aldosterone antagonist which is used as a potassium sparing diuretic in pediatric patients, but shows incomplete oral absorption because of its low solubility and slow dissolution rate. Therefore, spironolactone-loaded poly(ϵ -caprolactone) (PCL) nanocapsules were developed to increase the solubility of this drug [4]. Initially, a solubility study of spironolactone was performed in Labrafil®, Labrafac® CC, Labrafac® Hydro, Myritol®, and olive oil. The solubility was greater in a mixture of C₈/C₁₀ ethoxylated glycerides (Labrafac® Hydro), resulting in a higher encapsulation efficiency, a parameter which is influenced by the solubility of the substance in the oily core of nanocapsules. The percentage of dissolved spironolactone from nanocapsules was 100.51 % in 20 min after dilution of the formulation in simulated gastric fluid. In another study, to improve the intrinsic solubility of the poorly water-soluble drug efavirenz, polymeric micelles based on a mixture of poloxamine (Tetronic T304 and T904) and poloxamer (Pluronic F127) were developed [5]. Solubility factors were calculated by the relation of the apparent solubility of the drug in micelles and its intrinsic solubility in buffer. The highest values for this parameter were obtained with the T904/F127 mixture (75 : 25), since these were the most hydrophobic micelles.

The development of nanocapsules is advantageous not only for drugs but also for organochalcogen compounds. Diphenyldiselenide is a selenoorganic compound that is poorly soluble in water, presenting a log *D* value of 3.13 [6]. Thus, nanocapsule suspensions containing 1.56 and 5.0 mg ml⁻¹ of the drug were prepared by nanoprecipitation. Using canola oil as the oily core, the encapsulation rate was close to 100 %. The intragastric administration of the drug in mice induced concentrations in urine and adipose tissue higher for the diphenyldiselenide nanocapsules than for the free compound, and the opposite was observed in the feces. The results showed that organochalcogen solubilization in nanocapsules improved its bioavailability.

For class II compounds of the Biopharmaceutics Classification System (BCS) (low solubility and high permeability), nanotechnology has been extensively

applied allowing increased solubility of the compound, reclassifying it as class I (high solubility and high permeability) [7-9]. Celecoxib is a non-steroidal anti-inflammatory drug for pain and inflammation which is classified as a class II compound. Ethylcellulose:casein nanoparticles were prepared by microfluidization and subsequent spray-drying [8]. *In vitro* non-sink dissolution of celecoxib from nanoparticles in a fasted duodenal model solution showed rapid dissolution, reaching the terminal value within the first minute. On the other hand, bulk celecoxib crystals take at least 1 h to reach their maximum concentration. *In vivo* pharmacokinetic testing in dogs and humans showed that time of maximum concentration ($T_{\max}$) from nanoparticles and resuspended nanoparticles was twice as fast as the free drug. The bioavailability was 25 % and 75 % for free celecoxib and the nanoparticles, respectively. Since nanoparticles have a higher surface area and a shorter drug diffusion distance for release, high concentrations of the dissolved drug are found at the dissolution site, *i.e.* the gut.

Felodipine is a drug which exhibits poor oral bioavailability (15 %) due to limited aqueous solubility and extensive first pass metabolism. A 3^2 factorial design was performed to evaluate the influence of felodipine, poly(D,L-lactic acid) (PLA) and Pluronic F-68 on nanoparticle characteristics [9]. The optimized polymer (1 : 20) and surfactant concentration (1.5 %) resulted in nanoparticles presenting the better characteristics in terms of encapsulation efficiency, particle size and zeta potential. Around 60 % and 30 % of the free and nanoencapsulated drug, respectively, were released in intestinal medium, and mathematical modeling suggested a first order release. Intra-gastric administration of the free drug and felodipine nanoparticles in hypertensive rats revealed that the drug delivery system normalized blood pressure and maintained normal levels for up to 3 days. These results could be attributed to enhanced bioavailability due to direct uptake by Peyer's patches in the intestine, as well as the sustained release of felodipine from the polymeric matrix.

2.2.2. Improving drug permeability

Chitosan (CS) and its derivatives facilitate nanoparticle uptake and enhance the permeability of drugs *via* two mechanisms: *i)* mucoadhesion, by the interaction of their positive surface charge with the anionic components of the glycoprotein on the surface of epithelial cells and *ii)* tight junction opening, thus increasing paracellular transport [10,11]. Thiolated CS coated polymethacrylate nanoparticles were developed using a non-hazardous organic solvent method and two molecular weights of CS (20 and 50 kDa). The apparent permeability coefficient was similar for both formulations, and the nanoparticles were 30-fold better than the free drug regarding their ability to disrupt the membrane of Caco-2 cells [11]. The relative bioavailability of docetaxel-loaded nanoparticles prepared with 20 kDa CS was 68.9 % in comparison to non-encapsulated docetaxel after oral administration in Wistar

rats. The half-life and area under the plasma concentration time curve from zero (0) hours to infinity (∞) ($AUC_{0-\infty}$) values for the free and nanoencapsulated drug increased along with permeability and bioavailability for nanoparticulate docetaxel [12].

The mechanism of the absorption and permeability of 7-ethyl-10-hydroxycamptothecin (Sn38)-loaded CS-coated poly(lactic-co-glycolic acid) (PLGA) nanoparticles was investigated. Sn38 is a class IV compound in the BCS, and P-glycoprotein (Pgp) efflux contributes to its low permeability. CS-coated and uncoated nanoparticles were prepared by the oil-in-water emulsion solvent evaporation method and showed a similar particle size, encapsulation efficiency, and drug loading content, but the zeta potential values were influenced by coating with CS. The inhibition of Pgp by verapamil showed that the drug absorption rate was constant and the effective permeability coefficient was similar to the values obtained with CS-coated nanoparticles, suggesting that Sn38 was not recognized by Pgp, but other mechanisms may be involved. An investigation into transcytosis pathways in Caco-2 cells showed a specific decrease in uptake of 6-coumarin-labeled CS-coated PLGA nanoparticles by the use of sucrose (an endocytosis inhibitor), indicating that these particles use the clathrin-mediated endocytic pathway to escape from Pgp recognition [13].

Aiming to improve the paracellular permeability of hydrophilic compounds, 2-dodecyl-1-yl-succinic anhydride groups were attached to CS to form lauryl succinyl chitosan (C_{12} -CS). Insulin-loaded C_{12} -CS nanoparticles were prepared by ionic gelation. The transepithelial electrical resistance values were reduced in the presence of both the native and derivatized CS nanoparticles. The data were corroborated by confocal laser scanning microscopy images and showed that the interruption of ZO1-type tight junctions occurred with both nanoparticle formulations [14].

Curcumin also shows poor solubility and permeability which limits its therapeutic use, despite its numerous pharmacological activities. To counteract these limitations and improve its biological availability, PLGA nanospheres were developed. Curcumin-loaded PLGA nanospheres prepared by modified solid-in-oil-in-water solvent evaporation technique showed an increase in solubility and sustained drug release, especially in intestinal juice, which can be attributed to the fact that curcumin is primarily absorbed in the gut. In the rat gut, the residence time was shorter for nanospheres in comparison to free curcumin, due to the inhibition of Pgp by nanoparticles observed in the *in situ* single-pass intestinal permeability. After intragastric administration to rats, the AUC of curcumin and curcumin-loaded PLGA nanospheres were 367 and 2066 min $\mu\text{g mL}^{-1}$, respectively, resulting in a relative bioavailability of 563 % [15].

One of the pathways for nanoparticle uptake in the gut is through Peyer's patches, which is the gut-associated lymphoid tissue that contains M cells.

Through this way, nanoparticles can be delivered to the lymphatic system and then to the circulation. Gemcitabine chlorhydrate loaded PLGA nanoparticles were formulated by a multiple emulsification solvent evaporation method [16]. The permeability of the nanoencapsulated drug was 6.38 times higher than the free drug, evaluated using a Caco-2 cell monolayer. This increase can be attributed to uptake by Peyer's patches, which was confirmed by confocal microscopy in rat intestinal villi. In agreement, gemcitabine-loaded PLGA nanoparticles showed a 3.24-, 3-, and 21.47-fold increase in maximum drug concentration ($C_{\max}$), half-life, and relative bioavailability, respectively, in comparison to the drug solution after oral administration to rats. In another study [17], an *in situ* intestinal perfusion technique was used to assess the permeability of vancomycin-loaded PLGA nanoparticles. Different drug:polymer ratios were studied, and the permeability was superior for all these formulations in comparison to the free drug.

Active drug targeting is a strategy used to improve the bioavailability of paclitaxel, a BCS class IV compound, by folic acid functionalized PLGA nanoparticles [18]. Paclitaxel transport across Caco-2 cells was significantly increased by the nanoencapsulation (by approximately 8-fold). The intracellular accumulation was also higher for folic acid-PLGA nanoparticles than for free paclitaxel, and it was time dependent, with peak concentrations up to 6 h after incubation. Another strategy used to improve PLGA nanoparticle permeability is the presence of a stabilizer. Sonaje and co-workers used didodecyldimethylammonium bromide (DMAB) and poly(vinyl alcohol) (PVA) to improve the permeability of ellagic acid [19]. The positive zeta potential, influenced by the presence of DMAB, was substantial enough to improve rat intestinal permeability.

2.2.3. Improving drug stability in the gastrointestinal tract

To evaluate whether encapsulation in CS-tripolyphosphate (TPP) nanoparticles enhances the gastrointestinal stability of green tea catechin (-)-epigallocatechin gallate, nanoparticles were administered to mice and catechin concentrations were measured in gastric and intestinal samples [20]. The stability of catechin in nanoparticles were significantly increased by 1.5- and 2.5-fold in the stomach and in the intestinal juice, respectively, when compared with free catechin. The plasma level was 1.5 times higher for the encapsulated drug than non-encapsulated drug. These results can be attributed to enhanced exposure of catechin in the jejunum due to the stability increasing effect of CS-TPP nanoparticles.

In the same way, CS nanoparticles prepared by an ionic gelation method using TPP or hydroxypropyl methylcellulose phthalate as the complexing agent demonstrated the advantageous use of a pH-sensitive polymer to improve encapsulated insulin stability [21]. Insulin in solution undergoes total degradation within 5 min in simulated gastric fluid. In CS-TPP nanoparticles,

only 10 % of the insulin was protected after 30 min, while 40 % of the drug was protected after 120 min with CS-hydroxypropyl methylcellulose phthalate nanoparticles. The intestinal mucosal adhesion and uptake of these particles in rats showed higher values in comparison to the free drug. Both insulin-loaded nanoparticle formulations showed significant hypoglycemic effect in rats after oral administration. Insulin-loaded CS-TPP nanoparticles decreased glycemia by 3.5-fold, whereas for the insulin-loaded CS-hydroxypropyl methylcellulose phthalate nanoparticles, the decrease was 9.8-fold in comparison to the free drug.

Polymeric micelles have been developed to improve the stability, solubility, and bioavailability of docetaxel [22]. The mixed micelles were prepared with poly(ethylene glycol) (PEG)-PLA, D- α -tocopheryl, PEG 1000, succinate, and stearic acid grafted with CS oligosaccharide, using a thin film hydration method. After dilution of the micelles in simulated gastric fluid without pepsin (pH 1.6) and simulated intestinal fluid without trypsin (pH 6.5), the particle size showed little modification during 12 h of incubation. At higher pH, the size was significantly increased after 12 h. The pharmacokinetic parameters after oral administration of free docetaxel and docetaxel incorporated into micelles demonstrated a significant 3-fold increase in $C_{\max}$ after micelle administration, and the peak was reached in a quarter of the time compared to after administration of the free drug. The relative bioavailability was 2.5-fold higher.

2.2.4. Oral controlled release

Countless polymers can be used in the nanoparticle composition to provide sustained release. Among them, Eudragit® stands out. Eudragit® is the trade name of copolymers derived from esters of acrylic and methacrylic acids, which have numerous applications depending on their functional groups. This versatility makes them extremely used in the pharmaceutical industry. Polymethacrylates are non-biodegradable polymers and are therefore not suitable for parenteral use [23].

Eudragit®-based nanosuspensions were developed to control glimepiride release and improve its solubility [24]. Eudragit® RLPO was chosen because it forms positively charged submicron particles. The drug:polymer ratios used (1:5 – 1:40) influenced glimepiride release evaluated by the dialysis bag diffusion technique using phosphate buffer solution (pH 6.8) as medium. All formulations showed a biexponential release profile. In this case, initial release can be attributed to the burst effect, which is due to rapid release of the drug adsorbed on the particle surface, while the second phase is slow and depends on the characteristics of the polymeric matrix. Eudragit® RLPO is pH independent and undergoes swelling in aqueous media with a consequent increase in permeability and release of the drug. Formulations prepared with a 1 : 40 drug : polymer ratio showed a slower release rate with 95 % and 96 % of the drug released in 24 h, following a Fickian release mechanism.

Another example of a cationic polymer of the trade name Eudragit® is the Eudragit® RS. Considering its mucoadhesive features, Wu and co-workers [25] developed insulin-loaded PLGA-Eudragit® RS nanoparticles which were used to fill hydroxypropyl methylcellulose phthalate-coated capsules. The shake-flask method was used to evaluate insulin release in two media: simulated gastric fluid (pH 1.2) and simulated intestinal fluid (pH 7.4). The charge interaction between insulin and Eudragit® RS were responsible for total insulin release in acidic pH medium and only 50 % in pH 7.4 medium in less than 1 h.

Due to their ability to dissolve only in an environment where the pH is greater than 7.0 or 6.0, pH-sensitive Eudragit® S100 and Eudragit® L100, respectively, were used to prepare papain-loaded nanoparticles using the water-oil-water emulsion solvent evaporation method [26]. The release rate of papain was 20.71 % (Eudragit®L100) and 13.01 % (Eudragit®S100) at pH 6 and 100 % (Eudragit®L100) and 53 % (Eudragit®S100) at pH 7.4, according to Eudragit® solubility. The nanoparticles contributed to the dissolution and diffusion process of papain.

2.3. INTRANASAL DRUG DELIVERY

The nasal route has attracted great interest as an alternative route for the administration of diverse agents. Generally, the nasal route is used for the administration of decongestants, antibiotics, and mucolytics. However, the advancement of nanotechnology has enabled studies involving anticancer drugs, analgesics, central nervous system (CNS) drugs, peptides, and diagnostic agents.

The nasal mucosa presents many advantageous characteristics for the systemic absorption of drugs (epithelial microvilli, large surface area, rich vasculature, and a highly porous endothelial membrane) which facilitate drug permeation, such that drugs can be absorbed directly into the systemic circulation without the first pass effect. These characteristics lead to a fast onset of action, quickly reaching therapeutic plasma levels of the drug [27,28]. All these factors permit dose reduction, reduce side effects, and increase patient compliance to treatment. Additionally, the intranasal administration provides a non-invasive and painless alternative to the intravenous and oral routes, thus maximizing patient comfort.

Despite the advantages of this pathway, there are some barriers which limit the nasal absorption of drugs that must be considered during the discovery of new chemical entities intended for nasal therapy as well as during the development of nasal formulations. These barriers include mucociliary clearance, which rapidly removes the formulation from the nasal cavity. In addition, enzymatic degradation can occur in both the lumen of the nasal cavity and passing through the epithelial barrier [29]. Other factors that limit drug

nasal absorption are the low permeability of the epithelium which impedes the transference of polar drugs or high molecular weight substances such as peptides and proteins, the pH of the system which must be compatible with the nasal cavity, and the small volume that can be administered and the mucus layer.

Nanoparticulate systems, especially polymeric nanoparticles, show many advantages as systems for nasal administration, such as alteration of the mucus layer, alteration of tight junctions, erosion of the mucosal surface, increased drug contact time at the absorption site, the use of bioadhesive materials, and a reduction in the mucociliary clearance rate. [27,30]. The main applications of polymeric nanoparticles for nasal route include the delivery of peptides and proteins, vaccines, and brain targeting.

2.3.1. Systemic delivery of peptides and proteins

Peptides and proteins have great therapeutic potential; however, they lack appropriate characteristics for oral administration. Their instability in acid gastric medium, high molecular weight, and hydrophilicity hinder the permeation of peptides and proteins through the gut epithelium. Additionally, the general administration of these macromolecules involves the parenteral route, generating drawbacks such as the requirement for a qualified professional for administration, patient compliance, and high production cost. Thus, the nasal route has emerged as a route of interest to increase the bioavailability of these agents.

Insulin-loaded nanoparticles administered by the nasal route are an alternative to increase the biological half-life and improve the stability and therapeutic efficacy of this protein. Different polymers have been studied for this purpose, especially CS [31]. CS is an attractive material that can confer bioadhesion and increase the absorption of formulations intended for drug delivery in the nasal cavity. Among the methods used to prepare CS nanoparticles, it is highlighted the ionotropic gelation using tripolyphosphate (TPP) ions as the cross linking agent [32-35].

A simple one-step procedure to obtain CS-TPP and concomitant complexation with sodium alginate was developed, aiming to prepare transmucosal formulations for the absorption of insulin. [31]. The pharmacological evaluation in rabbits showed that nasal administration of insulin-loaded CS-TPP nanoparticles induced a rapid decrease in blood glucose, and the presence of alginate in the nanoparticles led to a prolonged hypoglycemic response for up to 5 h. According to the authors, these effects were due to the intracellular delivery of insulin. In addition to mucoadhesion of the nanoparticles due to the characteristics of CS, alginate may have contributed to this mechanism due to its high affinity for Ca^{2+} . Using the same preparation method, insulin-loaded nanoparticles were developed based on a copolymer formed of PEG-CS [33]. Pharmacodynamic/pharmacokinetic experiments in

rabbits showed that the nanoparticles led to a significant reduction in blood glucose levels that remained at a low concentration for, at most, 2–3 h. In addition, a rapid increase in plasma insulin concentrations occurred compared to that obtained with the copolymer suspension. Another example of hybrid CS nanoparticles are those constituted by CS-sulfobutylether-cyclodextrin [34]. These cationic insulin nanoparticles were able to enter in the nasal mucosa due to their permeation-enhancing properties. The transport of insulin across the nasal barrier led to a significant decrease in the plasma glucose levels.

A strategy to improve the mucoadhesive and permeation properties of unmodified CS is the covalent attachment of thiol-bearing groups. Krauland and co-workers [36] developed insulin-loaded CS-4-thiobutylamidine nanoparticles and showed that after nasal administration in non-diabetic rats, the plasma concentrations of insulin were higher than with unmodified CS-nanoparticles. Insulin-loaded CS-4-thiobutylamidine nanoparticles led to a more than 1.5-fold higher bioavailability and more than 7-fold higher efficacy in decreasing glycemia. In another study [37], insulin-loaded trimethyl-CS nanocomplexes were prepared. The ratio between insulin and the derivated polymer in the formulation influenced both the bioavailability and nasal epithelial integrity. The best absorption values were obtained using the proportion 1 : 30.6 (insulin:trimethyl-CS), but this formulation also led to severe damage to the nasal cavity in rats. In comparison, PEGylated trimethyl-CS copolymers were used to prepare insulin nanoparticles with similar efficacy but exhibited a mild level of epithelial damage with slight mucus secretion and goblet cell distension.

Nanoparticles based on polymers having characteristics of mucoadhesion and enzyme inhibition have been used to encapsulate insulin. Phenylboronic acid-functionalized glycopolymers exhibit potent inhibition activities against serine proteases such as trypsin, chymotrypsin, elastase, and leucine aminopeptidase and have been used to synthesize poly(3-acrylamidophenylboronic acid-ran-*N*-maleated glucosamine) nanoparticles [38]. These nanoparticles adsorbed high amounts of mucin, which was attributed to the interaction between the phenylboronic acid groups and sialic acid residues of the mucin. All formulations induced a decrease in blood glucose levels 9 h after nasal administration in rats. Images of the interaction of fluorescein isothiocyanate (FITC)-insulin-loaded nanoparticles with the rat nasal epithelium by confocal laser scanning microscopy indicated that endocytosis plays an important role in insulin transmucosal delivery. CS-*N*-acetyl-L-cysteine nanoparticles were prepared by *in situ* ionic gelation of CS with TPP [32]. The mucoadhesion of the nanoparticles prepared with *N*-acetyl-L-cysteine was around 2-fold higher than unmodified CS nanoparticles. In addition, the total decrease in plasma glucose levels was 16.2 % within 5 h, which was significantly higher than the value obtained with unmodified insulin nanoparticles (8.3 %) [39].

2.3.2. Vaccine delivery

Current immunization methods involve parenteral and intramuscular administration of antigen. Mucosal immunization has several advantages in comparison to these routes, such as needle-free administration and the possibility of self-administration. These features eliminate the necessity for trained personnel for vaccine administration and improve patient compliance in comparison to the parenteral route. The nasoepithelium has low enzymatic activity, pH values close to neutral, moderate permeability, and high availability of immune-reactive sites [40]. However, the viability of free antigens is low because of permeability problems and a reduced ability to stimulate the innate and adaptive immune system. Thus, the use of adjuvants, such as polymeric nanoparticles, is an alternative to bypass these disadvantages, thanks to improved residence time and contact with the mucosa, delivering the antigens directly to lymphoid tissues. Table 1 shows recent advances in the nasal delivery of vaccines based on nanoparticles.

2.3.3. Central nervous system delivery

The blood-brain-barrier (BBB) is located at the interface between the brain and the vessels of the circulation and is the most important structure connecting the central nervous systems with peripheral tissues. The main characteristic of this barrier is the existence of an endothelium with very restricted permeability as well as the presence of enzymes in large amounts. Only water, gases such as oxygen and carbon dioxide, and certain fat-soluble and very small hydrophilic molecules pass from the blood to the brain [41]. Therefore, the BBB functions as an interface that limits and regulates the exchange of substances between the blood and the central nervous system for 100 % of large molecule neurotherapeutics and more than 98 % of all small molecule drugs [42], making it difficult to reach therapeutic concentrations of drugs in the brain *via* the general circulation. Thus, the nasal route is an alternative to circumvent these limitations.

Designated nose-to-brain transport, the local key is the olfactory region, which is located at the top of the nasal cavity and is extremely porous, allowing the passage of neuronal bundles from the nasal region to the brain [43]. These nerves connect the nasal passages to the brain and spinal cord and, as well as the vasculature, cerebrospinal fluid, and lymphatic system, contribute to transportation of molecules to the CNS following adsorption from the nasal mucosa. Besides the olfactory nerve, the trigeminal nerve plays a fundamental role, facilitating the translocation of drugs *via* sensory fibers. The trigeminal nerve is formed by the ophthalmic branch that innerves the anterior and upper parts of the nasal cavity, the maxillary branch located on the respiratory nasal mucosa, and the parasympathetic fibers that accompany the sensory nerves to the sphenopalatine ganglion [29,44].

Despite the advantages of this pathway, transmucosal drug delivery is still limited by the physiological characteristics of nasal administration, such as mucociliary clearance and low permeability. Aiming to increase the availability of the drug by modification of the residence time and permeability, the use of bioadhesive agents and absorption enhancers in nanosized formulations for brain uptake have been used. In addition, nanoparticles are able to protect the encapsulated drug from biological and/or chemical degradation, and extra cellular transport by P-glycoprotein efflux. Their small size potentially allows nanoparticles to be transported by the transcellular pathway through olfactory neurons to the brain or *via* the various endocytic pathways of sustentacular or neuronal cells in the olfactory membrane. Thus, studies involving nanoparticles for nose-to-brain drug transport have increased over the last decade and represent a promising strategy for use in diseases such as schizophrenia, depression, epilepsy, meningitis, migraine, neuro-AIDS, brain cancer, and neurodegenerative diseases.

Taking account the advantages of the CS polysaccharide such as mucoadhesion and enhanced permeation, countless articles have studied the application of this atoxic polymer in CS nanoparticles for nose-to-brain transport. Thymoquinone-loaded CS nanoparticles were developed to avoid first-pass metabolism and improve its distribution to the brain with sustained action [45]. CS nanoparticles were prepared using the ionic gelation process. The optimized formulation containing 1.5 : 1.5 : 2 thymoquinone : CS : TPP was evaluated in an *ex vivo* permeation assay using goat nasal mucosa; it was found that the maximum permeation was 3-fold higher for nanoencapsulated thymoquinone in comparison to free drug. In the same direction, the relative bioavailability (nose-to-brain) evaluated in rats showed a 12-fold increase in comparison to thymoquinone intranasal solution.

Rivastigmine is a hydrophilic drug used in Alzheimer's disease because it is an inhibitor of acetylcholinesterase enzyme. Rivastigmine-loaded CS nanoparticles were prepared by the same method cited above [46]. A biodistribution study in rats found that the brain-blood ratios 30 min after administration were 0.790 and 1.712 for the rivastigmine intranasal solution and the drug-loaded nanoparticles, respectively, indicating direct nose to brain transport bypassing the BBB. Comparing to brain concentrations of nanoencapsulated rivastigmine after intravenous and intranasal administration, the results showed that the concentrations were higher for the extravascular in comparison to the intravascular route, highlighting the advantage of nose-to-brain transport. In another study, the plasma and cerebrospinal fluids of free estradiol and estradiol encapsulated in CS nanoparticles were investigated after intravenous and nasal administration in rats [32]. The concentration of estradiol in the cerebrospinal fluid after intranasal administration was higher than after intravenous administration. Besides, the time to reach this concentration was very fast by the nasal route

using nanoparticles, showing that CS allows for a rapid onset of action in the CNS.

The apparent permeability coefficient of tizanidine solution in a human nasal septum carcinoma cell line (RPMI 2650 cells) was strongly increased when the drug was encapsulated in thiolated CS nanoparticles [41]. The brain : blood ratio of tizanidine-loaded thiolated CS nanoparticles was 1.92 after 30 min and $C_{\max}$ was significantly higher than that obtained with the free drug. These results emphasized the importance of the mucoadhesion effect of thiolated CS, which increased the mean residence time in the nasal cavity. Another reason for the improvement in brain drug uptake may be the inhibition of CYP450 activity present in the nose, due to the ability of the thiol group to inhibit the metabolism of this enzymatic group. In another study, the permeability of free and leucina-enkephalin loaded *N*-trimethyl CS nanoparticles were evaluated in the porcine nasal mucosa, showing a 35-fold increase in comparison to the free peptide [47]. Furthermore, leucina-enkephalin was labeled with the fluorophore 4-fluoro-7-nitrobenzofurazan and instilled into the nostrils of mice to investigate brain penetration. The results showed that, after 60 min, significant fluorescence was visualized in brain sections. These observations were corroborated by antinociceptive tests that demonstrated a superior response with the use of nanoparticles. The authors suggested that the positive charge of *N*-trimethyl CS on the nanoparticle surface induced an electrostatic interaction with the anionic binding sites of the brain capillaries and transferred the labeled peptide into the brain. Moreover, CS formed a hydrophilic corona around the nanoparticles, preventing uptake by the mononuclear phagocytic system and increased the residence time of the drug in the body.

PEG surface modification is associated with improving nose-to-brain delivery of encapsulated agents in pegylated nanoparticles [48-52]. Likely, the PEG chains easily penetrate the mucus layer, thus preventing the degradation of particles and allowing access to epithelial cells in the olfactory region [43]. Other factors can influence the ability of PEG-coated nanoparticles to undergo nose-to-brain transport, such as the anchorage of ligands. Lactoferrin, a natural iron binding cationic glycoprotein of the transferrin family, is expressed in various tissues and also in the brain cells, such as brain endothelial cells and neurons. Using an emulsion-solvent evaporation technique, Liu and co-workers [52] developed lactoferrin conjugated PEG-*co*-PCL nanoparticles and encapsulated 6-coumarin as a model drug. PEG-*co*-PCL nanoparticles showed increased time-dependent uptake compared to naked nanoparticles within 6 h in the human bronchial epithelial cell line (16HBE14o-cells). Small interfering RNAs (siRNAs) have been extensively researched to treat CNS diseases, but the stability and cell penetration ability of these molecules are limited. To this purpose, another study [53] developed PEG-*co*-PCL nanomicelles conjugated with a cell-penetrating peptide named Tat-G. Concentrations in the cerebrospinal fluid were significantly higher for

conjugated nanomicelles in comparison to naked nanomicelles. In agreement, after nasal administration in rats, the nose-to-brain pathway involving the olfactory and trigeminal nerves showed the ability of these systems to permeate the nasal mucosa and to facilitate the brain delivery of nucleic acids.

Lectin surface modification is a way to improve brain delivery, since lectins are proteins or glycoproteins that have selective affinity for biological surfaces. One example of lectin is the wheat germ agglutinin (WGA) which presents the ability to specifically bind to *N*-acetyl-D-glucosamine and sialic acid present in the nasal cavity. WGA-PEG-co-PLA nanoparticles were prepared at a 1:3 molar ratio of WGA : maleimide by an emulsion-solvent evaporation technique. The cytotoxicity was evaluated *in vivo* in a rat nasal mucosa model and it was comparable to the negative control [49]. The immunogenicity and toxicity induced by WGA nanoparticles were evaluated *in vivo* in the rat nasal cavity. WGA and naked nanoparticles induced a significant increase in brain glutamate levels, but only conjugated nanoparticles exhibited lactate dehydrogenase activity in the olfactory bulb, indicating possible neurotoxicity. The levels of interleukin 8 (IL-8), tumor necrosis factor alpha (TNF- α), immunoglobulin G (IgG) and immunoglobulin A (IgA) in the rat olfactory bulb and brain remained similar to control and significantly lower than WGA alone [50]. However, the molar ratio of WGA : maleimide played an important role in these results, suggesting that an increase to 1:10 would be sufficient to provide the most efficient uptake and mild cytotoxicity [54]. The uptake occurred along olfactory nerves and trigeminal nerves within 2 h following intranasal administration and the cerebrospinal fluid pathway was not important in this delivery method [51].

Table 1. Recent studies for immunization *via* nasal route

Reference	Polymer	Antigen
[55]	CS	Anti-caries DNA
[56]	CS	DNA <i>Mycobacterium tuberculosis</i>
[57]	CS	Plasmid pVAXN
[58]	CS	Pneumococcal surface antigen A
[59]	CS	<i>Dermatophagoides farinae</i>
[60]	CS Mannosylated-CS	Plasmide DNA anti-gastrin releasing peptide
[61]	CS-PCL	H1N1hemagglutinin protein
[62]	CS Trimethylated chitosan	Hepatitis B surface antigen
[63]	Reacetylated <i>N</i> -trimethyl CS <i>N</i> -trimethyl CS	Ovalbumin
[64]	Trimethyl CS/ hyaluronic acid Thiolated trimethyl CS/ Thiolated hyaluronic acid	Ovalbumin
[65]	CS Trimethyl CS Tri-methylated CS	Hepatitis B surface antigen
[66]	Trimethyl CS	CpG DNA
[67]	Ovalbumin-Trimethyl CS	Ovalbumin
[68]	<i>N</i> -trimethyl-chitosan-mono- <i>N</i> - -carboxymethyl chitosan	Tetanus toxoid
[69]	PLGA CS-PLGA Glycol-CS-PLGA	Hepatitis B surface antigen
[70]	Poly(anhydride)	<i>Shigella flexneri</i>
[71]	Mannosylated Poly(anhydride)	<i>Brucella ovis</i> antigen
[72]	Poly(methylvinylether- <i>co</i> -maleic anhydride) Poly(anhydride)	<i>Brucella ovis</i> antigen

CS: chitosan; TPP: tripolyphosphate; PCL: poly(ϵ -caprolactone);
PLGA: poly(lactic-*co*-glycolide acid)

2.4. PARENTERAL DRUG DELIVERY

Generally, the therapeutic results achieved with orally administered drugs require the application of higher doses than those that would be required to achieve the same effect using the parenteral route. This is mainly because the parenteral pathway allows for the rapid and complete absorption of drugs, unlike what happens in the oral route where a large fraction of the dose is lost due to first-pass liver metabolism. Furthermore, the parenteral route allows the use of lower doses and has extended therapeutic effects in comparison to other routes [73,74]. Thus, it is also expected to lower the incidence of side effects compared to oral administration [74].

Drugs with poor dissolution properties are generally promising candidates to have their effect expanded by parenteral administration. These molecules are often difficult to formulate using conventional approaches and are associated with formulation-related performance issues, *e.g.* poor bioavailability, lack of dose proportionality, slow onset of action, and other attributes leading to poor patient compliance [73,75]. Other advantages of parenteral administration are the continuous infusion of drugs with a short half-life as well as drug administration to unconscious and comatose patients. [73,74].

Considering the features of the parenteral route, several studies using nanosystems have been conducted aiming to improve the therapeutic performance of the drugs and/or counteract the issues related to chemical or physical disadvantageous characteristic of the compounds. Countless molecules can be administered in nanoparticles. Cardiovascular agents, anticancer, anti-inflammatory, antibiotic, immunomodulatory, and immunostimulatory drugs, antiglaucoma compounds, and even peptides have been encapsulated in nanoparticles proposed for parenteral route. As a result, their pharmacological effects have been improved or their side effects have been reduced in comparison to the free drugs [75-78].

In one study, nanocapsules were labelled with technetium-99m (^{99m}Tc) and rhenium-188 (^{188}Re) and evaluated in terms of biodistribution parameters after intravenous injection in rats. The results obtained by dynamic scintigraphy showed predominant hepatic uptake, and an *ex vivo* evaluation indicated a long circulation time of labelled nanocapsules [79]. In another study conducted by Danhier and co-workers [80], it was hypothesized that nanosuspensions could be promising for the delivery of the poorly water soluble anti-cancer multi-targeted kinase inhibitor MTKi-327. The nanosuspension was administered by the parenteral and oral routes and it was observed that the highest regrowth delay of A-431-tumor-bearing nude mice occurred when the nanosuspension was administered intravenously. In this study, it was clear that nanoparticles can be used to increase drug efficacy.

Nanoparticles administered by the parenteral route can be quickly captured by the mononuclear phagocyte system (MPS). In this case, blood cells such as monocytes, leukocytes, and platelets, as well as resident phagocytes such as

the Kupffer cells of the liver and spleen macrophages, play an important role [81-83]. Thus, organs such as the spleen and liver prevent unrestrained circulation of nanoparticles in the blood, which could otherwise reach several sites in the body [83,84].

2.4.1. Stealth nanoparticles for the parenteral route

An approach to distributing nanoparticles to different sites of the body is the use of furtive (to the immune system) nanostructures. Thus, the rapid adhesion of plasma proteins and the consequent capture of nanoparticles by the MPS can be avoided by reducing the particle size, as well as using compounds with hydrophilic surface characteristics. In this way, the introduction of long hydrophilic polymer chains and non-ionic surfactants at the nanocapsule surface can lead to slow opsonization due to a steric effect, delaying essential electrostatic and hydrophobic interactions to bind opsonins onto the nanoparticle surface [85,86]. As a consequence, the half-life circulation of the nanoparticle in blood increases [84]. Steric shielding can be obtained using polymers such as polysaccharides, polyacrylamides, PVA, poly(*N*-vinyl-2-pyrrolidone), PEG, PEG-block copolymers, and PEG-containing surfactants (*e.g.* poloxamines, poloxamers and polysorbates). PEG is the most effective and commonly used strategy to obtain stealth nanoparticles, reducing or delaying the time of recognition and capture of nanoparticles by the MPS [80,84,86,87]. Nanoparticles with stealth ability allow for a reduction in drug dose, and consequently, decrease the adverse effects due to better delivery of the drug to the site of action [83,87,88].

A large amount of work has aimed at increasing the half-life of nanocarriers in the bloodstream, and changing their biodistribution, allowing them to reach other cells and tissues such as solid tumors and sites of inflammation [77,83,88-90]. One study prepared a nanoparticle formulation (nanoerythrosomes) containing the antimalarial drug pyrimethamine for intravenous application [81]. The biodegradable, long circulating carrier allowed for controlled and stable drug release, improving the treatment of malaria. In another study, the authors evaluated the antitumor effect, biodistribution profile, and tumor penetration of docetaxel-loaded PEG-PCL nanoparticles using a hepatic cancer model. The prepared nanoparticles were effectively transported into tumor cells by endocytosis and they accumulated around the nuclei in the cytoplasm. In addition, the *in vivo* biodistribution evaluation performed on tumor-bearing mice by real-time near infrared fluorescence (NIRF) imaging showed that the nanoparticles reached higher concentrations and were retained longer in the tumor than in non-targeted organs after intravenous injection [91].

The ability of nanoparticles to circulate in the bloodstream for a prolonged period of time is a prerequisite for successful therapy [85]. In this context, paclitaxel-loaded PLGA-CS-PEG nanoparticles have been investigated [87]. The

proposed nanosystem was able to encapsulate a hydrophobic drug and was taken up by phagocytosis. Thus, there was a reduction in opsonization by blood proteins, increasing the bioavailability of the drug. The results suggest that the PEG-CS coating may be a significant step in the development of long-circulating drug carriers for drug delivery into tumors.

A study conducted by Mosqueira and co-workers [92] evaluated the pharmacokinetics and efficacy of intravenously administered halofantrine-loaded nanocapsules prepared from a PLA homopolymer or PLA-PEG. The results showed that while the parasitemia decreased rapidly with the PLA nanocapsules, the effect was more sustained with PLA-PEG nanocapsules. In addition, nanocapsule administration resulted in a suitable halofantrine profile in the plasma, reduced the intravenous dose necessary for the therapeutic effect and, consequently, reduced the toxicity. Thus, these results demonstrate that the application of halofantrine-loaded nanoparticles by the parenteral route can be useful in severe malaria. In this case, is evident that nanoparticles were able to decrease uptake by the MPS because of steric stabilization afforded by PEG linked to the nanoparticle surface [83,92,93].

2.4.2. Active and passive targeting of nanoparticles

Different studies have evaluated the ability of nanoparticles to target several sites in the body after parenteral administration. Targeting ability is a major breakthrough in therapy because it is associated with many advantages such as reduced side effects. In passive targeting, the nanoparticles move freely through the vascular system and may randomly pass through the pores of the endothelium, especially in pathological situations. The plasma circulation half-life should be enough for them to reach the tissue passively. Colloidal systems administered intravenously may have a residence time in the bloodstream controlled by chemical changes on the surface of the particulate system [80,93]. Particles with furtive characteristics, such as those coated with PEG or biocompatible substances (*e.g.* peptides and lipids), present advantageous characteristics in this case [73,74,80,87,89].

Likewise, there is the possibility of surface functionalization in nanoparticles with substances such as antibodies, antibody fragments, carbohydrates, peptides, glycolipids, folic acid, mannitol, and genetic material. In the active targeting, the nanoparticle is selectively recognized by receptors on the surface of cells. Since ligand-receptor interactions can be highly selective, which would allow for a more precise targeting of a specific site in the body [76,77,90,93-96]. The approaches using active vectorization with nanoparticles are a quite challenging, and are currently one of the main goals of several studies in nanotechnology. The main findings in the literature show that surface functionalization with nanoparticles offers maximum therapeutic activity, prevents the degradation or inactivation of drugs while on route to the active site, and avoids several other inappropriate reactions [90,95,97]. Since

these kinds of nanoparticles have a specific binding site, they are able to prevent premature binding by plasma proteins and the consequent phagocytic capture more easily than other furtive nanosystems. Additionally, these nanoparticles present the potential to reform the drug development landscape since they can improve drug solubility, change undesirable pharmacokinetics, and increase drug accumulation in target organs and tissues [91,97,98].

The major focus of studies with active vectorization involves therapies and diagnosis for cancer. Considering the complexity of the cancer microenvironment and cancer immune responses, several anti-tumor strategies can be conjugated in order to induce a stronger and more complete anti-tumor immune response [80]. To improve the biodistribution of cancer drugs, nanoparticles have been designed with optimal size and surface characteristics to increase their circulation half-life in the bloodstream [89,91]. In addition, nanoparticles directed specifically to a site in the body can avoid systemic toxic effects and significantly enhance the maximum dose tolerated by patients [99].

Among the targeted ligands for cancer therapy, monoclonal antibodies are one of the most commonly used on the nanoparticle surface due to their high specificity and affinity for target antigens [75,100]. The efficiency of chemotherapeutic drugs or toxins targeted to the tumor is based on the binding and internalization of these conjugates into the target cell [75]. Torrecilla and co-workers [78] developed PEG-CS nanocapsules conjugated to the monoclonal antibody anti-TMEFF-2 for targeted delivery of docetaxel. In this study, free docetaxel exhibited a fast and short effect on tumor volume reduction, while bioconjugate nanocapsules with the monoclonal antibody showed delayed and prolonged action with no significant side effects.

Arias and co-workers [93] developed pentamidine-loaded nanoparticles based on PEG covalently attached to PLGA. This complex was coupled to a single domain heavy chain antibody fragment (nanobody) that specifically recognizes the surface of the protozoan pathogen *Trypanosoma brucei*. In the *in vitro* effectiveness assay, the results showed that the 50 % inhibitory concentration (IC_{50}) was decreased by 7-fold for the nanobody in comparison to the free drug. Furthermore, an *in vivo* evaluation using a murine model of African trypanosomiasis showed that the formulation healed all infected mice at a 10-fold lower dose than the minimal full curative dose of free pentamidine; for 60 % of the mice, this occurred at a 100-fold lower dose. These results show that an active vectoring system based on nanoparticles applied parenterally has the ability to improve conventional therapy.

In another study [94], a ligand metal-CS-lecithin complex was prepared as a new strategy to functionalize the surface of PCL nanoparticles. The results showed that the nanoparticulate complex was able to connect recombinant antibody fragments, known as anti-electronegative LDL single-chain fragment

variable [scFv anti-LDL(-)]. This complex was able to react with LDL(-) cholesterol molecules, making it an important therapeutic tool.

There are many advantages reported in studies evaluating the application of nanoparticles by the parenteral route in therapeutics and diagnosis [79,89,93]. However, basic assessments of compatibility of these nanoparticulate systems with the constituents present in the blood circulation are still somewhat deficient. Thus, nanoparticles that aim toward parenteral application should be evaluated with respect to biocompatibility. Polymeric nanoparticles employing biocompatible materials, when administered by the parenteral route, decrease the probability of incompatibilities [80,88]. But in any case, the concentration of the material, the presence of other incompatible materials, or unexpected reactions with the constituents of the formulation may make it unstable [82].

One study evaluated the hemocompatibility of the formulations of polymeric lipid-core nanocapsules stabilized with polysorbate 80-lecithin and uncoated or coated with CS. *In vitro* hemocompatibility studies were carried out with mixtures of nanocapsule suspensions in human blood at 2 % and 10 % (v/v). The results showed that the ability of plasma samples to activate the coagulation system was maintained in the presence of the lipid-core nanocapsule. The hemolysis values remained restricted to the recommended limits (1 %) when whole blood was incubated with either uncoated or coated CS-nanoparticles at 2 %. On the other hand, when the nanoparticles were added to blood at 10 %, hemoglobin was readily released into the extracellular environment. According to the authors, this result could be explained by a shift in polysorbate 80 from the colloids to cells or by the interaction of CS with cells due to the high concentration used (10 %), causing hemolysis [82].

Therefore, several molecular reactions may occur that destabilize nanoparticulate systems in a biological medium. The parenteral route is an excellent alternative for nanoparticle administration, but aspects of the chemical nature of the compounds used alone or in combination should always be taken into account.

2.5. DERMAL AND TRANSDERMAL DRUG DELIVERY

Nanoparticle application has been primarily focused on parenteral and oral applications. Nowadays, besides these uses, nanosystems applied to the skin are attracting more and more attention from researchers, considering the advantages of nanoparticles for dermal application such as the protection of incorporated active compounds against chemical degradation and flexibility in modulating the release of the compound [101]. Nanoparticles applied to the skin can have one of two desired effects: local activity within the skin (dermal drug delivery) or systemic activity after nanoparticle permeation through the skin (transdermal drug delivery) [102].

In both the dermal and transdermal routes, the *stratum corneum* is the main barrier of the skin that has to be overcome for suitable drug delivery [102]. The skin is composed of the epidermis, dermis, and subcutaneous tissue. The epidermis is again subdivided into four layers (the *strata corneum*, *granulosum*, *germinativum*, and *basale*). The cells in the *stratum basale* divide continuously to produce new keratinocytes that move to the outer layers and form the *stratum corneum*, which is a horny layer of dead cells. In addition to keratinocytes, the viable epidermis contains cells with roles such as melanin production (melanocytes), sensory perception (Merkel cells), immunological function (Langerhans) and the appendages. The appendages include the pilosebaceous units such as hair follicles and associated sebaceous glands, apocrine and eccrine sweat glands [103].

The main role of the skin is to exert defensive mechanisms (physical, immunological, metabolic, and UV-protective barriers) counteracting attacks by microbes, toxic chemicals, UV radiation, and particulate matter [103]. However, the large surface area and easy accessibility of the skin make it an attractive route for drug delivery. Three main routes in the skin have been identified for the penetration of substances. These penetration pathways into and through the skin are separated to the intracellular (across the corneocytes), intercellular (by the lipid bilayers that surround the corneocytes), and transappendicular (that includes hair follicles and sweating gland) routes.

The intercellular route is recognized as the most feasible pathway. However, substances with a molecular weight greater than 500 Da and ionic substances accumulate in appendicular organs, since these substances have great difficulty passing through the *stratum corneum* [104]. In fact, dominance amongst the three pathways depends on the drug (its solubility, diffuseability, molecular size and physico-chemical properties) and the system used for delivery of the substance [105]. Considering this statement, many researchers have made efforts to develop drug delivery systems for topical application able to target drug molecules to specific skin layers or to the systemic circulation.

2.5.1. Topical application of nanoparticles

Despite the benefits of transdermal drug delivery systems, including simple administration, avoidance of uncomfortable intravenous administration, escape from the first pass effect in the liver, and controlling the dose of drug delivery, the development of a transdermal delivery formulation has to consider two critical issues: the physical barrier of the *stratum corneum* and the hydrophilicity and numerous types of enzymes present in the chemical barrier of the epidermis [104]. Despite the efficiency of therapeutic agents using the transdermal route for both systemic delivery and local delivery, many techniques have been used to enhance the permeability of drug molecules, including nanoparticles.

Marimuthu and co-workers [106] developed PLGA nanoparticles intended for the transdermal application of encapsulated glucosamine, which is a highly hydrophilic and poor permeable drug. The nanoparticles were prepared by self-assembly of PLGA–glucosamine which was facilitated by probe sonication followed by reversible locking. The authors hypothesized that the nanoparticle's flexibility was due to its structure (hydrophobic PLGA assembly on the outer surface and hydrophilic glucosamine in the inner core). This flexibility helps the nanoparticles to permeate through the skin lipid membrane and release the drug in a sustained manner. In comparison to glucosamine solution, nanoparticles exhibited a better permeation profile and demonstrated a shorter lag time with a higher flux value in *ex vivo* transdermal permeation. In another work, polymeric nanoparticles were prepared using CS, PLA, and PCL by a solvent extraction method in an attempt to provide prolonged delivery of repaglinide, a hypoglycemic drug [107]. The optimized PLA-repaglinide nanoparticles loaded in transdermal patches induced a reduction in plasma glucose levels and were 76-fold more effective than conventional oral administration in diabetic rats.

In addition to transdermal patches, semi-solid vehicles make a suitable formulation for nanoparticles administration to the skin. Contri and co-workers [108] evaluated the effect of the encapsulation of capsaicinoids (capsaicin and dihydrocapsaicin) in nanocapsules, as well as the effect of the incorporation of capsaicinoid-loaded nanoparticles in a CS hydrogel, on skin adhesion and skin penetration/permeation. The *in vitro* skin adhesion experiments showed lower washability for the CS hydrogel containing capsaicinoid-loaded nanocapsules in comparison to the CS hydrogel containing the free drug and hydroxyethyl cellulose containing drug-loaded nanocapsules. The adhesion assay results predicted the skin penetration/permeation behavior, since the CS gel containing nanocapsules led to a higher amount of capsaicinoids in the epidermis and dermis. In a similar work, PLGA nanoparticles and lecithin/CS nanoparticles containing betamethasone-17-valerate enhanced the amount of the drug in the epidermis when compared with the commercial formulation [109]. When nanoparticles were diluted in CS gel, accumulation in skin layers from both gel formulations was higher than the commercial formulation. In addition, both formulations significantly improved anti-inflammatory and skin-blanching effects in comparison to the commercial cream. In another study, a Pluronic F127 hydrogel containing lidocaine-loaded PCL-PEG-PCL nanoparticles was prepared, aiming transdermal application [110]. The efficiency of the local anesthetic, evaluated by the tail-flick latency test in rats, was better for the nanoparticle based hydrogel in comparison to the conventional treatment (cream) with or without focal ultrasound pretreatment.

In some cases, it is necessary to increase the ratio of the drug in the target tissue relative to systemic exposure to ensure successful drug targeting. It is especially important for drugs that require chronic use or drugs that produce

significant side effects at other body sites [111]. In this context, CS nanoparticles containing hydrocortisone were administered percutaneously to improve transcutaneous absorption of the drug [112]. The hydrocortisone-loaded nanoparticles reduced the corresponding flux and permeation coefficient of the drug across mouse skin in *ex vivo* experiments, while they exhibited a higher epidermal and dermal accumulation of the drug in comparison to control groups.

In another study, Lee and co-workers [113] developed a core-shell nanoparticle (PLGA core and a positively-charged glycol CS shell) for use as a DNA carrier intended for transdermal delivery into the epidermis *via* gene gun. The *in vivo* evaluation using a mouse model demonstrated that bombardment of nanoparticles transfected DNA directly into Langerhans cells present in the epidermis, which migrated and expressed the encoded gene products in the skin draining lymph nodes. It is emphasized that Langerhans cell migration was detected by fluorescent quantum dots loaded into the core of the nanoparticle. Therefore, nanoparticles have the potential for use in immunotherapy and vaccine development and can be an important approach for monitoring functional aspects of the immune system.

2.5.2. Innovative approaches for cutaneous application of nanoparticles

Nanoparticle-based topical delivery systems have been demonstrated to be successful approach for topical (into the skin strata) and transdermal (to subcutaneous tissues or into the systemic circulation) delivery. Beside this, the targeted delivery of encapsulated drugs to hair follicle stem cells, such as iontophoresis and microneedle array technologies, has been employed [114].

Indomethacin-loaded PLGA nanoparticles were prepared with or without (bare nanoparticles) a PVA covering by an antisolvent diffusion method with preferential solvating or an emulsification-solvent evaporation method, respectively [104]. The authors evaluated the effectiveness of the nanoparticles for *ex vivo* iontophoretic transdermal drug delivery. Both nanoparticles presented an average diameter of 100 nm. Bare nanoparticles did not have a hydrophilic stabilizer on the surface, presenting high hydrophobicity and negative charges. The cumulative indomethacin amounts that permeated through rat skin were significantly increased by using either kind of nanoparticles when iontophoresis was applied. However, the bare nanoparticles presented significantly higher permeability in comparison to the PVA-coated nanoparticles, showing that the combination of a bare nanoparticle system with iontophoresis was the most effective at enhancing permeability.

Microneedles are another way to enhance the permeation of nanoparticles into the skin. The mechanism of transdermal delivery of nanoencapsulated across microneedle-treated skin was studied using the rhodamine B (Rh B) and FITC as model hydrophilic and hydrophobic small/medium-size molecules,

respectively [115]. Permeation of the model dyes encapsulated in PLGA nanoparticles through porcine skin pretreated with a microneedle array was affected by the physicochemical characteristics of nanoparticles and the encapsulated dyes. Confocal laser scanning microscopy images showed dye-rich reservoirs, suggesting a mechanism involving the influx of nanoparticles deep into microneedle-created channels. The results showed even dye flux was enhanced by nanoparticles with a smaller particle size, hydrophilicity, and a negative zeta potential.

Nanoparticles can interact with the skin at a cellular level, and this interaction can be used to enhance immune reactivity for topical vaccine applications [103]. Considering this statement, dissolving microneedle arrays loaded with nanoencapsulated (PLGA nanoparticles) antigen were evaluated regarding their efficacy in increasing vaccine immunogenicity by targeting the antigen specifically to contiguous dendritic cell networks within the skin [116]. The results showed that the antigen-encapsulated nanoparticles were delivered from skin dendritic cells to cutaneous draining lymph nodes, where they subsequently induced significant antigen-specific T cell proliferation. Antigen-encapsulated nanoparticle vaccination *via* microneedles induced antigen-specific cellular immune responses in mice. Furthermore, the activation of antigen-specific cytotoxic CD8⁺ T cells induced protection *in vivo* against both the development of antigen-expressing B16 melanoma tumors and a murine model of para-influenza.

Another proposal to increase the ratio of the drug in the target tissue is based on follicular drug delivery carriers. The hair follicle has been shown to be not only an important penetration route for nanoparticles, but also a significant long-term reservoir. In hair follicles, nanoparticles are surrounded by a dense network of blood capillaries, which is important for drug delivery and systemic uptake. Even differentiated targeting of specific follicular structures can be achieved, since the penetration depth of nanoparticles can be influenced by their size [117]. It is important consider that the movement of the hairs caused by massage push the nanoparticles deeper into the hair follicles. Therefore, massage after the application of nanoparticles may be necessary [102].

In this context, Raber and co-workers [118] quantified the uptake of fluorescently-labeled PLGA nanoparticles into hair follicles using *in vitro* (pig ear) and *in vivo* (human volunteers) models. The follicular uptake of the nanoparticles was dependent of the surface modifications (plain PLGA, CS-coated PLGA, or CS-PLGA coated with different phospholipids). Plain PLGA nanoparticles with a negative zeta potential, as well as dipalmitoyl phosphatidylcholine (DPPC) and DPPC : 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) (92 : 8)-coated CS-PLGA nanoparticles presented follicular uptake to a greater extent than CS-PLGA nanoparticles and DPPC : cholesterol (85 : 15)-coated CS-PLGA nanoparticles, which may indicate that a negative surface charge as well as lipophilic surface properties may facilitate follicular uptake.

The penetration and storage behavior of dye-containing nanoparticles in hair follicles and the dye in the non-particulate form were evaluated *in vitro* in porcine skin [119]. When massage was applied, the nanoparticles penetrated much deeper into the hair follicles than the dye in the non-particulate form. In addition, a differential stripping assay was carried out *in vivo* on human skin, showing that the nanoparticles were stored in hair follicles up to 10 days, while the free dye could only be detected for up to 4 days. These results indicate that hair follicles could be used as a reservoir for the topical administration of active molecules.

2.6. CONCLUSION

Drug-loaded nanoparticles have emerged as one of the most important applications in medicine. These innovative systems present physical properties that can be exploited to overcome anatomical and physiological barriers associated with drug delivery. When administered by the parenteral route, the pharmacological effects of the nanoencapsulated drug can be improved, the side effects can be reduced, and a specific site in the body can be reached using an active targeting approach. By oral administration, nanoparticles are able to enhance intestinal permeability, control drug delivery, and protect drugs in the gastrointestinal tract, whereas by nasal administration, nanosystems can improve local absorption on several levels. In addition, cutaneous application of nanoparticles can release the compound within the skin or allow for permeation through the skin, acting as a dermal or transdermal carrier system.

Thus, nanoparticles can be used to increase drug bioavailability, to induce drug accumulation at a specific site of the body, and to decrease drug side effects, leading to improved therapeutic effectiveness and increased patient adherence to treatment.

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NANO-BASED DRUG DELIVERY SYSTEM

Gamze Güney Eskiler^{1*}, Gökhan Dikmen², and Lütfi Genç³

¹ Department of Medical Biology, Medical Faculty, Uludag University, Turkey, 16059

² Central Research Laboratory, Eskisehir Osmangazi University, Turkey, 26480

³ Faculty of Pharmacy, Department of Pharmaceutical Technology, Anadolu University, Turkey, 26470

*Corresponding author: gamzeguney@anadolu.edu.tr

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3.1. INTRODUCTION

The conventional application of drugs presents challenging problems in the treatment of many diseases for example: therapeutic effectiveness, poor biodistribution, stability, solubility and intestinal absorption, lack of selectivity, side effects and fluctuations in plasma concentration [1-3]. Drug delivery systems (DDS) have been designed to overcome these limitations and drawbacks. DDS provide specific drug targeting and delivery, minimizing undesirable side effects, using lower doses of drug and protecting the drug from degradation. Recent developments in nanotechnology have indicated that nanoparticles (ranging in size from 1–1000 nm) can be successfully used as drug carriers with optimized physicochemical and biological properties (small size, increased drug accumulation and therapeutic effects, ability to cross cell or tissue barriers, controlled drug release, *etc.*). The use of nanoparticles as drug carriers can play an important role in eliminating the challenging problems associated with conventional drugs used for the treatment of many chronic diseases such as cancer, asthma, hypertension, human immunodeficiency virus (HIV), and diabetes [4-10].

Polymeric nanocarriers, dendrimers, polymeric micelles, liposomes, solid lipids nanoparticles (SLNs), metallic nanoparticles (magnetic, gold), carbon nanotubes, nanospheres, nanocapsules and nanogels are examples of nano-based drug delivery systems that are currently under research and development [11-15]. Some of them, especially cancer treatments, have been clinically used and approved by the Food and Drug Administration (FDA).

The use of drug delivery systems in cancer treatment consists of two strategies: passive and active targeting (Figure 1). Each targeting strategy aims to increase the accumulation of the drug within the tumor tissue while reducing the side effects [16,17].

Figure 1. The main characteristics of passive and active targeting

In this chapter, the production techniques, the characterization methods and applications particularly in cancer treatment of certain drug delivery systems including dendrimers, polymeric micelles, liposomes and SLNs will be discussed.

3.2. POLYMERIC MICELLES

Polymeric micelles that consist of amphiphilic co-polymers in the form of core/shell nanostructures can be designed to ensure selective delivery of drugs, especially water insoluble drugs, to their subcellular targets. Polymeric micelles comprise of two structures: an inner core and an outer shell. The hydrophilic shell of the copolymer consists of hydrophilic non-biodegradable polymers (such as poly(ethylene oxide) (PEO), poly(*N*-isopropylacrylamide) (PNIPA), poly(alkylacrylic acid), and poly(ethylene glycol) (PEG)) and sustains stability in the aqueous medium enabling interactions with plasmatic proteins and cell membranes. The hydrophobic core of the micelles encapsulates various non-polar drugs (such as paclitaxel, doxorubicin, tamoxifen, camptothecin, porphyrins, *etc.*) and provides their controlled release. The hydrophobic core may consist of biodegradable, non-biodegradable or poorly biodegradable water-soluble polymers such as poly(propylene oxide) (PPO), poly(β -benzyl-L-aspartate) (PBLA) [17], poly(L-lactic acid) (PLLA), poly(lactic-co-glycolic acid) (PLGA), poly(ϵ -caprolactone) (PCL) or poly(aspartic acid) (PASP), polystyrene (PST), poly(methyl methacrylate) (PMMA), and various polyacrylates [18-28].

Polymeric micelles range in size from 10–100 nm, each type having a narrow size distribution. This narrow size range is the most important property of polymeric micelles, ensuring high stability, sterility and long term circulation in the bloodstream. It is important to point out that polymer chains in the inner core of polymeric micelles affect their stability and it is a key point in drug delivery to avoid interaction of single polymer chains with the loading drug.

The structure of polymeric micelles plays an important role because it consists of different polymer and surfactant according to chosen applications [26-31].

Table 1. The advantages and disadvantages of polymeric micelles [18-31]

Advantages of Polymeric Micelles	Disadvantages of Polymeric Micelles
Small size (10–100 nm)	Difficult polymer synthesis
High structural stability (lipid-core micelles formed by self-assembled amphiphilic polymers)	Not stable (normal self-assembled polymeric micelles)
Large amount of drug loading and sustained drug release	No universal incorporation method
High water solubility when hydrophobic drugs loaded	Possible chronic liver toxicity due to slow metabolic process
Low toxicity	Sometimes toxic side effects exist due to a longer period impacts than free drug
Modification by various chemical species	Not produced in a large industrial scale
Protection of encapsulated drugs from degradation and metabolism	Only few polymeric micelles are approved by FDA and used in clinical applications

3.2.1. Production and drug incorporation into polymeric micelles

The production of polymeric micelles can be separated into two techniques: direct or indirect. What technique will be selected relies on the simple equilibration of the drug and the type of polymers. While the direct method includes the direct solubilization of the amphiphile in aqueous medium followed by encapsulation of the drug, the indirect method depends on the use of water-miscible organic solvents (*e.g.*, acetone, dimethylacetamide) to co-solubilize the co-polymer and the drug and then to separate organic solvents by evaporation or dialysis. However, these methods have been improved in recent studies to increase encapsulation capacity and stability and to control drug release kinetics [32-36].

Drugs can be loaded into micelles by chemical conjugation or by physical entrapment through dialysis or emulsification techniques. The drug loading procedure may influence the entrapment efficiency and the distribution of a drug within the polymeric micelles. Chemical conjugation enables the drug to be incorporated with the hydrophobic polymer by a covalent bond, such as an amide bond. Thus the entrapped drug is protected from enzymatic cleavage due to non-hydrolysis of chemical bonds. The dialysis or oil-in-water emulsion procedure is used in the physical entrapment of drugs. This technique depends

on the removal of the organic solvents used. Whereas soluble (*e.g.*, ethanol, *N-N*-dimethylformamide), non-toxic solvents are used in the dialysis method, water-insoluble solvents (*e.g.*, chloroform, chlorinated) are used in the oil-in-water emulsion method [26,37-42].

3.2.2. Characterization of polymeric micelles

Micelles are characterized by turbidity measurement, critical micelle concentration (CMC) and aggregate size. CMC and CMC ratio along with other components are very important for polymeric micelles. In order to determine CMC, there are various techniques, for example interfacial tension, conductivity, osmotic pressure, *etc.* However, these methods are not convenient for polymeric micelles because of their low CMC values.

In order to measure the size of micelles, dynamic light scattering is a very useful technique, because function of concentration is used as an indicator of the onset of micellization [43,44].

In addition, the hydrodynamic diameter of polymeric micelles can be determined by using dynamic light scattering (DLS). But, since the DLS method is not effective for measuring multimodal size distributions, atomic force microscopy is used for this purpose. Furthermore, DLS instruments often use a detection angle of 90° and this optical configuration may not be sensitive enough for the successful measurement of micelle surfactants [45-47].

The size of micelles is dependent on the encapsulation of the drug. That is, the process of surrounding of the drug in the hydrophobic core causes an increase in the size of the micelles. The size of micelles is generally measured using transmission electron microscopy (TEM) or scanning electron microscopy (SEM) [48,49].

The zeta potential (ZP) of micelles is measured using a zetasizer. ZP values give information about the surface charge of micelles and therefore their aggregation status. The zetasizer machine automatically calculates ZP values in the analyzer using the following Smoluchowski equation:

$$m = \frac{e \cdot z}{h}$$

where *z* is the ZP, *m* the mobility, *e* the dielectric constant and *h* the absolute viscosity of the electrolyte solution [50].

Stability testing of micelles consists of analytical measurements of drug content. Firstly, lyophilized and aqueous solutions of drug-loaded micelles are weighed under fixed and constant conditions and after then in order to determine the drug content, samples are taken at the beginning and at the end of particular time. Content of drug is measured spectrophotometrically and UV spectra are recorded [33,51].

3.2.3. Applications of polymeric micelles in cancer treatment

Different chemotherapeutic agent-loaded polymeric micelles consisting of different polymers have been studied in recent years. The developed formulations are potential drug delivery systems for the loaded agents because they increase the therapeutic efficacy and the accumulation of the drug within the tumor tissue, reduce systematic toxicity and demonstrate excellent multi drug resistant (MDR)-overcoming ability for anticancer drug delivery. These studies are summarized in Table 2 and 3 [52].

Table 2. Clinical trials of doxorubicin, paclitaxel, SN-38, DACH-platin, cisplatin, and epirubicin-loaded polymeric micelles

Product Name	Incorporation drug	Treatment	Trial phase	References
NK-911	Doxorubicin	Solid tumors	II	[53]
SP-1049C	Doxorubicin	Metastatic adenocarcinoma of the upper gastrointestinal tract, breast cancer and NSCLC	II/III	[54]
NK-105	Paclitaxel	Breast cancer	III	[55,56]
		Stomach cancer	II	
Genexol-PM	Paclitaxel	Breast, lung, pancreatic, recurrent breast cancer	Approved	[57-60]
NK-012	SN-38	Breast cancer	II	[61]
NC-4016	DACH-platin	Colorectal cancer (targeted future indication)	I	[62]
NC-6004	Cisplatin	Solid tumors	I/II	[53]
Nanoplatin®	Cisplatin	Solid tumors and NSCLC	I	[62]
		Pancreatic cancer	III	
NC-6300/ k-912	Epirubicin	Breast cancer (targeted future indication)	I	[62]

Table 3. Summary of incorporated drugs, polymer types and treatment of polymeric micelles in published papers

Incorporation drug	Polymer Type(s)	Treatment	References
Doxorubicin (Dox)/wortmannin			
Dox/17-hydroxethylamino-17-demethoxygeldanamycin	PEG- β -p(Asp-Hyd)a	MCF-7 breast cancer	[63]
	Alginate- γ -poly(<i>N</i> -isopropylacrylamide) (PNIPAAm)	<i>in vivo</i>	[64]
	PEG-poly(phosphoester)	MDA-MB-231 tumor model	[65]
	(PCL)2-[poly(2-(diethylamino)ethyl methacrylate- β -PEG methyl ether methacrylate]2 [(PCL)2(PDEA- β -PPEGMA)2]	HepG2 cells	[66]
	PCL β -poly(2-(diethylamino)ethyl methacrylate)- β -PEG methyl ether methacrylate) (4/6AS-PCL- β -PDEAEMA- β -PPEGMA)	HepG2 cells	[67]
Dox	pH-sensitive polyHis- β -PEG micelles	MDA_MB_231 breast cancer tumor model	[68]
	Methoxy poly(ethylene glycol)- β -poly(ϵ -caprolactone) copolymer with citraconic amide	HepG2 and 4T1 cancer cells	[69]
	PCL63- β -PNVP90 block copolymer	K-562, JE6.1 and Raji and mice lymphoma cells (Dalton's lymphoma, DL)	[70]
	PCL/PEG copolymer	Colon cancer mouse model	[71]
	Monomethoxy-PEG-S-S-hexadecyl (mPEG-S-S-C16)	HeLa cells	[72]
Parthenolide (PTL)-Dox	Poly(styrene-alt-maleic anhydride)- β -poly(styrene)	MDR ovarian cancer cells	[73]

	(PSMA- β -PS) and poly(styrene-alt-maleic anhydride)- β -poly(butyl acrylate) (PSMA- β -PBA)		
Dox and gene Msurvivin T34A	MPEG-PCL- γ -PEI labeled with (99)Tc	B16F10 tumor model and lung metastasis model	[74]
Dox/Etoposide	PEG- β -p(γ -benzyl L-glutamate)	CT-26 murine colorectal cancer	[75]
Mitoxantrone (MTX) and Dox	Polyethylene oxide- β -poly(acrylic acid) polymer	A549 NSCLC	[76]
Dox/Paclitaxel (PTX)	PEG- β -PLGA	A549 NSCLC, B16 mouse melanoma, HepG2 liver cancers	[77]
Elacridar- PTX	PEG-PE	PTX resistance in two cancer cell lines	[78]
PTX/17-AAG/rapamycin	PEG- β -PLA	A549 NSCLC, MDA-MB-231	[79]
Cisplatin (CDDP)-PTX	PEG, glutamic acid and phenylalanine (PEG-PGlu-PPh _e)	A2780 ovarian cancer cells	[80]
PTX /17-AAG/Etoposide	Poly(2-methyl-2-oxazoline)	MCF-7 and MDA-MB-231 breast cancer	[81]
PTX /17-AAG/Bortezomib	- β -poly(2-butyl-2-oxazoline)- β -poly(2-methyl-2-oxazoline)	PC3 prostate cancer, HepG2 liver cancer	[81]
PTX /cyclopamine/gossypol	PEG- β -PCL	ES-2-luc and SKOV-3-luc ovarian cancers	[82]
PTX /17-AAG	PEG-DSPE/TPGS	SKOV-3 ovarian cancer	[83]
PTX / CDDP	PEG- β -poly-(glutamic acid)- β -poly(phenylalanine)	A2780 ovarian cancer	[80]
PTX /17-AAG/rapamycin	PLGA- β -PEG- β -PLGA	ES-2-luc ovarian cancer	[84]
PTX -Lapatinib (LPT)	Pluronic F127	T-47D cell line	[85]
PTX- Curcumin (CUR)	PEG2000-PE and vitamin E	NCI-ADR-RES and SK-OV-3TR	[78]

		ovarian cells	
PTX	PEG750-p(CL-co-TMC) copolymer	CT26 colon carcinoma cells and <i>in vivo</i>	[86]
	Tocopherol succinate-chitosan-PEG-folic acid	4T1 mice breast cancer cell line	[87]
	Deoxycholic acid-modified chitooligosaccharide (COS-DOCA) and methoxy PEG - polylactide copolymer (mPEG-PDLLA)	A549 lung xenograft model	[88]
	PEG- β -poly(mono-2,4,6-trimethoxy benzylidene-pentaerythritol carbonate-co-acryloyl carbonate) (PEG- β -P(TMBPEC-co-AC)) and Gal-PEG- β -PCL (Gal-PEG- β -PCL)	HepG2	[89]
	PEG block-poly(2-methyl-acrylic acid 2-methoxy-5-methyl-[1,3]dioxin-5-ylmethyl ester) (PEG- β -PMME)	A549	[90]
	PEG-distearoylphosphatidylethanolamine (PEG-DSPE)	Hep-2, U-937 leukemic cancer cell line	[91]
	Hyaluronic acid-octadecyl (HA-C18) and Folate (FA) conjugated	MCF-7 tumor-bearing mice	[92]
	PEGylated P(CL-co-LLA) (poly(ϵ -caprolactone-co-L-lactide))	S180 tumor-bearing mice	[93]
Platinum	MeO-PEG- β -P(Glu) and Mal-PEG- β -P(Glu)	BxPC3 cells human pancreatic cancer	[94]
Phenformin	PEG-polycarbonate	H460 human lung cancer cell line	[95]
Cyclopamine/Gefitinib	PEG- β -poly(carbonate-co-lactic acid)	MIAPaCa-2 pancreatic cancers	[96]
Camptothecin (CPT)	Poly(cholesteryl acrylate-co-methoxy PEG methacrylate), poly[CHOL(y)-co-	MCF-7	[97]

mPEG(n,x)]			
4-(N)-stearoyl Gem (GemC18)	PEG-poly(d,L-lactide) (PEG-PLA)	Gem high-resistant AsPC-1 cells	[98]
Docetaxel (DTX)	PCL- β -PEG copolymers	LNCaP prostate adenocarcinoma cells	[99]
	Pluronic P105 and F127 copolymers	A549	[100]
	PEGylated poly(amine-co-ester) terpolymers	SK-BR-3 cancer cells	[101]
	Vitamin E TPGS -D- α -tocopheryl PEG 1000 succinate	NIH3T3,SK-BR-3,MCF-7, MDA MB 468, MDA MB 231 and HCC38	[102]
DTX- chloroquine (CQ)	PEG- β -PLGA	MCF-7	[103]
DTX-CDDP	α -tocopherol (Ve) and PEG to poly(L-glutamic acid) (PLG)	B16F1 mouse melanoma cells and <i>in vivo</i>	[104]
CUR	<i>N</i> -Benzyl- <i>N,O</i> -succinyl chitosan (BSCS)	HeLa, SiHa and C33a cervical cell lines	[105]
	Methoxy PEG-poly(lactide)-poly(β -amino ester) (MPEG-PLA-PAE) copolymers	MCF-7 cells and <i>in vivo</i>	[106]
	Poloxamers and D-alpha-Tocopheryl PEG 1000 succinate (TPGS)	Multidrug-resistant ovarian cancer (NCI/ADR-RES) cells	[107]
CUR-PTX	PEG-phosphatidylethanolamine (PEG-PE)/vitamin E	SK-OV-3-paclitaxel-resistant (TR) cells	[108]

3.3. DENDRIMERS

Dendrimers are hyperbranched globular shaped particles having a unique three-dimensional architecture. They can provide perfect control over molecular structure for a nanosized based drug delivery system due to their multiple functional surface groups.

The first poly(amidoamine) (PAMAM) dendrimer was synthesized by Tomalia in 1985 [109]. Recent studies have focused on improving their application and functional design as drug or gene delivery systems, especially in cancer treatment, in order to eliminate their disadvantages.

Dendrimers have three different separate components:

a core, at the center of the dendrimer, determines the size and shape of the dendrimer,

branches, constituted of repeat units lead to a monodisperse, treelike, star-shaped or generational structure,

terminal functional groups, generally located at the exterior surface of the dendrimer. These surface groups enable growth of the dendrimer so they determine the properties of dendritic macromolecules according to their chemical modification.

Dendrimers can be used as a suitable carrier for increasing drug solubilization, enhancing gene or drug delivery and increasing the therapeutic effectiveness of any drug, as well as enabling targeting to specific sites [110-119].

Table 4. The advantages and disadvantages of dendrimers [110-119]

Advantages	Disadvantages
Monodisperse molecular structure unlike linear polymers and providing control over its architecture	Three-dimensional structure and the design of dendrimers especially secondary-structural motifs within the branches are not well understood
Cross biological barriers to circulate in the body with small size and target specific site by modifying	Not suitable for all administration excluding parenteral
Terminal end groups possess positive, negative or neutral charges	Non-specific interaction with a variety of cells and toxic effects

3.3.1. Types of dendrimers

Different types of dendrimer having different functionalities have been developed using recent advances in synthetic chemistry and characterization techniques to overcome limitations and improve applications. The most commonly used types of dendrimer are mentioned in Figure 2 [110,120-133].

PAMAM Dendrimers

Synthesized by the divergent method,
Products up to generation 10 (G 10),
Due to the presence of positive charge on the surface, used in gene transfection.

PPI Dendrimers

Synthesized by divergent method,
Commercially available up to G5,
Applications in material science as well as in biology.

Liquid crystalline (LC) dendrimers

Limited application in the delivery of DNA,
Consist of mesogenic LC monomerase.

Core shell (tecto) dendrimers

Application in the field of nanomedicine including drug delivery,
Different compounds incorporated into dendrimers,
Potential use as diseased cell recognition and diagnosis, drug delivery

Chiral dendrimers

Potential use as chiral hosts for enantiomeric resolutions and as chiral catalysts for asymmetric synthesis

Peptide dendrimers

Produced by divergent and convergent methods,

Used in industry as surfactants, and in biomedical field as multiple antigen peptides (MAP), protein mimics and vehicles for drug and gene delivery.

Glycodendrimers

Encompass sugar moieties such as glucose, mannose, galactose and/or disaccharide,

Potential use as site-specific drug delivery to the lectin-rich organs.

Hybrid dendrimers

Obtained from various polymers,

Generated the compact, rigid, uniformly shaped globular dendritic hybrids,

Potential use as drug delivery.

PAMAM-organosilicon (PAMAMOS) inverted dendrimers

Potential applications for electronics, chemical catalysis, nano-lithography and photonics, *etc.*,

Unique properties; constancy of structure and encapsulate various guest species with nanoscopic topological precision.

Figure 2. Schematic illustration of dendrimer types and their properties [120-133]

3.3.2. Synthesis of Dendrimers

Dendrimers have three traditional macromolecular architectural classes: linear, cross-linked, and branched. Synthesis of dendrimers enables the possibility of generating monodisperse, structure-controlled macromolecular architectures [134].

Dendrimers can be produced by controlled branched chemistry. In the synthesis of dendrimers there are two main strategies: divergent and convergent. The divergent method is both costly and difficult. In this method, synthesis of dendrimers starts from the core. That is, the dendrimer grows outwards from a multifunctional core molecule. There is a disadvantage in that the extension of some branches may not be carried out smoothly causing deformations to occur during the production process. These deformations may impact the functionality of the dendrimer. Impurities are another problem for this method.

In the convergent method, the dendrimers are produced from the functional molecules. The branches then form inwards, eventually attaching to a core. This method is easier because impurities can be easily eliminated, but large quantities of dendrimers cannot be produced by this method [135-138].

The polymers used in dendrimer production are: PAMAM, poly(propylenimine) (PPI), poly(L-lysine), triazine, melamine, PEG and carbohydrate-based citric acid, poly(glycerol-co-succinic acid), polyglycerol, and poly[2,2-bis(hydroxymethyl)propionic acid]. Dendrimers can be based on monodispersed or polydispersed frameworks. Both of these methods influence the size of dendrimers because of the generation number. When dendrimers reach the maximum size, they form a tightly packed ball-like structure. The divergent and convergent methods are most frequently used for dendrimer synthesis. However, hypercores and branched monomer growth, double exponential growth and click chemistry methods have been improved in recent years [110,139,140].

3.3.3. Characterization of Dendrimers

There are many methods for the characterization of dendrimers. For example:

- Ultraviolet-visible spectroscopy (UV-VIS)
- Infrared spectroscopy (IR)
- Nuclear magnetic resonance (NMR)
- Mass spectrometry
- Raman spectroscopy
- Atomic force spectroscopy
- X-ray photoelectron spectroscopy (XPS)
- High pressure liquid chromatography (HPLC)

3.3.3.1. Nuclear Magnetic Resonance (NMR)

NMR is the most widely used technique for characterizing dendrimers. In fact NMR spectra are used during the synthesis of dendrimers but NMR has also been used for determination of dendrimer size and morphology in recent years. Special techniques including Cosy, HMBC, HSQC, ^1H , ^{13}C , ^{31}P , and ^{29}Si have used NMR. According to NMR technique, dendrimers or polymers are dissolved in any solvent, for example chloroform, dimethyl sulfoxide, methanol, *etc.* and then measured in NMR. However, sometimes dendrimers or polymers are not soluble. In that case, solid state NMR should be used [141-144].

3.3.3.2. IR spectroscopy

IR spectroscopy is generally used for the determination of synthesis and for determination of functional groups, conjugation and for the understanding of drug-dendrimer interactions. Moreover, infrared spectroscopy is used to understand the chemical transformations which occur at the surface of dendrimers. The appearance, disappearance, or chemical shift of characteristic peaks gives information about the progress of the synthesis. In general, nitrile groups, amine groups, aldehydes, *etc.* are examined in infrared spectroscopy for the characterization of dendrimers. Disappearance of nitrile groups or amine groups and the appearance or disappearance of hydrogen bonds indicates the synthesis and surface modification of dendrimers [145,146].

3.3.3.3. Ultraviolet-visible spectroscopy (UV-VIS)

UV-VIS spectrophotometry gives information about the synthesis and surface modification on dendrimer molecules by the observation of characteristic absorption maxima. Additionally, UV-VIS spectrometry can be used to determine the functional groups attached to the dendrimers by revealing shifts in the absorption peaks. UV-VIS spectra can be evaluated to measure reaction rates during the synthesis of dendrimers. The UV-VIS spectrum is easily recorded, however solvent selection plays an important role in correct measurement [147,148].

3.3.3.4. X-ray photoelectron spectroscopy (XPS)

XPS is a quantitative method and is used to measure the elemental composition, empirical formula, chemical, and electronic state and thickness of dendrimers. Aromatic rings, carbanile groups, *etc.* can be identified by using XPS [149].

3.3.3.5. Microscopy

Atomic Force Microscopy (AFM) and Transmission Electron Microscopy (TEM) are widely used for imaging dendrimers. AFM is used for surface examination whereas the inner layer and core of dendrimers can be examined by TEM.

Furthermore, the size, shape and structure of the core can be determined by using TEM and AFM [150].

3.3.3.6. Mass spectrometry

Mass spectrometry can only be used to characterize small dendrimers because there is an upper limit on the mass of molecules that can be determined in this way. Generally mass spectrometry is used to check for the presence of impurities in dendrimers. However, dendrimers generally have high molecular mass and so matrix assisted laser desorption ionization time of flight (MALDI-TOF) is used. In particular, PAMAM dendrimers cannot be characterized using mass spectrometry. In addition, it may be possible to determine chemical defects of dendrimers using mass spectrometry [151-154].

3.3.4. Applications of dendrimers in cancer treatment

The applications of dendrimers have been summarized as follows: cancer therapy (site-specific or passive targeting), gene delivery, photodynamic therapy, organ imaging, solubilization, drug delivery (ocular, transdermal, topical, pulmonary administration), vaccine delivery, diagnostic agents, and biomarkers; delivery of bioactives. Here we focus on the applications of dendrimers in cancer therapy.

Dendrimers have been extensively studied for use in cancer therapy owing to their unique properties. Different chemotherapeutic agents have been loaded into dendrimers to increase their therapeutic effects and it has been shown that they preferentially accumulate in cancer cells by passive targeting. On the other hand, dendrimers can be conjugated with a variety of cancer-targeting ligands (biotin, folic acid, amino acids, peptides, aptamers, and monoclonal antibodies) thus they can specifically treat cancer cells by active targeting. Furthermore, dendrimers are suitable as drug delivery systems for gene silencing [155-157]. Kesharwani and Iyer discussed some of the literature about dendrimer-mediated drug and gene delivery in their recent study [110]. The types of dendrimer and the loaded agents or genes which were published in the last year are summarized in Table 5.

Table 5. Summary of the incorporated drugs, and types and treatment of dendrimers in published papers

Incorporation drug	Types of dendimers	Treatment	References
Circulating cancer cells antibody conjugate	Poly(amido amine) (PAMAM) dendrimer	HUVECs	[158]
Diaminocyclohexyl platinum (II)	mPEGylated peptide dendrimer	SKOV-3	[159]

Doxorubicin (Dox)	PAMAM, modified with fluorescein isothiocyanate (FI) and LA (or polyethylene glycol (PEG)-linked LA, PEG-LA)	Liver cancer cells	[160]
	Dendritic poly(L-lysine)	<i>in vivo</i>	[161]
	PEG-PAMAM hybridized gold nanorod	HeLa, <i>in vivo</i>	[162]
	mPEGylated peptide dendrimer	4T1 breast tumor model	[163]
	PEGylated polylysine dendrimer	A syngeneic rat model of lung metastasised breast cancer	[164]
	PAMAM -coated iron oxide nanoparticles	MCF-7/Dox resistant breast cancer	[165]
	Cyclic arginine-glycine-aspartic acid (RGD) peptide-conjugated generation 5 (G5) poly(amidoamine) dendrimers	U87MG cells	[166]
Doxorubicin hydrochloride (Dox)	Poly(propyleneimine) (PPI) dendrimers	MCF-7 cell line	[167]
	Lactose-functionalized poly(amidoamine) dendrimers	A549, DU-145, and HT-1080	[168]
	PAMAM- folate-targeted dendrimer-polymer hybrid nanoparticles	BALB/c athymic nude mice bearing folate receptor (FR)-overexpressing KB xenograft	[169]
	Lysine dendrimers (G1-G3)		[170]
Viologen-based CXCR4 antagonists	-	U2OS human epithelial osteosarcoma and HepG2 cells	[171]
Tat peptide (Tat, T) conjugated	PAMAM dendrimer	S180 cells, Sarcoma 180-	[172]

		bearing mice	
Dicer-substrate siRNA (dsiRNA)	Peptide decorated dendrimer	PC-3 and CR prostatic cancer cells, MDA-MB431, MCF-7	[173]
Head shock protein 27 (Hsp27)- siRNA	PAMAM dendrimer	PC-3, MDA-MB431, <i>in vivo</i>	[174]
	PAMAM dendrimer	PC-3	[175]
Antisense oligonucleotide (ASO)	PAMAM dendrimer	A431, human epidermoid carcinoma cells, <i>in vivo</i>	[176]
siRNA enhanced green fluorescent protein (eGFP) gene	PAMAM dendrimer	A549 lung alveolar epithelial cells	[177]
dsDNA	PAMAM dendrimers conjugated with multiple folate (FA) or riboflavin (RF) ligands for cell receptor	KB cancer cell line	[178]
Akt siRNA- Paclitaxel (PTX)	Triethanolamine-core poly(amidoamine) dendrimer		[179]
Survivin – ASO	PAMAM dendrimer	Hepatic cancer model	[180]
Docetaxel (DTX)- PTX	Dendrimer-D- α -tocopherol PEG succinate (TPGS) mixed micelles	A549, MCF-7 and Chinese hamster ovary (CHO) cells	[181]
DTX	Dendritic copolymer H40-poly(D,L-lactide)	MCF-7	[182]
Methotrexate (MTX)	PAMAM dendrimers	MES-SA cells	[183]
	Folate conjugated-PPI dendrimers	HeLa and SiHa cells	[184]
	Octreotide-conjugated PAMAM	MCF-7 cells	[185]
	PAMAM dendrimer	LNCaP, PC3 cells	[186]
Melphalan	PPI dendrimers	<i>in vitro</i>	[187]

10-hydroxycamptothecin (10-HCPT)	PAMAM-long hydrophobic C ₁₂ alkyl chains, PEG chains and c(RGDfK) ligands	22RV1 cells	[188]
Follicle stimulating hormone receptor (FSHR)	PAMAM conjugated with the binding peptide domain of FSH (FSH33)	OVCAR-3, SKOV-3 cells	[189]

3.4. LIPOSOMES

Liposomes, which were first described by Dr Alec D. Bangham in 1961 (published 1964), are closed spherical vesicles composed of a natural or synthetic phospholipid lipid bilayer in which drugs or genetic material can be encapsulated (Figure 3). This structure presents a unique opportunity, since hydrophilic drugs tend to be encapsulated in the core; while hydrophobic drugs will be entrapped within the lipid bilayers. Liposomes deliver the drugs into cells by fusion or endocytosis mechanisms to provide controlled drug release, which is protected from degradation by plasma enzymes and has reduced side effects. Moreover, liposomes can be easily modified by coating the surface or attaching different specific surface biomarkers PEG, antibodies, and peptides to reach cancer cells by active targeting. The physical and chemical properties of a liposome (size, permeability, charge density, and steric hindrance, *etc.*) are determined by its constituent phospholipids [190-193].

Figure 3. The structure of liposomes [194-196]

Generally, liposomes can be classified as unilamellar or multilamellar (MLV, 100 nm – 20 μ m). Unilamellar liposomes are further divided into two categories: small size [small unilamellar vesicles (SUV), 25–100 nm] or large size [large unilamellar vesicles (LUV), 100–1000 nm]. SUV and LUV are both composed of a single lipid bilayer and a large aqueous core, and thus are suitable for loading hydrophilic drugs, while multilamellar liposomes (MLV) are composed of several lipid bilayers (up to 14 layers) and a limited aqueous space, thus being suitable for loading hydrophobic drugs [197,198].

Table 6. The advantages and disadvantages of liposomes [190-198]

Advantages of Liposome	Disadvantages of Liposome
Liposome are increased efficacy and therapeutic effects of drug by providing changes in the drug biodistribution.	Production cost is high.
Liposome is increased drug stability by control retention of entrapped drugs.	Leakage and fusion of encapsulated drug or molecules.
Liposomes are biocompatible, biodegradable, non-toxic, flexible and nonimmunogenic.	Sometimes phospholipid structure undergoes degradation.
Liposomes are reduction in toxicity of the encapsulated agent.	Short half-life due to chemical and physical instability.
Provides passive targeting or selective active targeting to tumour tissue.	Low solubility and limited encapsulation efficiency.
Liposomes can be generally used in the gene delivery and diagnostic imaging.	Unsterilization.

3.4.1. Liposome preparation methods

Different preparation methods have been used to control the properties of liposomes such as the size, structure, number of bilayers and interaction with drugs, *etc.* as shown in Figure 4. In these methods, drug loading into the liposomes is passive [199-203].

There are several considerations to be taken into account in selecting the loading method:

- 1) The physicochemical properties of the drug or agent to be entrapped must align with the liposome structure;
- 2) The preparation medium in which the liposomes are dispersed is important;
- 3) The effective concentration of the encapsulated drug or agent must be defined;

4) The size, type and delivery method of the liposomes must be determined in accordance with the desired target (active or passive targeting) for the intended application [204-206].

Figure 4. Schematic illustration of the main preparation techniques of liposomes [195,197-217]

Since the classical methods are limited for industrial-scale production of liposomes, a few liposome preparation methods have been improved to provide active targeting. These include the heating method, spray drying,

lyophilization, super critical reverse phase evaporation (SCRPE), the high-pressure extrusion method and the modified ethanol injection method as shown in Table 7 [195,205,218-221].

Table 7. Summary of new liposome preparation techniques [195,205,218-221]

Heating method	Based on the heating of liposome components in presence of glycerol due to providing an increase the stability No degradation lipids Particle size can be controlled Reducing the time and cost of liposome Not need any further sterelisation
Spray drying	Simple and industrially applicable method
Lyophilization	Based on removal of water from products at low pressures, Used to dry products, Provide sterilization, Submicron narrow sized liposomes, Long-term stability
Super Critical Reverse Phase Evaporation (SCRPE)	The SCRPE is based on using supercritical carbon dioxide A high trapping efficiency for both water-soluble and oil-soluble compounds
High pressure exterusion method	Converting MLv to SUV suspension Desirable diameters Homogeneous size distrubitions
Modified Ethanol Injection Method	
[1] The Crossflow Injection Technique	The control of the liposome size and the stability
[2] Microfluidization	Large scale continuous liposome preparation
[3] Membrane Contactor	

3.4.2. Characterization of liposomes

In order to determine the quality of liposomes and to obtain quantitative measures, the main characterization methods include observation of visual appearance and measurement, of size distribution, surface charge phospholipid content, lamellarity, composition, concentration of degradation products, encapsulation efficiency, and stability [195,215,222-224].

3.4.2.1. Determination of liposome size and zeta potential

The size and ZP of liposomes are important characterization parameters especially when the liposomes are used for therapeutic purposes including cancer or gene therapy. ZP gives information about the stability of colloidal systems. When the ZP value of a particle suspension changes within ± 30 mV, the nanoparticles are considered stable.

Some methods used for characterizing the liposomes include dynamic light scattering, static or size-exclusion chromatography (SEC) in conjunction with the aforementioned microscopy techniques, and field-flow fractionation. Additionally, NMR, flow cytometry, and capillary zone electrophoresis have been used in recent studies.

DLS, also known as photon correlation spectroscopy (PCS), is extensively used in drug carrier systems to measure size distribution and ZP. It is a fairly easy technique for obtaining data about low nanometer to low micrometer size particles in native environments with minimal sample volume.

HPLC using SEC is a simple but powerful method which can be used to detect and separate liposomes on the basis of size distribution and stability according to a time-based resolution of hydrodynamic size. Disadvantages of HPLC for liposomes size result from unwanted adsorption of lipids on the HPLC column and therefore, certain data cannot be obtained. However when the shape and chemical composition of liposomes are well matched with the column, liposome size and weight information can be measured. Field-flow fractionation (FFF) is a technique which is used to overcome some of the limitations of HPLC for liposome analysis. While large liposomes separate first in HPLC-SEC, small liposomes elute first in FFF because of its higher diffusion. However the carrier liquid used in FFF should be chosen carefully for the separation of small liposomes [225-228].

3.4.2.2. Encapsulation efficiency and *in vitro* drug release

Encapsulated and un-encapsulated drug fractions are present during liposome preparation. Therefore, it is important to determine the difference between the encapsulated drug within the liposomes and the free drug. Mini-column centrifugation, dialysis, spectrophotometry, fluorescence spectroscopy, HPLC, and FFF can be performed to determine encapsulation efficiency.

In vitro drug release can be measured using the dialysis tube diffusion technique. This technique is based on continuous magnetic stirring at 37 °C with samples of the dialysate taken at specified time intervals and assayed by HPLC or spectrophotometry [229-232].

3.4.2.3. Liposome stability

The liposomes stability is an important consideration for liposome preparation, storage and delivery. The applications of liposomes for drug

delivery are based on high molecular weight drugs loading into liposome suspension which consist of different lipids or polymers. The liposome stability affects the efficient delivery of the contents and successful loading into liposomes. Another important problem is that the average size distribution of liposomes changes upon storage resulting from vesicles affinity for each other leading to larger aggregates. This leads to drug leakage from the vesicles. The chemical stability of the liposomes is also changed by interactions of drugs with the phospholipid layer. Sterilization is a further important parameter to consider in providing microbial stability for therapeutic applications [233,234].

3.4.2.4. Lamellarity

The number of lipid bilayers, the lipid constitution and the preparation technique, impact on the encapsulation efficiency and drug release properties of liposomes as well as their cellular uptake. Liposome lamellarity is often measured by microscopic (TEM, cryo-TEM) or spectroscopic techniques (UV absorbance, NMR, small angle X-ray scattering (SAXS) [235,236].

3.4.2.5. Morphology of liposomes

The visual appearance of liposomes is changed in terms of the composition and particle size. TEM is a technique for size, morphology, and lamellarity characterization of liposomes, especially for small liposomes. However SEM is sufficient to visualize large vesicles. In recent years, cryo-transmission electron microscopy (cryo-TEM) has been used to eliminate structural changes resulting from the use of negative stains such as uranyl acetate or osmium tetroxide in TEM. Additionally, AFM has been utilized to show liposome morphology (especially for small liposomes), size and stability without sample manipulation. AFM analysis is a rapid and powerful technique to obtain information about liposome morphology and aggregation processes during their storage [226,237-239].

3.4.3. Clinical applications of liposomes in cancer treatment

Liposomes encapsulating a wide range of drugs (*e.g.*, doxorubicin, paclitaxel and cisplatin) and even approved by FDA, have been successfully applied for cancer treatment because of their unique properties including their size, their possession of both hydrophobic and hydrophilic structures, their reduced toxicity, increased therapeutic effects and biocompatibility/biodegradability, *etc.* Additionally, liposomes have been used in gene therapy research to encapsulate various genetic materials such as genes, miRNA, siRNA, *etc.*

The first liposomal pharmaceutical product “Doxil” was approved in 1995 and the latest “Marqibo” in 2012 [240]. However, some recent liposomal formulations have been developed to eliminate their disadvantages (degradation of lipids, size problems) and improve efficacy in targeting cancer

cells. The approved liposomal products and ongoing clinical trials are mentioned in Table 8.

In conclusion, new research has continued to achieve better formulations, extend the *in vivo* circulation time, improve tumor delivery and reduce toxicity for the clinical application of liposomes in cancer treatment [241,242].

Table 8. Clinical applications and certain approved liposome-based products currently on the market

Drug	Product Name	Treatment	Trial phase	Approved by FDA	References
Doxorubicin	Myocet	Metastatic breast cancer			[243]
	Lipo-dox	Kaposi's sarcoma, ovarian and breast cancer			[244]
	ThermoDox	Non-resectable hepatocellular carcinoma	III		[245-247]
Daunorubicin	DaunoXome	Blood cancer			[248]
Vincristine sulfate	Marqibo	Acute lymphoblastic leukemia			[249-251]
	LEP-ETU	Ovarian, breast and lung cancers	I		[252]
Paclitaxel	EndoTAG-1	Anti-angiogenesis, breast and pancreatic cancer	II		[253,254]
	SPI-077	Lung, head and neck cancers	I/II		[253,255]
Cisplatin and its analog	Lipoplatin	Pancreatic cancer, head and neck cancer, mesothelioma, breast cancer, gastric cancer and non-small-	III		[253,256-258]

		cell lung cancer.		
		Malignant pleural mesothelioma and advanced colorectal carcinoma	II	[199]
		Advanced or refractory solid tumours	I	[259]
Mitoxantrone	LEM-ETU	Leukemia, breast, stomach, liver and ovarian cancers	I	[252]
Topotecan	INX-0076	Advanced solid tumors	I	[199,253]
Vinorelbine	INX-0125	Breast, colon and lung cancers	I	[199,253,260]
Lurtotecan	OSI-211	Ovarian, head and neck cancers	II	[199]
BLP25 lipopeptide	Stimuvax	Non-small-cell lung carcinoma	III	[199,261]
All-trans retinoic acid	Atragen	Advanced renal cell carcinoma	I/II	[199,262]
Annamycin	Liposome Annamycin	Breast cancer	I/II	[199,263,264]
Cytarabine and daunorubicin	CPX-351	Acute myeloid leukemia	II	[199,265]
Irinotecan HCL and floxuridine	CPX-1	Colorectal cancer	II	[199,266]
	MM-398	Metastatic pancreatic cancer	III	[267]

3.5. SOLID LIPID NANOPARTICLES (SLNs)

SLNs which are in the size range of 10–1000 nm, are attracting major attention as a novel colloidal drug carrier with the potential to overcome limitations in drug delivery including poor drug loading capacity, size problems, unstable properties, uncontrolled drug release associated with polymeric nanoparticles, dendrimers and liposomes. SLNs offer unique properties such as small size, large surface area, high drug loading and entrapment efficiency, low toxicity, excellent physical stability, controlled drug release, protection of drugs from degradation, and avoidance of the use of organic solvents.

The structure of SLNs based on lipids that are solid at room temperature. The lipid structure of SLNs is important to determine whether or not a loaded molecule can be strongly encapsulated within a delivery system. Therefore, the lipids that form the SLNs core structure could be important for high-rate drug loading. Additionally, most lipids that form the structures of SLNs are biodegradable, so SLNs show excellent biocompatibility and have less toxic effects. When the use of SLNs is investigated, it seems that SLNs have often been selected for lipophilic and hydrophilic drug compatibility with lipids that do not have toxic effects as carriers. However, in recent years, increasing attention has also been paid to the coating of SLNs in order to load lipophobic and hydrophilic drugs in the lipid structure and also to provide gene delivery.

Table 9. The advantages and disadvantages of SLNs [268-283]

Advantages of SLN	Disadvantage of SLN
Long-term stability, controlled drug release and targeting.	Poor drug loading capacity.
Decreasing the danger of acute and toxicity of drug or compound.	Degradation of drug in the SLNs during storage.
Enhancing the bioavailability of entrapped drug or compound.	Having high water content.
Chemical protection of labile incorporated compound.	Low capacity to load hydrophilic drugs.
Easily large scale production.	Sometimes burst release of incorporated drugs.

Different methods are being developed to prepare SLNs, such as high pressure homogenization (hot and cold homogenization), microemulsion, solvent emulsion diffusion, solvent evaporation, ultrasonication/high speed homogenization, and spray drying methods. However it is very important to choose the best methods to avoid drug degradation and lipid crystallization according to the properties of the incorporated substances. After production, the second most important step is the characterization of nanoparticles.

Analytical methods can be used to characterize SLNs in terms of particle size, ZP, PCS, surface characteristics (TEM/SEM), crystallinity [differential scanning calorimetry (DSC), XRD], chemical shift, NMR, drug entrapment efficiency, and *in vitro* drug release studies (UV spectroscopy, HPLC). After SLNs are characterized, the third most important step is their application according to the desired properties. Different applications have been studied in the literature, especially in cancer treatment: (i) chemotherapeutic drugs incorporated into SLNs to eliminate their disadvantages and overcome MDR, (ii) gene therapy (delivery of peptides, genes, miRNA, siRNA into cells), (iii) drug targeting, new adjuvants for vaccines, treatment of infectious diseases, cosmetic and dermatological preparations and agricultural applications [268-283].

3.5.1. Production methods of SLNs

Figure 5. Schematic illustration of SLN preparation methods

3.5.1.1. High-pressure homogenization

High-pressure homogenization comprises two basic production techniques for SLNs: hot homogenization and cold homogenization. In both techniques, the drug is dissolved in molten lipid.

3.5.1.1.1. Hot homogenization technique

This technique is the most common method for producing SLNs. In this method the lipid phase is first melted at 80 ± 1 °C with stirring (above the melting point of the solid lipid) and the temperature is set to 80 °C in a thermostated water bath. The drug (in solid state) is added into the molten lipid at the same temperature. Later, a surfactant is added slowly to the mixture and homogenized by ultra-turrax at 20000–25000 rpm. Finally, the obtained mixture is homogenized using a high-pressure homogenizer at a pressure ranging from 100–1500 bar. Three to five homogenization cycles at 500–1500 bar is sufficient. The mixture is then cooled at room temperature and put into glass vials.

Due to the fact that the loss of hydrophilic drugs to the water medium can be considerable, the temperature should be carefully selected. This technique can generally be applied to lipophilic drugs [284-289].

3.5.1.1.2. Cold homogenization

The cold homogenization process is similar to hot homogenization. Initially, the solid lipid phase is melted and the temperature is set to 80 °C in a thermostated water bath with stirring. The drug (in solid state) is added into the molten lipids at the same temperature. Surfactant is then slowly added to the mixture. The mixture is cooled rapidly using liquid nitrogen or dry ice to distribute the drug homogeneously in the lipid matrix. Finally, the obtained mixture is powdered to micro and nanoparticle size using various methods (for example ball milling). It is exposed to high-pressure homogenization at room temperature or below room temperature. At this stage, it is important that the high-pressure homogenization is maintained at an appropriate temperature. In appropriate conditions, the obtained particles have micrometer diameters.

Many heat-sensitive drugs can be incorporated into lipid nanoparticles using this technique, which is more suitable for hydrophilic drugs [270,290].

3.5.1.2. Microemulsion method

A high surfactant/lipid ratio is used in this method. Microemulsions are composed of an inner and outer phase (e.g. oil-in-water). In this method, lipids are melted and surfactant is heated to the same temperature as the molten lipids. The solutions are mixed and a microemulsion is obtained. This mixture is at approximately 60–80 °C and is transparent. Next, the microemulsion is

dispersed in cold water at 2–3 °C with stirring. Liquid droplets are solidified by the cold water because of the high temperature gradient. In this method, the risk of aggregation is very low and the method is easy to perform. However, it has several disadvantages. For example, microemulsions have very low concentrations. The high concentration of surfactants/co-surfactants is another drawback [291-294].

3.5.1.3. Solvent emulsification/evaporation

Another method for the production of nanoparticle dispersions is solvent evaporation. Initially, lipid and drug are dissolved in organic-immiscible solvent such as chloroform, *etc.* The organic solution is emulsified in an aqueous phase using surfactant. Solvent is evaporated from nanoparticulate dispersions and the lipid is precipitated as dispersed nanoparticles. The main advantage of this method is that obtained nanoparticles have diameters between 25–100 nm. However, this method has some disadvantages such as limitation of the lipid concentration [295-297].

3.5.1.4. Supercritical fluid (SCF) technique

This method is performed above the critical temperature and critical pressure of a substance. Under these pressure and temperature conditions, the substance can exist in equilibrium between vapor and liquid form. In order to increase the amount of dissolved compound, the pressure can be increased while viscosity remains constant. Under these temperature and pressure conditions, the fluid can act like an organic solvent and so it simply dissolves lipids and other components. Generally, carbon dioxide is used as a super critical fluid because it is safe, cheap and non-irritating. However, obtained nanoparticles using this method have large diameters (micrometer range). Therefore this method is applied along with other methods *e.g.*, homogenization methods [297-299].

3.5.1.5. Ultrasonication

SLNs can also be produced by sonication. Whereas this method is easily applied in any laboratory, the particle sizes of the obtained SLNs are in the micrometer range. Additionally, physical instability and the potential for metal contamination are cited as other disadvantages of ultrasonication [300].

3.5.1.6. Spray-Drying

The spray-drying method of obtaining an aqueous SLN dispersion can be used as a less expensive alternative to lyophilization. The drawback of this method is that particle aggregation can be caused by the high temperatures, shear forces and partial melting of the particle [273,278,301].

3.5.2. Characterization of SLNs

3.5.2.1. Particle size

There are various techniques for the measurement of the particle size of nanoparticulate systems such as PCS (also known as dynamic light scattering), laser diffraction (LD) and the Coulter counter method. However the easiest and most useful techniques are PCS and LD. As particles move stably in the dispersion, they scatter light passing through the solution. PCS measures fluctuations in the intensity of this scattering light. PCS is an especially good method for nanoparticulate systems because of its working range from 1 nm to 3 μm . The LD method is more effective and useful method than PCS because it is related to the diffraction angle of the particle radius. Thus, small particles give rise to intense scattering whereas big particles cause less scattering. The working range of this method is from 1 nm to 1000 μm . The Coulter counter is hardly used in comparison with other methods because it is quite difficult to measure small particles. However, for electrolytes, which destabilize dispersive systems, it is necessary to use this method. Polydispersity index (PDI) is measured using PCS and is used for measurement of particle size distribution [270,278,297].

3.5.2.2. Zeta potential

ZP has been generally used to characterize colloidal dispersions. ZP, which measures surface charge, gives information about the storage stability of SLNs and their surface morphology. Therefore, ZP is generally used in product stability studies and surface adsorption research. ZP measures the magnitude of the electrostatic repulsion or attraction between particles. If nanoparticle systems show higher surface charge, a higher ZP value can be measured. On the other hand, aggregation of particles occurs when particles have low ZP because there is little electrical repulsion between the particles.

ZP value less than -60 mV is excellent, if it is less than -30 mV there is good electrostatic stabilization, so it can be said that particles show good physical stability. Colloidal systems which contain steric stabilizers are an exception because they can have good long-term stability even though ZP is 0 mV. Various factors such as particle size and shape, molecular weight, pH, surfactants, water impurities and water conductivity affect the ZP value [276,301-303].

3.5.2.3. Differential scanning calorimetry (DSC)

DSC is used to determine the sample structure and interactions between the components such as lipids, surfactants, *etc.* especially in colloidal dispersions. DSC compares the melting enthalpy of the bulk material with the melting enthalpy of the dispersion. That is, firstly, it measures amount of heat required to raise the temperature of a sample, then it determines amount of heat

required to raise temperature of a reference material. Finally, it evaluates differences (positive or negative) between them in heat transfer. During the phase transition, there are some physical changes or variations in the sample compared with known materials, because different lipids have different melting enthalpies and melting points [304].

3.5.2.4. Nuclear magnetic resonance (NMR)

NMR gives information about the physicochemical status of components within the nanoparticle. Other methods give information about mobile or dissolved constituents in colloidal systems, whereas solid state NMR is suitable for characterizing solid particles because of its ability to determine molecular mobility, rotational diffusion of particles, and phase transition of the particle matrix. Spin-lattice in rotating frame, only on proton spin measurement, should be applied to determine particle homogeneity and heterogeneous colloidal systems, especially the different chemical shifts which belong to each specific molecule. For example, methyl protons of lipids give signals at 0.9 ppm whereas protons of PEG chains give signals at 3.7 ppm. As a result NMR experiments provide confidence in the data because of their repeatable results [291,305,306].

3.5.2.5. Drug release

There are three different drug incorporation models (Figure 6). Drug release of solid lipid nanoparticles depends on the lipid matrix, the surfactant concentration and the temperature. In addition, drug release also depends on particle size and particle shape. In general, SLNs have a circular shape and an average 100–500 nm diameter and so drug release is controlled and sustained over a long period. Drug release from solid lipid nanoparticles produced by the hot homogenization method is faster than from those produced by cold homogenization [307,308]. Drug release studies are generally carried out using UV-VIS spectrometry or HPLC.

Figure 6. Three drug incorporation models

3.5.2.6. Entrapment efficiency (EE) and drug loading capacity (DL)

EE is the percentage of active ingredient within the nanoparticle core. EE is determined by measuring the concentration of free active ingredient in the dispersion medium. There are various methods to determine the EE. The most commonly used method is UV-spectrophotometry, because it is applied quickly and easily. The entrapment efficiency is calculated by the following equation [307,309]:

$$EE\% = \frac{M_{\text{initial drug}} - M_{\text{free drug}}}{M_{\text{initial drug}}} \times 100$$

where “ $M_{\text{initial drug}}$ ” is the mass of initial drug and the “ $M_{\text{free drug}}$ ” is the mass of free drug.

Drug loading capacity (DL) is calculated by the following equation:

$$DL\% = \frac{\text{Amount of drug in the particles}}{\text{Amount of drug + excipients added in total to the formulation}}$$

3.5.2.7. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM)

SEM and TEM are used to visualize colloidal dispersions by providing information about the size, shape and morphology of the particles. The surface of nanoparticles can be examined by SEM because SEM detects scattered electrons from the surface of the particles. Inner layers of nanoparticles can be examined by TEM which determines transmitted electrons. There are some disadvantages of both methods such as heating of the sample and difficulty in sample preparation [310-312].

3.5.2.8. X-ray scattering (XRD)

X-ray scattering is a good method for examining the lipid lattice structure of particles. That is, measurement of spacing in the lipid lattice can be performed by X-ray scattering. An advantage of this method is that samples are measured in their native state after production, and there is no process causing changes to the chemical environment. A disadvantage of this method is lack of sensitivity and long measurement time. The method consists of two techniques. The first one is SAXS, the other one is wide angle X-ray scattering (WAXS). Both methods can be used for the characterization of colloidal dispersions [310-314].

3.5.2.9. Powder X-ray diffraction (PXD)

PXD is a commonly used technique whereby the crystal structure of colloidal dispersions can be examined. In this technique, X-rays of fixed wavelength are passed through the sample. Inter-atomic spacing is calculated using the obtained data. However, samples have to be solid form in order to use this method. If a sample is in liquid form, it must be solidified by spray drying or lyophilization [290,310].

3.5.3. Applications of SLNs in cancer treatment

MDR and the disadvantages of chemotherapeutic agents such as adverse side effects, toxicity to normal tissues, poor specificity and stability, *etc.* remain major barriers to the success of anticancer chemotherapy. MDR is often mediated by drug efflux transporters such as P-glycoprotein (P-gp), which are overexpressed in cancer cells and therefore reduce cellular accumulation of the drugs. In addition, cancer cells tend to be more resistant to chemotherapeutic agents because of various permeability barriers, which prevent the accumulation of high drug concentrations. Another problem stems from the different physicochemical properties and highly diverse molecular structures of anti-cancer agents.

Nano based drug delivery systems have been improved to overcome these limitations to the delivery of therapeutic agents. SLNs have attracted attention in the field of cancer biology for the delivery of anti-cancer drugs in targeted organs or tissues. They can provide controlled drug release and reduce the associated toxicity, thus eliminating some disadvantages in the therapy. Additionally, SLNs have improved the stability of drugs, improved the encapsulation of chemotherapeutic agents with different physicochemical properties, enhanced drug effects on tumor cells by enhanced permeability and retention effects (EPR) and improved the pharmacokinetics of drugs. The EPR effect is based on the extra-vascular space which results in accumulating high drug concentration because the EPR effect helps to carry the nanoparticles inside the cancer tissue. The passive targeting of cytotoxic drugs enables their incorporation into SLNs. Furthermore, SLNs can target drugs to specific sites by surface modification.

Different therapeutic agents have been incorporated into solid lipid nanoparticle-based systems and their effects on various cancer cell lines have been evaluated as shown in Tables 10, 11 and 12. When the use of SLNs as drug delivery systems have been investigated, SLNs have been shown to increase the cytotoxic effects of the drugs as well as reducing toxic effects on normal cells. The ability to overcome the resistance of P-gp based MDR has been attributed to increased cellular accumulation of the drug by passive/or active targeting [297,315-319].

Table 10. The incorporation drug, treatment and preparation methods of SLNs associated with MDR

Drug / Agent	Cell Lines	Method of preparation	References
Doxorubicin (Dox)	-EMT6/AR1 (Murine breast cancer cell line)-MDA435/LCC6/MDR1 (human breast resistant cancer cell line)	Dox loaded polymer lipid hybrid nanoparticles (PLN)	[318-320]
Dox	-MCF-7/ADR cells (Dox resistant cell line)	Solvent emulsification-diffusion methods	[321]
Dox	-MCF7-MCF7/ADR	Solvent emulsification-diffusion method	[310]
Dox and Paclitaxel (PTX)	-NCI/ADR-RES (human ovarian adriamycin resistant carcinoma cell line) -OVCA8-8 (human ovarian carcinoma cell line) -MDA-MB435/ LCC6 /MDR1	Warm o/w microemulsion precursors	[322]
PTX and Dox	-MCF-7 -MCF-7/ADR -SKOV3 (human ovarian cancer cell)-SKOV3-TR30 (human ovarian resistant cancer cell)	Solvent diffusion method	[323]
PTX and Verapamil (VP)	-MCF-7/ADR resistant cells	Hot sonication method	[324]
GG918 (Elacridar) Dox	-MDA-MB 435/LCC6/WT-MDA-MB 435/LCC6/MDR1		[325]
Dox and mitomycin C (MMC)	-MDA-MB 435/LCC6 /WT -MDA-MB 435/LCC6 /MDR1		[326]
Idarubicin and Doxorubicin	-P388 -P388/ADR -HCT-15 cell lines	Co-precipitation method	[327]
Antisense	-NCI/ADR-RES	Microemulsion	[328]

Glucosylceramide Synthase Oligonucleotide (MBO-asGCS)		method combining shear and ultrasonic homogenization techniques
Dox and MBO- asGCS	-NCI/ADR-RES	[329]

Table 11. The incorporated drugs, treatments and preparation methods of SLNs in published papers

Drug / Agent	Cell Lines	Method of preparation	Reference(s)
Cholesteryl Butyrate	-HUVEC (Epithelial cell lines), -HT29, -HCT116 -CaCo-2 Human colon adenocarcinoma -MCF-7, -PC-3 (Human prostate carcinoma), -M14 and LM cells (Human melanoma)	Warm microemulsions	[330]
Hyaluronic Acid (HA) and Vorinostat (VRS)	-A549, -SCC7 (Human alveolar adenocarcinoma cell line), -MCF-7	HA was attached onto the surface of cationic SLNs by electrostatic attraction	[331]
Zanamivir	-Caco-2	A double emulsion (W/O/W) solvent evaporation method	[332]

5-Fluorouracil (5-FU)		Temperature-modulated solidification process	[333]
Docetaxel	-TC-1 cells (murine lung cancer cell line), -M-Wnt cells (murine mammary gland cell lines), -Human breast adenocarcinoma cells (MDA-MB-231)	Emulsion/solvent evaporation method	[334]
	-MCF-7	High-pressure homogenization method	[335]
	-BEL7402 hepatocellular carcinoma	Homogenization method	[336]
	-hCMEC/D3 a primary human brain microvascular endothelial cell line -NO3 glioblastoma cell line	Coacervation technique	[337]
	-A549	Solvent diffusion method	[338]
Paclitaxel (PTX)	-Sarcoma 180 ascited tumor and tumor bearing mice	Solid-liquid lipid nanoparticle (SLLN) modified with folate and PEG	[339]
	-A549	Film dispersion methods modified SA-R8	[340]
	-OVCAR-3 human ovarian cancer cells and -MCF-7	Homogenization	[341]
	-The MXT variant B2 (MXT-B2) was obtained through the isolation of epithelial cells representing different stages of the disease from mice breast adenocarcinoma (murine mammary	Melted homogenization technique	[342]

adenocarcinoma-MXT)			
Paclitaxel (PTX), Methotrexate (MTX), Mitoxantrone (MTO)	-MCF-7		[343]
Butyrate, Dox, PTX	-HT-29	Microemulsion technique	[344]
Podophyllotoxin (PPT)	-HeLa (HL-60) cells	Solvent emulsification- evaporation method	[345]
Mitotane	-Adenocortical carcinoma	Hot high pressure homogenization	[346]
2- methoxyestradiol	-MCF-7, -PC-3, -Glioma (SK-N-SN)	Hot homogenization- ultrasonication method	[347]
Curcumin (CU)	-HCT-116	Coacervation technique	[348]
	-HL-60, -A549, -PC3		[349]
	-MCF-7	Homogenization method	[350]
	MCF-7 cells	High-pressure homogenisation	[351]
	-A549 and xenografts.		[352]
Ferritin	-MDA-MB-468 breast cancer cells		[353]
Methotrexate	-EAC (Ehrlich ascite carcinoma) berain mice	Micro emulsification solidification method	[354]
Tamoxifen	-MCF-7	Microemulsion and precipitation techniques	[355]
		Temperature- modulated solidification methods	[356]

	-MCF-7	Hot high pressure homogenizer technique	[357]
Tamoxifen citrate		Solvent injection method	[358]
Camptothecin(CPT)	-MCF7	Supercritical fluid technology	[359]
Trilaurin (TL) and (PTX)	-Human ovarian cancer cells (SKOV-3)	Hot-melt high-pressure homogenisation	[360]
Carmustine	-The human GBM U87MG cells	Microemulsion catanionic solid lipid nanoparticles (CASLNs) with surface anti-epithelial growth factor receptor	[361]
Etoposide (VP16)	-SGC7901 cells.	Emulsification and low-temperature solidification methods	[362]
Etoposide	-The Dalton's lymphoma tumor cells were maintained in the peritoneum of Balb/c mice	Melt emulsification and homogenization technique	[363]
	-B16F10 melanoma colonization in lung	Hot homogenization	[364]
Doxorubicin (Dox)	-A549, -MCF-7	Solvent injection method	[365]
	-B16F10	Cationic solid lipid nanoparticles	[366]
	-A549	Hot melting homogenization method	[367]
Resveratrol	-Cells from immortalized human keratinocyte cell line NCTC2544	Melt-emulsification process	[368]
Emodin	-MCF-7, -MDA-MB-231, -MCF-10A	High pressure homogenization (HPH).	[369]
Rhodamine 123	-A172, U251, U373 U87	Emulsification	[370]

(R123)	human glioma cell lines -Macrophage cell line (THP1)	process	
Mitoxantrone	-MCF-7 -Model of P388 lymph node metastases in Kunming mice	Dispersion- ultrasonication method	[371]
Shikonin-Act	-A549 cells	Hot homogenization method	[372]
Histone deacetylase inhibitor, vorinostat (VOR),	-MCF-7, -A549, -MDA-MB-231	Hot homogenization using an emulsification- sonication technique	[331]
Retinoic acid	-MCF-7	Hot melting homogenization method using an emulsification- ultrasound	[373]

Table 12. The incorporated drugs, treatments and preparation methods of SLNs associated with gene delivery

Drug/ Agent	Cell Lines	Method of preparation	Reference (s)
c-MeT siRNA	-U-87MG glioblastoma and xenograft model	Cationic solid lipid nanoparticlesbconjugated Pegylated c-Met siRA	[374]
Anti-microRNA for miR-21	-A549	AMO-CLOSS (Cationic lipid binded oligonucleotide (AMO)) loaded SLNs)	[375]
Green fluorescence protein plasmid (pEGFP) and Dox	-A549 and mice beraing A549 tumors		[376]
Pre-miR-107	-SCC15, -SCC25, -CAL27, -UMSCC74A head and neck squamous cell carcinoma	Cationic lipid nanoparticles	[377]
Kinesin spindle protein (KSP) targeting siRNA	Murine endothelial cell line MS1-VEGF - HUVEC	DOPC(DOPC/DOTAP/DOPE- PEG2000) or DOPE (DOPE/DOTAP/DOPE-PEG2000) based preparations the thin film and hydration method.	[378]
miRNA-34a- PTX	-B16F10 -C57BL/6 mice	a film ultrasonic method	[379]
PTX and siRNA	human epithelial carcinoma KB cells	emulsification solidification methods.	[380]

3.6. CONCLUDING REMARKS

The applications of nanotechnology in drug delivery will be a potential priority research area for the pharmaceutical and biotechnology industries in the future due to its unique potential to overcome the limitations and drawbacks of conventional drugs. It may be possible to develop new strategies providing target-specific drug therapy through newly developed drug delivery systems in cancer treatment.

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CHARACTERIZATION OF DRUG-LOADED NANOPARTICLES

**Irina Kalashnikova¹, Norah Albekairi²,
Sanaalarab Al-Enazy², and Erik Rytting^{1,2,3*}**

¹ Department of Obstetrics & Gynecology, University of Texas Medical Branch, Galveston, Texas, USA

² Department of Pharmacology & Toxicology, University of Texas Medical Branch, Galveston, Texas, USA

³ Center for Biomedical Engineering, University of Texas Medical Branch, Galveston, Texas, USA

*Corresponding author: erik.rytting@utmb.edu

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4.1. INTRODUCTION

Advantages of nanoparticles for drug delivery applications include drug targeting, controlled drug release, protection of therapeutic payload, and improved bioavailability [1]. In the development of drug-loaded nanoparticles, it is imperative that the nanoparticles are adequately characterized in terms of size, surface charge, encapsulation efficiency, and drug release. These characteristics will guide the scientist in the selection of an optimal nanoformulation to enhance drug delivery. Targeting ligands on the nanoparticle surfaces can promote cellular uptake and transport. These include peptides, small molecules, transferrin, and antibody fragments [2].

Several materials have been utilized for the development of drug-loaded nanoparticles, including polymeric nanoparticles, polymeric micelles, dendrimers, liposomes, and solid lipid nanoparticles [3]. Examples of these nanoparticle types will be reviewed, followed by definitions and equations related to typical steps for the characterization of drug-loaded nanoparticles. As this chapter encompasses a wide variety of nanoparticle types, readers are referred to specific citations for details regarding methods for the synthesis of each type of drug-loaded nanoparticle.

4.1.1. Polymeric nanoparticles

Table 1 lists a number of synthetic and natural polymers that have been utilized in the preparation of nanoparticles for drug delivery [4-9]. Methods for the preparation of nanoparticles from synthetic polymers include emulsion-solvent evaporation/extraction, nanoprecipitation, emulsification/solvent diffusion, salting out, dialysis, emulsion polymerization, spray drying, supercritical fluid technology, electrospraying, sonoprecipitation, and aerosol flow reactor methods [4,6,8,10]. Production of nanoparticles from natural polymers includes methods such as coacervation/precipitation, emulsification, emulsion cross-linking, spray drying, ionic gelation, emulsion-droplet coalescence, sieving, and a reverse micellar method [7,11]. The best nanoparticle synthesis method for drug encapsulation will depend on the desired particle characteristics, the type of polymer, and the physicochemical properties of the drug.

Table 1. Examples of natural and synthetic polymers used in the development of drug-loaded nanoparticles

Natural Polymers	Synthetic Polymers
Chitosan	Poly(caprolactone)
Gelatin	Polylactide, Polyglycolide
Sodium alginate	Poly(alkylcyanoacrylates)
Pullulan	Poly(ethyleneimine)
Starch	Poly(methylidene malonate)
Heparin	Polyanhydrides
Proteins	Poly(ortho esters)
	Poly(methyl methacrylate)
	Poly(vinyl alcohol)

A number of polymeric nanotherapeutics have proceeded to clinical trials, including CALAA-01 (a cyclodextrin and poly(ethylene glycol) (PEG) containing anticancer siRNA delivery platform) and BIND-014 (a poly(lactic-co-glycolic acid) (PLGA)-PEG nanoplatform for the delivery of doxorubicin to treat prostate cancer) [12].

4.1.2. Polymeric micelles

Polymeric micelles can be produced by a direct dissolution method, an evaporation method, or nanoprecipitation/dialysis methods [13-16]. Several applications for drug-loaded polymeric micelles have been investigated, such as anticancer therapy, drug delivery to the brain, delivery of antifungal agents, and polynucleotide therapies [17-29]. Table 2 displays some anticancer polymeric micellar formulations that have progressed to clinical trials. The stability of polymeric micelles is dependent on the critical micelle concentration (CMC). If *in vivo* administration of the micelles results in dilution to below the CMC, this will lead to rapid dissociation of the micelles [30].

Table 2. Some polymeric micelle formulations that have been in clinical trials [19,31-41]

Name	Drug	Polymeric composition
NK105	Paclitaxel	PEG-polyaspartate
Genexol-PM	Paclitaxel	PEG-poly(D,L-lactic acid)
NC6004	Cisplatin	PEG-poly(glutamic acid)
NK012	SN-38	PEG-poly(glutamic acid)
SP1049C	Doxorubicin	Mixture of pluronic block copolymers L61 and F127
NK911	Doxorubicin	PEG-polyaspartate

4.1.3. Dendrimers

Dendrimer materials commonly used in nanomedicine include poly(amidoamine) (PAMAM), poly(L-lysine), polyesters, poly(2,2-bis(hydroxymethyl)propionic acid), and amino-bis(methylenephosphonic acid) [42]. Dendrimers have been investigated as drug delivery systems for nonsteroidal anti-inflammatory drugs, antivirals, antimicrobials, anticancer agents, and antihypertensive drugs [43]. Synthetic approaches include divergent synthesis, convergent methods, click chemistry, solid-phase synthesis, self-assembly, and supramolecular synthesis [42-47]. Dendrimers can accommodate small molecules in their interiors, internal cavities, and surface channels. Drugs can be entrapped by chemical conjugation or by complexation through hydrophobic or electrostatic interactions [48]. The dendrimer structure (generation number, branch components, functionality of external termini and the core) will also affect drug entrapment.

4.1.4. Liposomes

Liposomes consist of phospholipid bilayers with amphiphilic properties and have been investigated as drug carriers since the 1960s. Hydrophobic agents can be solubilized within the phospholipid bilayers, and hydrophilic compounds can be entrapped within the aqueous core [49,50]. Liposomes are biocompatible and have been developed for the delivery of anticancer drugs, antibiotics, anti-inflammatory drugs, genes and proteins [49-54]. Liposome synthesis and control over particle size can be achieved by the thin film method, sonication, and extrusion [55-58]. Food and Drug Administration (FDA)-approved liposomal formulations include DepoCyt®, AmBisome®, Doxil®, Marqibo®, and DaunoXome® [59].

4.1.5. Solid lipid nanoparticles

Solid lipid nanoparticles have gained attention due to advantages such as increased drug stability, high drug payload, avoidance of organic solvents, and ease of preparation [60]. Solid lipid nanoparticles may enhance oral drug absorption, lymphatic uptake, and bioadhesion [61]. Dermal applications of solid lipid nanoparticles are possible due to improved skin hydration, viscoelasticity, and ultraviolet (UV) protection [62-64]. Solid lipid nanoparticles have been investigated for anticancer drug targeting, gene delivery, ocular delivery, pulmonary delivery, and brain targeting [65-68]. Methods for preparing solid lipid nanoparticles include high shear homogenization and ultrasound, high pressure homogenization, solvent emulsification/evaporation, solvent injection, and dilution of microemulsions or liquid crystalline phases [69-73].

4.1.6. Metal and metal oxide nanoparticles

The multiple properties of gold nanoparticles make them good candidates for theranostic applications. Depending on their size and shape, gold nanoparticles may absorb light in the near infrared region, thereby avoiding potential interference from tissue autofluorescence. Functionalization of gold nanoparticles is possible by means of gold-thiol covalent bonds, which allows for the conjugation of drug molecules to nanoparticle surfaces [74]. Release of the therapeutic cargo can be triggered by glutathione displacement. Gold-amine binding and other non-covalent strategies have also been explored. Gold nanoparticles may facilitate the delivery of antibiotics, anticancer agents, and oligonucleotides for gene therapy [74]. Mesoporous silica and zinc oxide nanoparticles have porous cores amenable to drug loading. Drug release from the pores could be triggered by pH changes or ultrasound [75]. Drugs may also be loaded onto metal oxide nanoparticle surfaces by electrostatic interactions between a positively-charged drug and a negatively-charged citrate surface on Fe_3O_4 magnetic nanoparticles, for example. Such particles could be directed to a diseased site by an external magnetic field [75].

4.2. NANOPARTICLE CHARACTERIZATION

4.2.1. Particle size and shape

In vitro and *in vivo* studies have shown that the interactions of nanoparticles with cells are correlated with particle size, shape, and surface characteristics [76-82]. It has been demonstrated that the cellular uptake of nanoparticles is size-dependent, with smaller particles being taken up more easily than larger particles [83]. Nanoparticles with dimensions between 250 nm and 3 μm can be internalized within cells *in vitro* via phagocytosis and micropinocytosis. Nanoparticles smaller than 200 nm, on the other hand, are more likely to involve other cellular uptake routes, such as clathrin- or caveolin-mediated endocytosis, independent endocytosis mechanisms, or passive transport [76,82]. Nevertheless, the internalization pathway may not adhere to these typical size guidelines if there are specific ligands on the nanoparticle surface [76,84]. The enhanced permeability and retention effect can be observed with nanoparticles with diameters ranging from 40–400 nm [76]. Particle size has been shown to affect the circulation time of liposomes [85]. Likewise, the circulation time of dendrimers depends on their size, as only dendrimers with a low generation number (G5 or less) and a hydrodynamic radius less than 3.5 nm are likely to be eliminated into the urine [86].

Nanoparticle shape can influence intracellular nanomaterial trafficking. Hexagonal shapes were shown to be retained in the cytoplasm, whereas rod-like particles could move towards the nucleus by microtubules [80]. The

circulation time of elliptical discs has been shown to be longer than that of spherical particles, and differences in cellular internalization have been observed for cylinders, cubes, and particles of varying rigidity [4,79,80,82,87].

Particle size and shape can be examined by electron microscopy or atomic force microscopy. With scanning electron microscopy (SEM), particles are coated with a thin layer of gold or platinum. However, the particles may shrink during the drying step, causing an under-estimation of actual particle diameters [88]. Since the individual sizes of a large number of nanoparticles must be tallied to determine average size and polydispersity index (PDI), it is usually more convenient to measure the particle size of nanoparticles in a liquid suspension by dynamic light scattering (also known as photon correlation spectroscopy). Nevertheless, it is still wise to confirm light scattering data by a microscopic method [88]. With light scattering, the Brownian motion of particles is related to particle size (small particles move faster than larger particles), and when the temperature and viscosity of the liquid are known, the hydrodynamic diameter can be determined using the Stokes-Einstein equation [89]. Particle size is usually reported as the Z-average diameter (also referred to as the cumulants mean). The standard deviation from multiple measurements should also be reported. Characterization of particle size is incomplete without the PDI, which describes the width of the particle size distribution. PDI values will range between 0 and 1. If the majority of particles are the same size, the PDI will be close to zero. On the other hand, a sample containing a diverse mixture of particle sizes will have a larger PDI value. PDI values less than 0.40 can be deemed acceptable [90], PDI values less than 0.25 are favorable, and values under 0.10 are excellent.

4.2.2. Zeta potential

Surface charge can also affect nanoparticle biodistribution, opsonization, and toxicity [81]. Typically, larger and negatively charged nanoparticles exhibit less toxicity compared to smaller and positively charged polymeric particles [81]. Surface properties (such as charge and coating) are more likely to affect cellular internalization pathways than polymer type [77,78]. Negatively charged liposomes can have prolonged circulation times compared to neutral liposomes, which may be more readily recognized by macrophages and cleared from the body [91]. A number of surface coatings can prevent nanoparticle aggregation and opsonization, improve cellular uptake, increase wettability, and affect degradation rates. Examples of such nanoparticle surface coatings include poly(vinylpyrrolidone), PEG, dextran, chitosan, pullulan, sodium oleate, dodecylamine, polysorbate 80, poloxamer 188, poly(vinyl alcohol), poly(2-methyl-2-oxazoline), poly(sulfobetaine methacrylate), and α -tocopherol PEG-1000 succinate [6,79,80].

The zeta potential (ζ) of a particle is a quantitative measure of its surface charge, and the value is typically reported in units of mV. This is an important

parameter with regards to the stability of a nanosuspension. If the absolute value of ζ of nanoparticles in suspension is less than 10 mV (in other words, if ζ is less than +10 mV or greater than -10 mV), then the nanoparticles are more likely to aggregate [92]. Due to the relatively neutral surface charge when ζ is close to zero, there is insufficient charge repulsion to prevent aggregation. On the other hand, if ζ is sufficiently positive or negative (*e.g.*, $|\zeta| > 25$ mV), then charge repulsion of individual particles with similar charge will make aggregation less likely, leading to a more stable nanosuspension [93]. It is important to note that the numeric examples of 10 or 25 mV mentioned in this paragraph are only guidelines that will not apply to every nanomaterial; stability will also depend on surface coatings. For example, nanoparticles coated with PEG may have $|\zeta| < 10$ mV but still be stable [94]. ζ can be determined by laser Doppler velocimetry. When an electrical field is applied, the nanoparticles move towards the electrode of opposite charge. The quantification of this movement — referred to as electrophoretic mobility — permits the determination of ζ through the Henry equation when the dielectric constant and the viscosity are known [95].

4.2.3. Encapsulation efficiency

The encapsulation efficiency of a nanoparticle formulation is defined as the fraction of the amount of drug used in the nanoparticle preparation process that was actually encapsulated within the nanoparticles. It is most often calculated indirectly by determining the amount of free drug that was not encapsulated, as described with the following equation [96]:

$$\text{Encapsulation efficiency} = \frac{\text{mass of drug added to the nanoparticles} - \text{mass of free drug}}{\text{mass of drug added to the nanoparticles}}$$

Encapsulation efficiency is often presented as a percentage, in which case the fraction on the right side of the equation would be multiplied by 100 %. The determination of encapsulation efficiency requires separation of the nanoparticles from the surrounding medium in order to analyze the concentration of free, unencapsulated drug present in the aqueous nanosuspension. This can be achieved by high-speed centrifugation or by filtration. Caution is necessary in both cases. For instance, if high-speed centrifugation is applied to obtain a pellet of nanoparticles and a particle-free supernatant, care must be taken to ensure that handling of the samples does not cause any resuspension of nanoparticles into the supernatant prior to its analysis. In addition, one must ensure that the centrifugation was sufficient to collect all of the nanoparticles. The smaller the particles, the more difficult this may be. A simple method to check whether the supernatant is free of nanoparticles is to make a particle size measurement of the supernatant by dynamic light scattering. If the measurement provides particle diameter results, the centrifugation was not adequate. On the other hand, if the result

matches the measurement of pure water (which may result in an instrumental error or warning), one can be confident that the supernatant is free of particles. In our laboratory, centrifugation at $110,000 \times g$ has been deemed successful for this purpose. If one uses filtration to separate the nanoparticles from the surrounding medium, care must first be taken to ensure that the filter membrane does in fact restrict the passage of nanoparticles into the filtrate. (This can be checked by dynamic light scattering as just described.) In addition, control experiments must be performed to determine whether the encapsulated drug itself binds to the filter membrane. If so, corrections will be necessary to account for such binding. The concentration of free, unencapsulated drug in the nanosuspension (quantified in the supernatant or filtrate) is usually determined by a validated high performance liquid chromatography (HPLC) method.

Additional definitions applicable to the discussion of encapsulation efficiency include theoretical drug loading and actual drug loading. The theoretical drug loading indicates the percentage of total nanoparticle mass that is drug, assuming 100 % encapsulation efficiency. The theoretical drug loading thus represents the amount of drug introduced to the nanoparticle formulation. The actual drug loading is the actual percentage of drug mass contained within the nanoparticles once they have been formed and the encapsulation efficiency has been determined. It is important to note that although the actual drug loading can be approximated by the product of encapsulation efficiency $\times$ theoretical drug loading, this is only an approximation. The exact calculation of actual drug loading must take into account the fact that the total nanoparticle mass (*e.g.*, drug mass plus polymer mass) is reduced by the amount of unencapsulated drug. Table 3 illustrates these points with several examples.

Table 3. Examples of the relationships between theoretical drug loading, actual drug loading, and encapsulation efficiency based on a simple nanoparticle formulation comprised of a single polymer and a single drug without additional excipients or stabilizers. In these examples, it is assumed that the entire polymer mass is recovered as nanoparticles.

Polymer mass added	Mass of drug added	Theoretical drug loading	Unencapsulated drug mass	Actual mass of drug encapsulated	Actual drug loading	Encapsulation efficiency	Ratio (actual drug loading /theoretical drug loading)
99 mg	1 mg	1 %	0.2 mg	0.8 mg	0.80 %	80 %	0.802
95 mg	5 mg	5 %	1 mg	4 mg	4.04 %	80 %	0.808
90 mg	10 mg	10 %	1 mg	9 mg	9.09 %	90 %	0.909
90 mg	10 mg	10 %	2 mg	8 mg	8.16 %	80 %	0.816
90 mg	10 mg	10 %	3 mg	7 mg	7.22 %	70 %	0.722
90 mg	10 mg	10 %	4 mg	6 mg	6.25 %	60 %	0.625
90 mg	10 mg	10 %	5 mg	5 mg	5.26 %	50 %	0.526
80 mg	20 mg	20 %	4 mg	16 mg	16.67 %	80 %	0.833

The question is sometimes raised as to how many drug molecules are encapsulated within a single nanoparticle. To illustrate this point, we will use an example of drug-loaded polymeric (PEGylated PLGA) nanoparticles recently formulated in our laboratory. The particle size was approximately 100 nm, the theoretical drug loading was 10 %, the encapsulation efficiency was 95 %, and the PDI was less than 0.25. For simplicity, we will assume a uniform particle size of 100 nm (PDI = 0), that the particles are spherical, and that the nanoparticle density is 1.33 g cm^{-3} (the density of PEG is 1.2 g cm^{-3} [97], the density of PLGA is 1.34 g cm^{-3} [98], and the density of the drug was approximately 2 % greater than the density of PLGA). In a sample of nanosuspension containing $20 \text{ }\mu\text{g}$ of nanoparticles, the number of nanoparticles (N_{NP}) is given by the equation $N_{\text{NP}} = \frac{W \cdot 6}{\rho \cdot \pi \cdot d^3}$, where W is the nanoparticle mass in the sample, and ρ is the density of a single nanoparticle. In this example, $N_{\text{NP}} = 2.87 \times 10^{10}$ particles. The mass of a single nanoparticle ($mass_{\text{NP}}$) is calculated as $mass_{\text{NP}} = \frac{W}{N_{\text{NP}}}$, or in this case, $6.96 \times 10^{-10} \text{ }\mu\text{g}$. Since the encapsulation efficiency was 95 %, the actual drug loading is 9.548 %, so the mass of drug in a single nanoparticle ($mass_{\text{drug}}$) is given by the product of the actual drug loading $\times mass_{\text{NP}}$, which in this example is equal to $6.65 \times 10^{-11} \text{ }\mu\text{g}$. Assuming a drug molecular weight (MW) of 500 Da, the number of moles of the drug in a single nanoparticle (n_{drug}) is calculated as $n_{\text{drug}} = \frac{mass_{\text{drug}}}{\text{MW}}$. In this example, a single nanoparticle contains $1.33 \times 10^{-10} \text{ nmol}$ of drug. Finally, the number of drug molecules encapsulated in a single nanoparticle is calculated as the product of $n_{\text{drug}} \times N_{\text{A}}$ (Avogadro's number, 6.022×10^{23}), which in this example is approximately 80,000. Although at first glance this might seem like a large number, it is important to consider the capacity of a three-dimensional spherical nanoparticle. A drug molecule with MW of 500 Da might have a molecular diameter in the range of 10–15 Å (1–1.5 nm). To simplify the discussion, we will assign the diameter of the drug molecule to be 1 nm (10 Å). As mentioned above for this example, the diameter of a single nanoparticle is 100 nm. One might initially express a two-dimensional reaction and state that only 100 drug molecules could possibly fit within this diameter. However, a 100 nm nanoparticle loaded with 1 nm drug molecules is not the same as a 100 cm bookshelf holding 100 books having a thickness of 1 cm each. Rather than picturing a bookshelf that is 100 cm wide, imagine instead a football stadium surrounding a field that is 100 m long. Continuing this analogy, assume that each spectator at the stadium needs 1 m of sitting space. A single sphere with a diameter of 100 m would take up an enormous amount of space in the center of a soccer field (which is approximately 100 m long and 70 m wide). Considering that the sphere's height would also extend 100 m upwards, it should not be so difficult now to imagine that 80,000 spectators in the stands might fit within 10 % of the space of such a sphere. (A sphere with a diameter of 100 nm has a volume that is 1 million times greater than the volume of a sphere with a diameter of 1 nm.)

4.2.4. Drug release

An encapsulated drug must be released from the nanoparticle in order to exert its pharmacologic effects. Drug release kinetics are greatly influenced by the nature of the drug, nanomaterial composition, and the localization of the drug within the nanoparticles (*i.e.*, whether the drug is encapsulated within the nanoparticle matrix, adsorbed to the nanoparticle surface, or covalently linked to the particle) [6,99]. Some nanoparticle formulations will display gradual or controlled release properties, which will offer a number of advantages with regards to dosing frequency. Mechanisms of drug release from within nanoparticles include diffusion, polymer degradation, and stimuli-responsive elements. Stimuli-responsive nanoparticles can increase the amount of drug released in response to either an endogenous or an external change in temperature, pH, light, polarity, redox potential, ionic strength, enzymes, ultrasound, electromagnetic radiation, chemical or biochemical agents, and oxidative stress [47,99-103]. For polymeric nanoparticles, smaller particles are generally more prone to exhibit an initial burst release in comparison to larger particles; on the other hand, larger particles tend to exhibit a faster degradation rate *in vivo* [6,84,104]. Polymer degradation depends on location within the body, nanoparticle size and shape, and the MW of the polymeric matrix [7,104]. High MW polymers are more likely to degrade more slowly than low MW polymers [105].

Drug release kinetics are typically determined in a well-stirred physiologically relevant medium, such as phosphate buffered saline at pH 7.4 and at 37 °C. Sink conditions must be maintained, which means that the volume of medium used must be sufficient so that at the point at which 100 % of drug release has occurred, the final concentration of the drug in the medium must not exceed 20 % of the saturated concentration of the drug in that medium [106]. At time zero, the nanoparticles are added to the medium and samples of the medium are taken at specified time points for the duration of the study. A release study could last hours to weeks, depending on the kinetics of drug release for the nanoformulation in question. An initial sample should be taken at time zero (or, practically speaking, a few seconds after the nanoparticles have been added to the medium). At time zero, the percent of drug release should match closely with the percentage of unencapsulated free drug from the determination of encapsulation efficiency. Initial burst release is usually characterized at the 30- or 60-min time point (unless the kinetics of release are gradual over several weeks, in which case the sample after 1 day might serve as a more appropriate measure of burst release). Individual samples from the release study can be processed as described above for the determination of encapsulation efficiency; *i.e.*, at each time point, the nanoparticles will be separated by high-speed centrifugation or filtration. The supernatant or filtrate will then be quantified by a validated HPLC method (or another suitable method applicable to the drug of interest) to determine the concentration of drug released from the nanoparticles at each time point. Alternatively, dialysis

bags can be used to study drug release. In this case, the nanoparticle suspension will be mixed with release medium when it is placed inside dialysis tubing or a floating dialysis device that has a suitable pore size to prevent the nanoparticles from escaping the dialysis tubing or device, but which permits the released drug molecules to pass through the pores to be sampled from the external medium at the designated time points. Dialysis tubing pore sizes are often defined using molecular weight cutoffs (MWCO). As an example, a 12–14 kDa MWCO corresponds to a pore size of 2.4 nm [107]. Drug release data are often fit to a descriptive model. One of the most common is the Higuchi model, which relates the fraction of drug released to the square root of time, as indicated by the following equation: $\frac{M_t}{M_\infty} = K\sqrt{t}$, where M_t is the mass of drug released at time t , M_∞ is the total cumulative mass of drug released at infinite time, and the constant K depends on experimental variables [108].

4.2.5. Time-resolved small-angle neutron scattering (TR-SANS)

Time-resolved small-angle neutron scattering can be considered an effective method to study the size, structure, development, and morphological changes of lipid/surfactant-based constructs, such as liposomes, micelles, and nanoparticle dispersions [109]. For example, TR-SANS was utilized to explore the effect of charge density, salinity, and temperature on the morphology of unilamellar vesicles (ULVs) [110,111]. Moreover, the TR-SANS technique provides data regarding coalescence, growth, and transformation of lipid vesicles. The kinetics of cylinder-to-sphere morphological transition have been studied in the block copolymer micelle system [112]. This technique can examine alterations in rigidity and the influence of nanomaterials on structural changes in biological systems [112].

4.2.6. X-ray diffraction

X-ray diffraction (XRD) is used to characterize crystallinity, crystal and molecular structure variations, non-crystalline periodicity and size, nanoformulation orientation (crystalline/amorphous), polymorphisms, and phase transitions [113,114]. XRD is a non-destructive technique that can examine the state of encapsulated drug [115]. Optimally, successfully encapsulated drug will be in an amorphous state, evenly distributed within the nanoparticles. Nanoparticle size can be calculated using the Debye-Scherrer equation: $d = 0.89\lambda/\beta\cos\theta$, where 0.89 is Scherrer's constant, λ is the X-ray wavelength, θ is the Bragg diffraction angle, and β is the full width at half-maximum of the diffraction peak corresponding to the plane [116,117]. Bragg's Law is applied to value of the interplanar spacing between the atoms (d): $2d\sin\theta = n\lambda$, where n is a positive integer and λ is the wavelength of incident wave. The dislocation of crystallographic defects within a crystal structure (dislocation density, δ) can be determined using the expression:

$\delta = 15\beta\cos\theta/4aD$, where θ is Bragg's diffraction angle, a is the lattice constant and D is the crystallite size [117].

4.2.7. Differential scanning calorimetry

Differential scanning calorimetry (DSC) can be used to quantify and investigate the following: solid and amorphous phases of nanomaterial and payload, percent crystallinity of the sample, polymorphic transitions, drug loading efficacy, conformational changes, self-assembly behavior, and stability [118,119]. Thermograms are typically recorded at a scanning rate of 5–10 °C min⁻¹ from 25–250 °C under nitrogen flow (30 mL min⁻¹) [113, 120–122]. DSC analysis should usually be performed for each component of the drug delivery system [113,118,120–123]. The disappearance of the melting peak of the loaded drug or bioactive agent indicates its encapsulation into the nanoparticles [113,122]. The presence of a drug's peak on the thermogram may indicate the physical adsorption of the drug on the nanoparticle's surface [118]. Decoration of a nanoparticle's surface *via* brushing, grafting, coating and covalent coupling of specific ligands can be confirmed and characterized by this technique as well [113,118,120,121]. The stability of drug-loaded nanoformulations can be verified by comparing thermograms obtained immediately after its fabrication to thermograms after long-term storage [96].

4.2.8. Fourier transform infrared spectroscopy (FTIR)

Chemical composition, the presence of chemical bonds, and functional groups within or on the surface of nanoparticles can be determined by Fourier transform infrared spectroscopy (FTIR). FTIR as well as XRD can characterize the interactions between a drug and the nanoparticle matrix [124]. Infrared-spectroscopic nanoimaging with a thermal source (nano-FTIR) offers improved capabilities in the application of conventional FTIR to nanomaterial characterization [125]. With nano-FTIR, the chemical identification of nanomaterials, quantitative assessment of mobility in doped nanostructures, and the presence of any contaminants are possible in ultra-small quantities and at ultra-high resolution [126].

4.3. SUMMARY

The prospects for nanomedicine to improve human health are tremendous. A number of drug-loaded nanoparticle formulations have already reached the market, many are currently in clinical trials, and new developments will continue to advance the field. As scientists work to create future formulations, the characterization of drug-loaded nanoparticles will guide the optimization of new products. When *in vitro* characterization data are confirmed *in vivo* [127], the team gains confidence to apply the benefits of

nanotechnology to improve the pharmacokinetics, pharmacodynamics, and safety profile of medications.

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NANO-DRUGS THERAPY FOR HEPATOCELLULAR CARCINOMA

Florin Graur^{1,2*}

¹ University of Medicine and Pharmacy “Iuliu Hatieganu” Cluj-Napoca Str. Victor Babeş Nr. 8, 400012 Cluj-Napoca, Romania

² Regional Institute of Gastroenterology and Hepatology
“Octavian Fodor” Cluj-Napoca Str. Croitorilor 19-21, Cluj-Napoca, Romania

*e-mail: graurf@yahoo.com

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5.1. INTRODUCTION

Hepatocellular carcinoma (HCC) represents one of the principal causes of cancer deaths (4th) worldwide with an approximate 500,000 deaths per year and a 5 year survival rate of below 5 % [1].

HCC is the fifth most common solid tumour worldwide and is caused when hepatocytes are turned into cancerous cells. It occurs more frequently in cirrhotic patients and in those with hepatitis B virus (HBV) and hepatitis C virus (HCV) infections, but varies significantly by region, with a predominance in Middle Africa and Eastern Asia [1]. For patients with HCV infection it is a major cause of death.

Liver resections can be performed only in a limited number of cases, mainly due to the development of liver cirrhosis, when liver transplantation is feasible if the patient is within the Milan criteria. Other therapies for HCC such as *in situ* ablation (radiofrequency and microwave ablation) and chemoembolization are considered palliative therapies and have poorer outcomes compared to surgical resection or liver transplantation. Systemic chemotherapy is toxic, does not accumulate specifically in the tumour and has a relatively rapid elimination. In addition many tumours develop resistance to chemotherapy [2,3].

The results of the various forms of treatments available are unsatisfactory in the long term, which is why a new therapeutic strategy is becoming necessary.

In the last 10 years a number of nanostructures have been used in imaging and therapy, preparing the foundations of a new field: nanomedicine [4].

In this chapter we will review the latest nano systems used in the treatment of HCC. Given the exponential momentum that we have in this research field, this chapter will not cover all the developed therapeutic modalities of the treatment of HCC, future research validating only those variants applicable in human pathology.

Nanotechnology can be used in malignant liver pathology in several directions: imaging, diagnosis and therapy. Combining modern therapy with diagnosis through the use of nanotechnology in medicine led to the development of a new field called theragnostics. The reason for using nanotechnology in medicine is due to the properties of the nanostructures used, structural, optical, magnetic, and radiant, which are not so far found in other materials [5,6].

Using nanostructures in HCC therapy can be developed in several directions depending on the nanostructure type and mode of action. Nanoconditioned treatments could have a better potential to treat multicentric or metastatic tumours compared to surgical procedures or local / loco-regional therapy used currently.

The mode of action varies depending on the type of nanostructure and the functional groups or molecules transported. Carriers release therapeutic agents at the tumour site; and thermal ablation systems destroy the tumour cells through a thermal effect produced by the irradiation of these molecular complexes with different types of radiation, including magnetic field or ultrasound waves [7-10].

These techniques demonstrate that there is no consensus about the direction research should take in this fight against HCC. The best therapies will evolve and be validated in human treatment (as some already are). Unfortunately, these treatments are still very expensive and only some highly specialized clinics can afford to treat highly selected patients. Maybe the future will allow more patients to benefit from this research, as the costs will most probably decrease in time.

5.2. NANO SYSTEMS USED AS CARRIERS OF THERAPEUTIC AGENTS FOR THE TREATMENT OF HEPATOCELLULAR CARCINOMA (HCC)

Among the types of nanosystems used as carriers, carbon nanotubes, silica or metal nanostructures, and micelles-based polymers have been widely used.

For the experimental treatment of subcutaneous H22 line tumours in murine subjects, docetaxel loaded on multi-layered [poly(ethylene glycol) (PEG)]ylated silica nanoparticles (NPs) was used (Li *et al.*). The result was a halving of tumour volume after four intravenous infusions compared to subjects receiving chemotherapy alone [11].

For the transport of interleukin 12 (IL-12) into the tumour, Diez *et al.* (2009) created lipopolymeric cationic micelles which combined the polymer and dioleoyloxytrimethylammonium propane (DOTAP) lipids. These complexes carrying the gene IL-12 were administered to murine subjects with a murine undifferentiated subcutaneous HCC. Survival was up to 60 days compared to administration of IL-12 without a carrier, with complete tumour regression of 75 % in the group with IL-12 transported in nano-complexes [12].

Kim *et al.* targeted peptide RGD-4C in a mouse model of hepatoma to carry the targeted doxorubicin (DOX) in the tumour, at the same time decreasing the cytotoxicity of free DOX. DOX-RGD-4C complex showed a better suppression of tumour growth than free DOX [13].

Barraud *et al.* proposed the encapsulation of doxorubicin in poly(isohexylcyano acrylate) (PIHCA) polymeric NPs which doubled the percentage of apoptotic cells compared to unencapsulated DOX administration in subjects with murine liver tumours [14].

PEGylated recombinant human arginase deaminated (rhArg) has been used with significant cell lines HepG2 and Hep3B [15].

Maeng *et al.* used a system of iron oxide NPs that contained folate-targeted DOX interlaced with poly(ethylene oxide) polymer chains. Infusing this drug in subjects with murine liver tumour resulted in significant reduction of tumour volume compared to subjects who received only DOX and tolerance was better in the group that received the nanoconditioned system [16].

For a more selective attachment to liver cells, Kopecek *et al.* proposed galactosidase-targeted (Gal-targeted) *N*-(2-Hydroxypropyl) methacrylamide (HPMA)-DOX conjugates that bind to cell-surface asialoglycoprotein receptor (ASGPR) – intensely expressed on the surface of liver cells. Subsequently, receptor-mediated endocytosis internalised nano-complexes in liver tumour cells. Studies have shown a selective biodistribution of nano-complexes in liver tumours and a significant decrease in systemic toxicity [17-19].

The styrene-maleic anhydride neocarzinostatin (SMANCS) system (poly(styrene-*co*-maleic acid) (SMA) polymers with neocarzinostatin (NCS) proposed by Maeda *et al.* was the first nano-conditioned therapy approved for clinical use in HCC. This treatment has resulted in minimal inhibitory concentrations of antitumour protein 100 times higher at 2–3 months after administration with tumour reduction in 95 % of patients [20-23].

Zhou *et al.* prepared a system of 5-fluorouracil (5-FU) encapsulation in polysaccharide amphiphilic nano-micelles {5-FU / dextran-graft-poly(lactic acid) [DEX-g-(PLA)]}. These systems have been administered *in vitro* and *in vivo* to HepG2 cell line. 5-FU concentration was increased in the group with 5-FU / DEX-g-PLA compared to free 5-FU and *in vivo* tumour growth inhibition was also more intense in 5-FU / DEX-g-PLA group [24].

Malarvizhi *et al.* developed a dual system combining sorafenib in a protein nano-shell with DOX in a poly(vinyl alcohol) nano-core with an affinity for transferrin. This therapeutic complex has demonstrated increased uptake in the liver tumour and synergistic cytotoxicity against it [25].

Ling *et al.* used pH sensitive nanoconditioned triptolide coated with folate for the treatment of tumours with increased expression of folate receptor. Triptolide has a cytotoxic effect on tumour cells and pH-sensitive nano-formulation reduce systemic toxicity and specific uptake in the tumour [26].

Zhou *et al.* demonstrated that mitoxantrone-loaded poly(butylcyan acrylate) (PBCA) nanoparticles (DHAD-PBCA-NPs) are effective in unresectable HCC in humans and prolong the median survival rates [27].

Thermally sensitive liposomes containing DOX (ThermoDox®) is a combination between radiofrequency ablation and liposome enveloped DOX. The DOX is released at temperatures above 39.5 °C and is stable up to 73 °C [28,29]. This system also caused obstruction of the vessels of the tumour.

The limitations of the delivery systems for anti-tumour agents are:

- reduced load of antitumoural agent on the carrier system, leading to an increased demand for transport system with consequent increases in systemic toxicity;
- low specificity and slow release of antitumour agent with consequent reduction of anticancer activity;
- removing nano-complexes by the liver, spleen and lungs which leads to an increase in toxicity to these organs. Kupffer cells preferentially capture nanostructures marked with Gal, thus decreasing the effect on tumours and increasing non-tumour liver toxicity by non-specific distribution.

5.3. NANO SYSTEMS FOR GENE TRANSFER

The challenge for nanotechnology is gene therapy, by introducing deoxyribonucleic acid (DNA) using certain vectors, and targeting "repairs" of each cell containing a nonfunctional gene. So far the most effective vectors for DNA transfer are of viral origin, but often their use raises safety concerns. Non-viral vectors such as liposomes and polymers have therefore been used, but they have a smaller capacity for transport. Virosomes seem to be the solution to this problem, due to their ability to internalise and encapsulate the DNA, and have proved to be as efficient as viral vectors in the gene expression process. There are NPs under research based on synthetic nucleotides, which can be combined with bioactive components such as peptides, to increase the transfer capacity across the cell membrane. They are used to inhibit gene expression at the level of messenger RNA (mRNA), and do not require administration to the nucleosome, but in the cytosol, and have a low cellular toxicity.

The possibility of treating cancer, a disease defined by genetic defects, through the introduction of genes that target these changes, has led to an intense interest in cancer gene therapy.

This therapy can be included in nano systems for the transport of therapeutic agents, but the effect is radically different because the gene carried by that nano system usually acts in the cell genome by replacing the defective gene that led to cancer.

The mechanism of actions used to treat HCC are [30]:

- Restoration of suppressor genes: especially used for mutations of gene p53.

- Inhibition of oncogenes (*i.e.* pituitary tumour transforming gene 1 (PTTG1), urokinase-type plasminogen activator (u-PA), p28-GANK).
- Gene-directed enzyme / pro-drug therapy (GDEPT): thymidine kinase gene from HSV-1 with prodrug ganciclovir; yeast Cytosine Deaminase with antifungal drug 5-fluorocytosine (5-FC); sodium iodide symporter (NIS) gene.
- Targeted expression of cytotoxic/pro-apoptotic genes: adeno-associated virus (AAV) vector expressing soluble tumor necrosis factor-related apoptosis-inducing ligand (sTRAIL) fused with a human insulin signal peptide.
- Immunogene therapy: immunomodulatory cytokines, vaccines.
- Anti-angiogenic gene therapy: adenoviral vector carrying the endostatin complementary DNA (cDNA); blocking the endothelium-specific receptor Tie2; pigment epithelium derived factor (PEDF); NK4 – a fragment of the hepatocyte growth factor (HGF).
- Oncolytic viruses: ONYX-015, NV1020, G207, rRp450 HSV-1.

There are viral vectors and non-viral vectors used for gene transfer. Among the non-viral vectors, the most commonly used are nanostructures.

NPs are intensely investigated vectors with unique functional properties that increase the efficiency of intracellular gene penetration. The gene transfer takes place at a reduced level in case of non-viral vectors. The non-viral categories of vectors which can transport the DNA are: cationic lipids (Lipoplex), the cationic polymers (Polyplex) and the mixture of these two categories (Lipopolyplex) with recombinant peptides or proteins (conjugate molecules) and, recently, NPs. In order to increase the affinity of nanoparticles for tumorous cells, some various proteins (antibodies, etc.) could be bound on its surface.

The characteristics of an ideal gene delivery system are that it is:

- stable
- biocompatible
- non-toxic
- cost-effective
- able to transfer genetic material strongly anionic in specific places
- targeted to specific cells by binding to specific receptors
- guided release (ultrasound, laser, magnetic field)
- facilities to remove non-toxic compounds

The negative charge of DNA prevents passage through the lipid membrane and sinusoidal endothelium fenestration which is 50 nm; the carrier nano-structures should meet the above characteristics.

Internalisation mechanisms for nucleic acids are the following:

1. Microinjections
2. Passive diffusion
3. Endocytosis
 - a) receptor mediated
 - b) fluid phase pinocytosis
 - c) absorptive endocytosis
4. Artificial internalisation
 - a) liposome
 - b) micro / nanoparticles
 - c) dendrimers

The mutations of the genes which codifies the p53 protein are frequently involved in human cancers (more than 50 % of them demonstrate this defect). The gene p53 has a role in the detection of any alteration in the DNA and blocks the cell cycle in the G1 phase, so that the repair of the defect will occur before the DNA replication and transmission of the defects to the daughter cells. The p53 protein binds at the DNA level and activates the genes involved in the DNA repair, and hence controls the cell cycle. The cell cycle is not blocked in the cells with a mutation of p53 and this progresses to the synthesis phase (S-Phase) and hence transmits the DNA alteration to the daughter cells.

Reactivated p53 can induce apoptosis, and can cause reduced proliferation or cellular senescence. p53 is a tumour suppressor gene that plays an important role in cell cycle regulation and loss of function is considered "wild-type" p53 – a promoter of carcinogenesis. *In vitro* and *in vivo* studies have demonstrated that the reintroduction and expression of "wild-type" p53 mutations in the p53-mutated tumour cells have slowed down tumour growth and the induction of apoptosis. One of the limitations of this gene therapy is finding a suitable input vector in order for the wild type of p53 to be carried into the tumour cell. Restoring the normal activity of anti-oncogene p53 causes tumour regression.

On an international level regarding the treatment of HCC there have been attempts to introduce the "wild type" of p53 through the use of various vectors as an intermediary: viruses such as recombinant adenoviruses [31], oncolytic viruses [32,33], liposomes [34-37], polisine-DNA complexes [38].

The results are encouraging for the use of gene therapy in HCC. In an experimental study where hepatic tumours were induced in mice, amelioration was attained in addition to an increased sensitivity to chemotherapy.

The international research in this domain includes the insertion of p53 gene into the tumour cells with the help of viral or non-viral vectors. The advantages of the nanostructures might be their stability, the possibility of binding to some of the diverse adhesion molecules found on the surface and also offering protection to the internal DNA sequence.

Carbon nanotubes have proved to be a more rapid and a safe alternative for delivering therapeutic molecules, genes and peptides. They can transport the molecules of interest through cytoplasmic and even the nuclear membrane, and it was demonstrated through a dynamic molecular study [39] that the molecule of DNA can be inserted spontaneously in the carbon nanotubule in a watery solution. The van der Waals type of binding and the hydrophobic forces are important factors in the process of insertion, of which the first plays an important role in the interaction between DNA and the carbon nanotube. The encapsulation of the DNA molecules marked with platinum in the multilayered carbon nanotubes was realised at a temperature of 400 K and a pressure of 3 bars [40,41]. The DNA molecules attached to the surface of the tube can be easily detached through gel electrophoresis. It is presumed that the van der Waals type of interaction between nanotube and the DNA is the moving force of the phenomenon of insertion.

Non-viral vectors have advantages compared to viral vectors because they are standardised and do not involve the risk of viral dissemination and immunogenicity. Non-viral vectors are also easily configurable, thus increasing efficiency, specificity and their control in time. The transferred gene (plasmid type) is attached inside or on the surface of non-viral nano vectors. There were various transport systems imagined for various classes of genes by modifying non-viral nano vectors to improve their qualities, however, non-viral vectors have achieved a reduced expression of the gene carried.

Tada *et al.* [42] injected naked plasmid DNA in rats with HCC induced with diethylnitrosamine. Those whose injection was performed in the hepatic artery showed a significant increase of transgene expression in cancer cells.

Other authors have used plasmid DNA incorporated in polyelectrolyte multilayers synthetic and degradable structures that showed effective gene transfection in human HCC cell lines [43].

Dai *et al.* synthesised antisense oligonucleotides (ASODNs) of midkine (MK) packaged with NPs that had been inhibited *in vitro* and *in vivo* growth of HCC [44].

Chen *et al.* EA4D selected a variant of the alpha-fetoprotein (AFP) promoter (which has the highest activity) and fused it with truncated BID (tBid) and

coupled with nano structure H1, thus forming pGL3-EA4D-tBid. This drug inhibited the growth of the AFP producing HCC [45].

Reduced toxicity, an absence of pathogenicity and relatively easy pharmacological production, favour non-viral vectors in competition with viral vectors, however, gene transfer is reduced with non-viral vectors.

5.4. NANO THERMAL ABLATION SYSTEMS USED IN THE TREATMENT OF HCC

Thermal ablation of HCC could be performed intravenously or directly into the liver delivered by nanostructures followed by the application of laser energy, high intensity focused ultrasound (HIFU) or a magnetic field.

Peptide-targeted gold nanoshells have been used for photothermal therapy. These were obtained by coating silica NPs with gold and then attaching A54 targeting peptides to the created system surface. Near infrared light was administered to the liver tumour cell line BEL-704 treated with the created complex, resulting in the thermal destruction of cancer cells [46]

Chen *et al.* administered magnetic nanoparticles (MNPs) Fe_3O_4 in a murine model of BEL-704 hepatoma, to which was subsequently applied a static magnetic field with extremely low-frequency, altering the electric magnetic field. NPs were crowded in the liver tumour under the action of a static magnetic field, and apoptosis was increased in the group exposed to the variation of the magnetic field. This mechanism is due to thermal production of the NPs exhibited in the magnetic field, but also due to the action of the variable magnetic field [47].

NPs of Ce(IV)-doped TiO_2 induced apoptosis in a study by Wang *et al.* on the BEL-7402 hepatoma cell line after being exposed to visible light with wavelength between 400 and 450 nm [48], however, this mechanism is more difficult to use in solid tumours due to the reduced tissue penetration of the visible light spectrum.

Liu *et al.* have used high intensity focused ultrasound on a model of HCC in rabbits after intravenously administering nano-hydroxyapatite. These NPs were absorbed in the reticuloendothelial system and applying an HIFU ablation then led to hydroxyapatite-enhanced hyperthermia resulting in coagulation necrosis area [49].

Li *et al.* prepared Carboplatin-Fe@C-loaded chitosan NPs which were injected into the hepatic artery of a hepatic tumour rat model. The exposure to alternating magnetic fields led to marked tumour apoptosis, the mechanisms involved were both hyperthermia and drug release into the tumour [50].

In an attempt to combine the diagnosis with therapy of HCC, Hu *et al.* proposed a novel theragnostic system based on cubic Au nano-aggregates, which were acting on the one hand as photo-acoustic agents for use in imaging, and on the other hand absorbing laser radiation of 808 nm releasing heat [51].

5.5. OTHER NANOSTRUCTURES USED IN THE THERAPY OF HCC

There are some nanostructures that may directly reduce tumour growth or cause even its destruction through the direct cytotoxic effect, without being bound by chemotherapeutic agents or gene activity.

Selenium nanoparticles (SeNPs) have the effect of inducing apoptosis in HCC. Ahmed *et al.* administered SeNPs to a murine model of HCC. In the study they found that in the group to whom were administered SeNPs, apoptosis was increased. AKr1b10 and ING3 gene expression were also increased, and there was low Foxp1 gene expression in the group with SeNPs. This suggests the action of SeNPs at the molecular level [52].

Zheng *et al.* synthesised ultrasmall SeNPs coated with PEG – PEG-SeNP – which have demonstrated antitumour effects on HepG2 lines resistant to chemotherapy by altering mitochondrial membrane potential and the production of superoxide anions [53].

Yin *et al.* used gambogic acid-loaded particles (GA-Ps) for the treatment of HCC with significant results compared with controls [54].

5.6. CONCLUSIONS

Nanotechnology enables the development of molecular systems with well-defined properties, which act either directly or release the active agent specifically to the desired site. Ideally, these systems are stable, preferentially accumulate in increased concentrations in the tumour, are not toxic to the body, and are removed quickly and easily from the body after their effect.

The possibility of treatment for cancer, a disease defined by defective genes, through the introduction of a gene which targets the modifications, has led to enormous interest in gene therapy for cancer.

The reduced toxicity, absence of pathogenicity and a relatively easy pharmacologic production favours the use of the non-viral vector in the competition over the viral vectors.

Nanodrugs used for hepatocellular treatment act as carriers of chemotherapy, of genes, as thermal ablation systems activated by energy fields (laser, magnets, ultrasound), and with a direct apoptosis effect.

Newly developed nanostructured drugs are being used to treat patients with HCC unsuitable for conventional therapies. Nanotechnology demonstrates the very versatile properties of developed molecules, which can treat HCC through various approaches.

The rapid development of such numerous alternatives suggests that the future will provide powerful therapies for a deadly disease, and currently radical therapies will have little room in HCC treatment.

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**APPLICATIONS OF NANOPARTICLE-BASED
DRUG DELIVERY SYSTEMS
IN BONE TISSUE ENGINEERING**

Junjun Fan and Guoxian Pei*

Department of Orthopaedic Surgery, Xi Jing Hospital, Fourth Military
Medical University, Xi'an, China

*Corresponding author: peiguoxiansci2@163.com

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6.1. INTRODUCTION

Large bone defects caused by trauma, infection, tumour, or other factors are still a big challenge for surgeons to repair. Autologous bone and allograft bone grafts are widely used for the clinical treatment of bone defects; however, there are many drawbacks including limited supply, bone graft resorption, instability in large bone defects, high risk of infection, high failure rates in difficult *in vivo* environments and immunological rejection, all of which impede clinical success [1-6]. Therefore, the urgent need to repair large bone defects has prompted the rapid development of biomaterials and bone tissue engineering. There are three basic elements for bone tissue engineering: biomaterial scaffolds, seed cells and bioactive factors [7]. By combining biomaterials, cells and bioactive factors, tissue engineered bone grafts can provide a native template for promoting the regeneration of bone tissue and finally achieve repair the bone defect. Although lots of basic and clinical researches have shown the feasibility and effectiveness of tissue engineered bone grafts to repair bone defects, there are still many limitations which greatly impede its wider use clinically, such as the uncontrolled release of bioactive factors and insufficient bone formation. Controlled drug delivery of bioactive factors at the bone defect site is essential for triggering and enhancing angiogenesis and osteogenesis of the biomaterial scaffolds and seed cells [8-10]. However, these bioactive factors such as bone morphogenetic protein (BMP), vascular endothelial growth factor (VEGF), transforming growth factor (TGF), and platelet-derived growth factor (PDGF) generally have a short biological half-life, and a rapid inactivation *in vivo*. Repeated administration of these bioactive factors may lead to unexpected side effects including carcinogenicity and toxicity because of uncontrolled drug distribution and accumulation in other tissues or organs [11-13]. An ideal tissue engineered bone graft should have biological properties with a biomimetic local microenvironment and the controlled release of bioactive factors. Thus, a drug delivery system with targeted and controlled release of bioactive factors to the bone defect site is critical to obtain a satisfying therapeutic effect of tissue engineered bone.

To get a controlled and targeted drug delivery system, it should be able to overcome the related limitations to maximise bioactivity while minimising the side effects of bioactive factors [14,15]. Traditional drug delivery systems have difficulty achieving long-term targeted drug release and retaining the stability of bioactive factors *in vivo*. The burst release of drugs makes it hard to achieve continuous and stable drug delivery to simulate osteogenesis by mimicking the natural process [16]. In order to overcome these drawbacks, novel drug delivery systems have been developed with the development of nanotechnology and nanoscale materials. A novel nano-based drug delivery system can achieve site-specific drug delivery and the controlled release of

bioactive factors in the bone defect sites to enhance the bone regeneration. Among these nano-based drug delivery systems, nanoparticles are the most widely used drug carriers, which can control and target the release of drugs into the bone defect sites [17]. The small size of nanoparticles enables better biocompatibility *in vivo*, and its high surface to volume ratio increases drug loading ability and provides better drug bioavailability [18]; meanwhile, the biomaterial property of the nanoparticle can serve as a temporary matrix with which to enhance the mechanical properties of tissue engineered bone. In many researches, the nanoparticle-based drug carrier itself promotes bone regeneration [19,20]. These nanoparticle-based drug delivery systems have been widely used to carry and release bioactive factors to stimulate bone formation in the bone defect site.

With the development of nanomaterials and nanotechnology, this opens up new opportunities in bone tissue engineering with a targeted and controlled drug delivery system to induce and promote bone regeneration. In this chapter, we try to introduce the current developments and applications of nanoparticle-based drug delivery systems used in bone tissue engineering.

6.2. THE PRINCIPLES OF NANOPARTICLE-BASED DRUG DELIVERY SYSTEM

Nanoparticles are usually defined as submicron-sized particles between 1–100 nm in size. These are the most widely used drug carrier vectors, are simply made and have high reproducibility. Many kinds of materials and technological methods can be used to synthesise nanoparticle-based drug delivery systems. Several critical principles need to be considered in order to make intelligent use of a nanoparticle-based drug delivery strategy in bone regeneration.

The first principle is to choose the most suitable material according to the actual situation of clinical application. The inherent physical or chemical properties of the nanoparticle, including the material, size and surface properties, will influence the loading and release of drugs to nanoparticles, as well as their biocompatibility and degradability [21]. There are many kinds of nanoparticle-based drug delivery systems according to different materials which have respective advantages and disadvantages. Liposome nanoparticle-based drug delivery systems have a high drug loading capacity, but their release behaviour is difficult to control [22]. Polymer nanoparticle-based drug delivery systems can be synthesised to generate specific molecular weights and compositions, but their drug loading capacity is low [23]. Particle size is also very important to the biological properties of loaded bioactive factors [24]. Nanoparticle size variation within the nano-scale range can exhibit distinctive physical, mechanical and bioavailability properties, which are very different from in the macroscopic size. Surface properties such as

hydrophobicity also have a significant influence on drug delivery. Surface properties can be modified to improve the loading and release behaviour of drugs, cells and tissue responses *in vivo* [25]. Besides, suitable materials for constructing nanoparticles should have good biocompatible properties, no immunogenicity or toxicity, and suitable biodegradable properties [26].

The second principle is that it is essential to make sure the nanoparticle-based drug delivery system with optimal and controlled drug release behaviour meets the temporal and spatial demands during the process of bone regeneration. Because of the longer period of bone formation compared with other tissues, it needs to make sure that the released bioactive factors can be maintained at the local bone defect area within therapeutic concentrations for a long time as well as the temporal and spatial optimal distribution. The release behaviour of drugs from nanoparticles may be affected by several factors, such as the degradation rate of the nanoparticle, the physiological diffusivity of the loaded drug, the methods used to load the drug and other factors. Biodegradable properties are critical for the release of bioactive factors and should ensure the slow release of bioactive factors *in vivo* to accommodate to the long duration of the bone regeneration process [27].

The third principle is that when the nanoparticle-based drug delivery system is prepared, it needs to ensure the biological activity of the drug when loaded onto the nanoparticle. The conditions of preparation should be mild without harsh solvents, high temperatures or pressures, and extreme pH. After the drug is incorporated into the nanoparticle, the bioactivity of the drug should be examined carefully to avoid any undesirable changes [28].

To achieve better therapeutic outcomes and bone formation, these basic principles should be carefully considered when designing and preparing nanoparticle-based drug delivery systems in tissue engineered bone. Also, we will introduce different kinds of nanoparticle-based drug delivery systems which are used in bone regeneration.

6.3. POLYMER NANOPARTICLE-BASED SYSTEM

Many synthetic polymers have good biodegradability and biocompatibility, and the property of drug loading and release behaviour can be easily improved by changing the molecular mass and surface functional groups. Thus, polymeric nanoparticles are widely used as the best candidates for drug delivery vectors used in bone tissue engineering.

Among these polymeric materials, poly(D,L-lactic-co-glycolic acid) (PLGA) exhibits good drug loading property because of its high molecular weight, biologically compatible degradation and free carboxylate end-groups. Therefore, PLGA nanoparticles have been widely used for the sustained release of encapsulated drugs or genes. PLGA nanoparticles have shown a great

therapeutic effect as a drug delivery system in bone tissue engineering [29]. For example, a bone scaffold with controlled releasing recombinant human bone morphogenetic protein-7 (rhBMP-7) was developed to enhance bone regeneration. The rhBMP-7-containing PLGA nanospheres were loaded onto nano-fibrous poly(L-lactic acid) (PLLA) bone scaffolds. *In vitro* results showed that this PLGA nanosphere-immobilised bone scaffold could release rhBMP-7 in a controlled manner. Also, *in vivo* results showed rhBMP-7 delivered from PLGA nanosphere scaffolds induced significant ectopic bone formation, while the passive adsorption of rhBMP-7 into the PLLA bone scaffold without PLGA nanospheres resulted in the failure of bone regeneration at 6 weeks [30].

Besides loading the proteins of growth factors, PLGA nanoparticles can also be used to load bioactive molecules which can enhance bone regeneration. For example, the PLGA nanoparticles were loaded with dexamethasone (DEX) which was a bioactive molecule for bone regeneration. These DEX-loaded PLGA nanoparticles were prepared using the method of water-in-oil standard emulsion and then immobilised onto the surface of a collagen membrane; the release behaviour of DEX from PLGA nanoparticles showed a sustained release of DEX. Also, the *in vivo* results showed this collagen membrane with DEX-loaded PLGA could repair the calvarial bone defects of rats and result in significantly more new bone formation compared with other bone defects that were unfilled or filled with collagen membrane alone [31].

Owing to the good biodegradability of PLGA nanoparticles, it can also be used as a favourable vector for non-viral gene delivery in bone tissue engineering applications. PLGA nanoparticles can offer the protection of genes to nuclease degradation and increase DNA uptake with the sustained release of encapsulated DNA. For example, PLGA containing alkaline phosphatase (ALP) plasmid DNA (pDNA) exhibited high encapsulation efficiency and sustained release behaviour. *In vivo* results of transfection in a rat tibial muscle showed that this gene delivery system based on PLGA nanoparticles allowed 28 days of sustained gene transfection with increased ALP expression levels [32]. Blood vessel growth is necessary for bone regeneration and polymeric nanoparticles have also been used for gene delivery to promote vascularisation. For example, VEGF pDNA-loaded PLGA nanoparticles were prepared and used for *in vitro* cell transfection and *in vivo* gene transfer. The result showed that it could enhance *in vivo* angiogenesis and increase the density of new capillaries [33].

Surface modification of polymer nanoparticles has also been reported to improve the targeting effect of drug delivery in bone regeneration. For example, tetracycline has good adsorption to calcium phosphate and can be used as a targeting adjunct for bone tissue drug delivery. Thus, tetracycline-modified PLGA nanoparticles were prepared and showed a great affinity with natural bone tissue. This surface modification of PLGA nanoparticles can be used as a targeting drug carrier for bone regeneration [34]. Alendronate is also a targeting moiety that has a strong affinity for bone, and other PLGA nanoparticles modified with both alendronate and poly(ethylene glycol) (PEG)

were prepared by the dialysis method. The results showed that this alendronate-modified PLGA nanoparticle had a strong and specific adsorption to hydroxyapatite (HA) which was the main content in natural bone tissue [35]. Besides surface modification of polymer nanoparticles with bone-specific moiety to improve targeting drug delivery in bone, other materials and methods were also used to modify the surface properties of polymer nanoparticles to improve drug release behaviour. For example, heparin was used to modify the surface of nanoparticles to sustain growth factor release. Several growth factors were known to bind heparin tightly, such as bone morphogenetic protein 2 (BMP-2), and transforming growth factor β (TGF- β) [36]. A heparin-functionalised nanoparticle combined with fibrin gel was prepared and used as a bone scaffold with the sustained release of BMP-2. The formation of new bone was significantly enhanced and more mature bone was obtained by using heparin-functionalised nanoparticles in a rat calvarial bone defect model [37]. Other heparin-conjugated PLGA nanoparticle-loaded BMP-2 were prepared and co-cultured with undifferentiated bone marrow-derived mesenchymal stem cells (BMMSCs). *In vitro* and *in vivo* testing found that these heparin-conjugated PLGA nanoparticles loaded with BMP-2 could induce significantly more new bone formation than control group [38].

6.4. LIPOSOME NANOPARTICLE-BASED SYSTEM

Liposome nanoparticles are also widely used as drug-delivery vehicles to load the bioactive factors. Since liposome nanoparticle-based drug delivery systems can be prepared with phospholipids, which form the natural structure of cell membranes, they are regarded as biocompatible and non-toxic. Because of their phospholipid bilayer membrane, they can also pass through the cell membranes and get into the cells [39]. There are many reports about using this liposome nanoparticle-based drug delivery system for bone tissue engineering.

For example, BMP-2 complementary DNA (cDNA) plasmids were loaded with the liposome nanoparticles and used in the repair of cranial defects of a rabbit model. In this study, the BMP-2 Liposome nanoparticle-loaded system showed great bone repair effect. After 6 weeks, the cranial defects of rabbits were filled with new bone after using BMP-2-loaded liposome nanoparticles [40]. Another study showed that the liposome vector could also be effective for *ex vivo* cell-mediated BMP-2 gene transfer. After pre-treatment with BMP-2 cDNA-loaded liposome vehicles, the bone marrow stromal cells were transplanted into critical bone defects in rats. After 6 weeks, the bone defect area was completely repaired with new bone formation [41].

When combined with magnetic particles, the magnetic liposomes can be modified to increase retention of the drug at the target site under magnetic force. For example, magnetic egg phosphatidylcholine liposomes were prepared with the addition of magnetite particles for TGF- β 1 delivery to

stimulate new bone formation in animal models [42]. The magnetic liposomes system loaded with recombinant human bone morphogenetic protein 2 (rhBMP-2) has also been used to treat bone defects in rats [43]. The bone defect was filled with complete bone bridge formation when treated with rhBMP-2 magnetic liposomes. These magnetic liposomes have better bone formation than conventional liposomes due to the longer persistence of BMP at the bone defect site under magnetic force. It may provide a new method for bone defect treatment with the method of using topical magnetic force to control magnetic drug-loaded liposomes at the injured site.

However, there are also some limitations when liposomes are used as drug delivery vectors in bone repair; the drug loading and release behaviour of liposome nanoparticles is relatively difficult to control. Other limitations include low dissociation efficiency, quick degradation of the drug, and instability of the injected liposome drug complex in solvent [44].

6.5. INORGANIC NANOPARTICLE-BASED SYSTEM

Besides the polymer and liposome nanomaterials, ceramic nanomaterials such as calcium phosphate, HA, and bioactive glass are also used for drug delivery and provide mechanical support in bone tissue engineering. There are chemical similarities to natural bone which provide suitable mechanical strength. These inorganic nanomaterials have much longer biodegradation times and special properties such as electrical, mechanical and magnetic functions. These distinct nanomaterials can be used for specific drug delivery systems in bone repair.

For example, a calcium phosphate nanoparticle was prepared to load BMP-2 and then encapsulated in PLGA microspheres. The controlled release of BMP-2 was obtained for over 7 weeks and higher osteocalcin was expressed when using this calcium phosphate nanoparticle [45]. Another plasmid DNA-loaded calcium phosphate nanoparticle was also proven to be an effective non-viral vector for gene delivery and functioned well for odontogenic differentiation through BMP-2 transfection [46]. HA nanocrystals were also used and cross-linked with collagen to control the release of BMP-2. The animal result showed both good mechanical strength and the formation of new bone using these HA nanocrystals [47]. Another bioactive glass nanoparticle (BGn) with loading of ampicillin or siRNA has been prepared and gained potential application in bone regeneration. The results showed that these bioactive glass nanoparticles had good cell viability and excellent apatite-forming ability. While the ampicillin released relatively rapidly, the loaded siRNA could be released for 3 days with almost zero-order kinetics. The siRNA-nanoparticles were also easily taken up by the cells with a transfection efficiency of up to 80 %. It may be a promising drug release system in bone tissue regeneration [48].

6.6. COMPOSITE NANOPARTICLE-BASED SYSTEM

Organic nanoparticles such as polymeric and liposome nanoparticles have good biodegradability and biocompatibility, while inorganic nanoparticles have many other special properties. Combining different organic and inorganic materials into a composite nanoparticle can provide a synergic function to benefit bone regeneration.

For example, a kind of magnetic liposome with incorporated rhBMP-2 was prepared, and the efficiency for bone formation after topical injection was evaluated in a rat bone-defect model. The results showed that the combined treatment of topical magnetic rhBMP-2 liposomes and magnetic implantation at the injury site was effective for the treatment of bone defects [43]. A novel bone cement pellet with sustained release of vancomycin was prepared by combining mesoporous silica nanoparticle and calcium sulphate alpha-hemihydrate. This composite pellet showed a strongly drug-sustained release effect and *in vitro* cell assays showed high biocompatibility and suitability to be used as bone cement in the treatment of open fractures [49]. Another study showed a new tigecycline-loaded calcium-phosphate/PLGA nanoparticles for controlled drug delivery with a double effect. In the first step, a drug was released from PLGA nanoparticles; in the second stage, after the resorption of PLGA nanoparticles, non-bioresorbable calcium phosphate remained the chief part of the particle and took the role of a filler, filling a bone defect. The average size was from 65–95 nm. This composited nanoparticles proved to be an adequate system for local and controlled drug release [50]. Another group of novel composite nanoparticles combining glycidyl methacrylate derivatised dextrans with gelatine was reported. Also, *in vitro* drug release studies showed that the efficient BMP release from this nanoparticle was maintained for more than 12 days under degradation conditions, and more than 90 % of the loaded BMP was released. No obvious cytotoxicity was found in this composite nanoparticle system [51].

6.7. OTHER NANOSTRUCTURE MATERIALS-BASED SYSTEMS

Although the above-mentioned nanoparticles are generally considered the spherical particulate, other nanostructures, such as dendrimers, nanofibres, nanogels and nanotubes, can also be considered generalised nanoparticles [14]. As a result of the different nanostructures, they may offer unique interfacial and functional advantages compared with spherical nanoparticles when used as drug delivery vectors in bone tissue engineering.

Dendrimers have a highly branched dendritic architecture which can be greatly controlled, as can their shapes, sizes, densities and surface properties. The drug can be physically entrapped by the dendritic architecture or

chemically attached to the surface by electrostatic interactions [52]. These advantages make the dendrimers attractive drug-delivery systems used in bone tissue engineering. For example, folate-poly(amidoamine) (PAMAM) dendrimer was used to carry the human bone morphogenetic protein-2 (hBMP-2) gene-containing plasmid for *in vitro* transfection of mesenchymal stem cells (MSCs). All osteogenic markers such as alkaline phosphatase activity, osteocalcin secretion and calcium deposition were significantly stronger in transfected cells. This study showed the possibility of PAMAM dendrimers used for inducing *in vitro* differentiation of MSCs to osteoblast phenotype [53]. Another dexamethasone-loaded carboxymethylchitosan / PAMAM dendrimer was also used to prompt the proliferation and osteogenic differentiation of rat bone marrow stromal cells *in vitro*. The results showed that this drug loaded dendrimer was a promising drug delivery system in bone tissue engineering [54]. The dexamethasone-loaded dendrimer was also evaluated *in vivo* by being implanted subcutaneously on the back of rats and the results showed that it could promote superior ectopic *de novo* bone formation *in vivo* [55].

Nanofibres are defined as fibres with diameters less than 100 nm. Nanofibres can be used to enhance the mechanical strength for tissue engineered bone and mimic the architecture of natural bone tissue [56]. Besides, the high surface-to-volume ratio and high porosity of the nanofibres combined with their nanostructure make them suitable drug carriers, and the drug release rate can be controlled by changing the morphology, porosity and composition of the nanofibres [57]. For example, BMP-2 was immobilised on a membrane surface made of chitosan nanofibres and half of the initial BMP-2 was attached to the membrane surface. This BMP-2-conjugated chitosan nanofibre membrane significantly promoted cell proliferation, alkaline phosphatase activity, as well as calcium deposition, indicating significant and localised bone formation [58]. DEX was also loaded into PLLA nanofibrous scaffolds by electrospinning. This drug-loaded nanofibre not only increased the mechanical strength in comparison with pure PLLA nanofibres, but also showed a sustained release profile for over 2 months. The cell proliferation and osteogenic differentiation of human mesenchymal stem cells cultured with these drug-loaded nanofibres were both improved compared to the scaffolds without drugs [59]. Rifampicin was also reported to be loaded onto the nanofibre meshes by depositing rifampicin-containing PLGA micro-patterns onto the PCL/chitosan biomimetic nanofibre meshes *via* ink-jet printing. This drug-loaded nanofibre mesh not only prevented the biofilm formation, but also favoured the attachment, spreading and osteogenic differentiation of pre-osteoblasts by up-regulating the gene expression of bone markers. This drug-loaded nanofibre mesh provides a feasible multifunctional surface for enhancing bone tissue formation while controlling infection [60].

A nanogel is a nanoparticle composed of a nano-scale hydrogel which presents a cross-linked hydrophilic polymer network with tens to hundreds of

nanometres in diameter. Like hydrogels, the pores in nanogels can be filled with drugs with a high drug-loading capacity for its high surface-to-volume ratio and heterogeneous nanostructure [61]. With regard to their properties of swelling, degradation and chemical functionality can be controlled when chemically or physically cross-linked. As drug vectors, nanogels display extended stability, sustained release and low cytotoxicity when used in bone tissue engineering [62]. For example, prostaglandin E2 (PGE2), a bone anabolic agent, was loaded onto a nanogel of cholesterol-bearing pullulan (CHP) and injected on to the calvariae of mice. This PGE2-loaded CHP nanogel induced new bone formation, while PGE2 alone or CHP alone did not induce any new bone formation. The results showed that this nanogel-based delivery system is efficient for promoting bone regeneration [63]. Another cholesterol-bearing pullulan nanogel-crosslinking hydrogel (CHPA/Hydrogel) was used to deliver BMP and implanted into the calvarial defects. The results showed that BMP loaded nanogel could induce osteoblastic activation and new bone formation *in vivo* [64]. Cholesteryl group- and acryloyl group-bearing pullulan (CHPOA) nanogels were aggregated to form fast-degradable hydrogels by cross-linking with thiol-bearing PEG. Also, two distinct growth factors, BMP-2 and recombinant human fibroblast growth factor 18 (FGF18), were loaded onto the nanogels and then implanted into a critical-size skull bone defect. The results showed that the drug-loaded hydrogel treatment strongly enhanced and stabilised the BMP-2-dependent bone repair by inducing osteoprogenitor cell infiltration inside and around the hydrogel. This report showed the successful delivery of two different proteins to the bone defect to induce effective bone repair by nanogel-based drug delivery systems [65].

A nanotube is a nanometre-scale tube-like nanostructure nanoparticle which can offer advantages over spherical nanoparticles for some applications. Many kinds of materials such as polymers, metals and inorganic materials can be used to fabricate nanotubes [66]. For its special nanostructure, it has large inner volumes which can be filled with kinds of drugs with different sizes, and the open-mouthed structure of nanotubes makes the drug loading process much simpler [67]. The nanotube can be used as a suitable drug vector in bone tissue engineering with its biocompatibility, low cytotoxicity and ability to promote bone formation. For example, DEX was used to be loaded onto the rosette nanotubes and the results showed for the first time that the drug could be easily encapsulated into nanotubes and released for a long time to promote osteoblast function [68]. Another TiO₂ nanotube was also used as a drug vector for loading bone morphogenetic protein 2 and then constructed on titanium substrates covered with gelatine and chitosan. The result showed that BMP-2-loaded nanotube was able to stimulate proliferation and promote the osteoblastic differentiation of mesenchymal stem cells [69]. Another *N*-acetyl cysteine (NAC)-loaded nanotube titanium (NLN-Ti) implant was also prepared as a potential drug delivery system to promote bone formation. *In vitro*, NAC was released in a sustained manner from NLN-Ti implants. Cell viability was

increased and inflammatory responses were decreased when mouse osteoblastic cell line (MC 3T3-E1) cells were co-cultured with the drug-loaded implant. *In vivo* results also showed increased newly formed bone volume and bone mineral density when the drug-loaded nanotubes implanted in the mandibles of rats [70].

6.8. SUMMARY AND FUTURE CHALLENGES

Nanoparticle-based drug delivery systems have generally been used in bone tissue engineering and bone regeneration with its outstanding characteristics. It provides a more effective and efficient method with which to deliver growth factors, genes or other bioactive factors to induce and enhance the process of bone regeneration. Different materials and methods used to prepare the nanoparticles have different properties with respective advantages or disadvantages. Combining different materials and methods to obtain composite nanoparticles as a drug delivery system can have a synergic function and benefit in the form of a better effect of bone regeneration. With the development of nanomaterials and nanotechnology, this drug delivery strategy may be a promising approach with which to overcome the previous limitations of bone tissue engineering when used in the clinic.

However, it is necessary to realise that most nanoparticle-based drug delivery systems are still in the early phases of laboratory research, and their toxicity and safety when used in patients are still lacking. A precise understanding about how different bioactive factors influence the bone regeneration process is still unclear. Thus, the wide use of nanoparticle-based drug delivery systems in clinical application is faced with more challenges.

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NANOPARTICLE-MEDIATED siRNA DELIVERY FOR LUNG CANCER TREATMENT

**Anish Babu^{1,2}, Narsireddy Amreddy^{1,2}, Ranganayaki
Muralidharan^{1,2}, Anupama Munshi^{2,3}, and Rajagopal Ramesh^{1,2,4*}**

¹Department of Pathology

²Stephenson Cancer Center, University of Oklahoma Health Sciences
Center, Oklahoma City, Oklahoma 73104, USA

³Department of Radiation Oncology, University of Oklahoma Health
Sciences Center, Oklahoma City, Oklahoma 73104, USA

⁴Graduate Program in Biomedical Sciences, University of Oklahoma
Health Sciences Center, Oklahoma City, Oklahoma 73104, USA

*Corresponding author: rajagopal-ramesh@ouhsc.edu

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7.1. INTRODUCTION

RNA interference (RNAi) is a process by which the function of a specific messenger RNA (mRNA) is blocked by introducing a short fragment (21–23 nucleotides) of RNA with complimentary sequences into the cell cytoplasm. The cellular machinery involving the RNA-induced silencing complex (RISC) then processes this short RNA (shRNA) into antisense RNA fragments, which ultimately bind to the complimentary mRNA and prevent the translation of mRNA into specific proteins [1]. Small interfering RNA (siRNA) is a double-stranded RNA helix that has been heavily investigated as an RNAi tool for anti-cancer gene therapy [2]. siRNAs are designed to specifically target genes, causing effective downregulation of proteins that are involved in cancer pathology and progression. In the past decade, tremendous achievements have been made in the development of siRNA therapeutics for cancer therapy, as demonstrated by a number of human clinical trials in progress [3]. Successful application of RNAi depends on the effective intracellular delivery of siRNA, bypassing a number of biological barriers. In the laboratory, siRNA is more stable than native mRNA; however, siRNAs are prone to degradation by nucleases and have a short half-life of less than 1h in the presence of plasma proteins. The macromolecular size, hydrophilicity, and negative charge of siRNA prevent effective transportation of naked siRNA across the cellular membrane. Therefore, an effective delivery vector is required for siRNA to be transported across physiological barriers to the desired cell cytoplasm. Viral vectors were the first siRNA delivery systems investigated thoroughly for their efficiency in targeted gene knockdown. Despite their high transfection efficiency, viral vectors were identified as potential elicitors of immune reactions in humans. Other safety concerns, such as the possibility of viral mutations, recombination, and oncogenic effects, also limited the use of viral vectors for siRNA therapeutic delivery [4]. Recent advancements in nanotechnology have driven the revolution in developing nanoparticle carriers for highly challenging siRNA delivery [5]. Current siRNA delivery systems can be categorized into two general classes: organic and inorganic nanoparticles. Commonly used siRNA nanocarriers, such as liposomes and polymer nanocarriers, are included in the class of organic nanoparticles, whereas metal-based nanoparticles, Quantum dots (QDots), carbon nanotubes, and mesoporous silica nanoparticles are inorganic nanoparticles. In this chapter, we will provide an overview of the current strategies of siRNA delivery using nanoparticle formulations and discuss our efforts in formulating nanoparticle-based siRNA delivery systems toward a recently recognized molecular target of cancer, the Human antigen R (HuR).

7.2. LIPOSOMES AS siRNA NANOCARRIERS

Liposomes or lipid nanoparticles are commonly used for siRNA delivery to mammalian cells. Liposomes are unilamellar or multilamellar micro-vehicles consisting of a phospholipid bilayer. Liposomal nanocarriers have been extensively used to enhance efficient drug delivery since they are biocompatible [6]. These amphiphilic phospholipids have a hydrophobic tail and a hydrophilic polar head. They form a bilayer in water, with the hydrophobic tails facing each other and the hydrophilic side facing towards water. Because of their shape, size, and surface characteristics, these liposomes have the ability to deliver different payloads, including chemotherapeutic drug, DNA, siRNA, shRNA, and proteins. Liposomal carriers offer several advantages. Liposomes prevent enzymatic degradation of siRNA, support high siRNA loading, allow preferential accumulation of siRNA at the tumor site, promote endosomal escape resulting in efficient cytoplasmic delivery, and provide a safe and effective systemic delivery [7]. Molecules containing several amines per head group, with slight spacing between the amine groups, can bind to the negatively charged backbone of siRNA more efficiently than the lipids containing a single positive charge per head group. The stability of the positively charged lipids may be enhanced by the addition of the neutral lipid (helper lipid) to reduce the repulsion between similar charges in the lipid bilayer. The addition of cholesterol, which takes up residence in the hydrophobic region in the bilayer, improves the stability of the carrier and facilitates the cellular uptake of siRNA.

7.2.1. Cationic lipids/liposomes

Cationic liposomes are non-viral delivery systems that are extensively used to deliver RNAi [7]. Cationic lipids can self-assemble with negatively charged DNA and siRNA to form lipoplexes by electrostatic interaction, and enhance transfection efficiency. Almost two decades ago, Malone and colleagues demonstrated the use of cationic lipids in nucleic acid delivery towards mammalian cells [8]. Later, the same group described the process of lipofection in detail, using cationic lipid *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium chloride (DOTMA) to deliver DNA and RNA into mouse, rat, and human cancer cells [9]. Since then, liposomes have been used as gene delivery vehicles for many biomedical applications. Optimizing the lipid composition, size, charge, payload-to-lipid ratio, and the targeting moiety will provide an efficient liposomal system for siRNA delivery. The most successful commercial transfection agent is a liposomal formulation, Lipofectamine 2000, or its advanced version, RNAiMAX [10]. siRNA can rapidly complex with Lipofectamine 2000 to form lipoplexes because of the strong anionic-cationic interaction. While possessing a strong positive charge for efficient complexation with siRNA and promoting an enhanced gene silencing effect, cationic lipids/liposomes are rapidly cleared from the circulation and are toxic to cells [11]. Anionic lipids or neutral lipids show better stability in the

circulation; however, the weak interaction between nucleic acid-anionic/neutral lipids may result in the premature release of nucleic acids into the circulation. Thus, cationic lipids are still the leading choice for siRNA delivery formulations.

Studies from our lab have shown that the cationic lipid carrier *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium chloride: Cholesterol (DOTAP:Chol) can deliver the tumor suppressor genes *p53*, *FUS1*, and *MDA7/IL-24* to lung tumor models [12-14]. Our studies demonstrated that DOTAP:Chol nanocarriers were selectively taken up by lung tumors without causing toxicity to the surrounding lung tissues, and resulted in increased transgene expression. In a typical study, the specificity of DOTAP:Chol liposomes was heightened by modification with the targeting moiety anisamide, enhancing the cellular uptake in lung cancer cells overexpressing sigma receptors [15]. A Phase I clinical trial was conducted using DOTAP:Chol-TUSC2 tumor suppressor gene complexes for treating human lung cancer [16]. The DOTAP-Chol-TUSC2 treatment resulted in transgene and gene product expression, specific alterations in TUSC2-regulated pathways, and antitumor effects. Besides our studies, numerous cationic liposomal systems have been investigated for gene delivery applications in cancer *in vitro* and *in vivo*. Some excellent reviews summarized the recent advances in cationic lipids/liposomes in gene delivery [17-19].

The efficiency of cationic liposomes in gene delivery can be further improved by the addition of poly(amino acid)-conjugated poly(ethylene glycol) (PEG) chains. For example, poly(L-arginine)-conjugated PEG-DOTAP/DOPE:Chol liposomes demonstrated enhanced intracellular uptake and low cytotoxicity compared with unmodified cationic liposomes in cancer cells [20]. PEG modification of liposomes has many advantages, such as preventing aggregation, enhancing shelf life, prolonged blood circulation time, reduced opsonization, slow clearance, and acting as a linker for the further modification of liposomes. Targeting ligands can be attached to the extremity of the PEG chains and interact with antigens or receptors overexpressed on the surface of the cancer cells. Incorporation of excess PEG-phospholipids will disrupt the integrity of the lipid membrane, due to its detergent-like properties, which will increase premature drug release and membrane permeability [21]. However, the degree of surface PEGylation is usually less than 5 mol %, in order to preserve the liposomes' integrity [22]. Various formulations of sterically stabilized PEG liposomes have been used for the systemic delivery of nucleic acid for gene silencing [23]. Atu027 is a DSPE-PEG-chains-stabilized, lipoplexed siRNA targeting protein kinase3 (PKN3). Atu027 underwent Phase I clinical trials in patients with cancer and was demonstrated to have a safe clinical profile, which might be partly attributable to the presence of the DSPE-PEG component [24]. This silencing therapeutic is currently undergoing Phase 2 trials as a treatment for advanced solid and metastatic cancers [25].

7.2.2. Lipid nanoparticles

Lipid nanoparticles have been proposed as alternative siRNA carriers [19]. Structurally, lipid nanoparticles are slightly different from liposomes, as lipid nanoparticles are composed of emulsified solid-lipid particles in aqueous dispersion. Lipid nanoparticles effectively protect the incorporated nucleic acids from nuclease attack and are known to modulate its releasing properties [26]. They are usually prepared from physiologically well-tolerated lipids, such as triglycerides like Tristearin, Compritol 888 ATO, and Dynasan 112, carnauba wax, cetyl alcohol, cholesterol, and cholesterol butyrate. Recently, solid tristearin lipid nanoparticles have been tested for sustained release of siRNA *in vivo* [26]. The siRNA was loaded into tristearin nanoparticles using an ion pairing approach, with the help of the cationic lipid DOTAP. The tristearin-DOTAP nanoparticle demonstrated extended release (10–13 days) of siRNA in a mouse model. Another study investigated the efficacy of a lipid nanoparticle-siRNA formulation in silencing androgen receptor (AR) protein in human prostate cancer cell lines and xenograft models [27]. Researchers screened a panel of cationic lipids and found that lipid nanoparticles that contain ionizable cationic lipid 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-KC2-DMA) showed the highest gene silencing efficiency *in vitro*. In the next step, they demonstrated its transfection efficiency in xenograft tumors, producing good gene silencing efficiency. This study claimed to be the first *in vivo* use of a lipid nanoparticle delivery system for silencing AR gene expression. siRNA-incorporated lipid nanoparticles have also been used to achieve synergistic therapeutic effects in anti-cancer combination therapy with co-loaded drug. A typical strategy used QDots incorporating siRNA/drug-loaded lipid nanoparticles for combinatorial therapy of human lung cancer cells [28]. The nanoparticles were able to co-deliver paclitaxel and BCL2 siRNA, and exhibited synergistic anticancer effects. In addition, the fluorescence exhibited by the QDots was helpful in intracellular localization of lipid nanoparticles in cancer cells. Such an application of lipid nanoparticles holds great promise in the field of cancer theranostics.

7.3. POLYMERIC NANOPARTICLES FOR siRNA DELIVERY

Polymeric nanoparticles have been widely investigated for siRNA delivery systems. siRNA is either electrostatically bound with the positively charged functional groups of polymers, or encapsulated within the polymer matrix of nanoparticles. Some commonly used polymers include poly(ethyleneimine) (PEI), PLGA (poly(lactic-co-glycolic acid)), cyclodextrin polymers, branched dendritic polymers, and chitosan. The unique properties of each of these polymers allowed researchers to investigate even the combined benefits of two or three polymers for efficacious siRNA delivery.

7.3.1. Poly(ethyleneimine)-based nanodelivery systems

The most studied polymer for siRNA delivery systems is PEI. PEI is a synthetic polymer with the ability to electrostatically interact with negatively charged siRNA due to its cationic ability to form nanoscale complexes. Both linear and branched PEIs have been used as siRNA nanocarriers. These PEIs demonstrate a high buffering capacity to bypass the endosomal barrier. However, the clinical application of PEI is limited, because its membrane-permeabilizing nature is toxic for cells [29]. Tremendous efforts have been devoted to modifications of PEI without affecting its nucleic acid condensing ability, in order to reduce toxicity and to achieve biodegradability. Recently, the introduction of disulfide bonds (S–S) in PEI has been shown to increase its biodegradability and reduce its toxicity, rendering this polymer more promising for efficacious gene delivery [30]. Moreover, due to the siRNA condensation ability of PEI, it has been used as a major component of many gene delivery systems. Combination with other biocompatible polymer(s) not only reduced PEI's toxicity, but also improved gene delivery efficacy [31,32]. For example, a phospholipid dioleoyl-phosphatidylethanolamine (DOPE) conjugated with PEI has been investigated for P-glycoprotein gene-targeted siRNA delivery to reverse drug resistance in breast cancer cells. The transfection efficiency of the PEI carrier, which was otherwise ineffective against doxorubicin-resistant MCF-7 breast cancer cells, was enhanced upon conjugation with DOPE [31].

7.3.2. Poly(lactic-co-glycolic acid) nanoparticles

In contrast to PEI, PLGA is recognized as a safe polymer and is FDA-approved for human use. PLGA is one of the most highly investigated polymers for drug and gene delivery applications [33]. PLGA nanoparticles are known for their biodegradability and biocompatibility, and employ mild formulation techniques. A simple interfacial deposition or double emulsion technique can be followed to prepare siRNA-loaded PLGA nanoparticles. However, the poor siRNA loading efficiency is a matter of concern, mainly because PLGA cannot electrostatically interact with siRNA [34]. Moreover, PLGA nanoparticles show moderate efficiency in endosomal escape and cytoplasmic release of siRNA, which ultimately lead to low transfection efficiency. Nevertheless, researchers have shown that modification of PLGA nanoparticles with positively charged components enhance the efficiency of siRNA delivery. Poly(ethyleneimine), cationic lipids, and chitosan are important cationic agents that are used for PLGA nanoparticle modification. A recent study showed that coating PLGA nanoparticles with lipid improves siRNA encapsulation efficiency [35]. Another group investigated PLGA modification housing PEI for co-delivery of signal transducer and activator of transcription 3 (STAT3) siRNA and paclitaxel to drug-resistant lung cancer cells. This PLGA-PEI combination was effective in co-delivering STAT3 siRNA and paclitaxel, resulting in downregulation of STAT3 expression and controlled release of paclitaxel [36].

7.3.3. Cyclodextrin-based nanocarriers

Cyclodextrin, a natural polymer derived from cellulose by bacterial enzymatic digestion, is an attractive delivery system due to its structural characteristics, excellent biocompatibility, and water solubility. While the typical structure, which is truncated and cone-like with a cavity in the center, helps to form inclusion complexes with hydrophobic anticancer drugs, the amidine functional groups allow for the efficient condensation of nucleic acids. The large number of functional groups in cyclodextrin polymer allow for the bioconjugation of ligands or antibodies for targeted delivery. Cyclodextrin was approved for clinical trials examining siRNA delivery in cancer treatment, before any other cationic polymer [3]. The targeted cyclodextrin containing that polymer-siRNA delivery system, denoted as CALAA-01, is currently being investigated in clinical trials for cancer treatment [37]. CALAA-01 has four different components: cyclodextrin, siRNA, steric stabilization agent poly(ethyleneglycol) (PEG), and human transferrin (Tf) as a targeting ligand. These components self-assemble to form a nanocomplex. The CALAA-01 nanoparticle carries siRNA for targeted disruption of the M2 subunit of ribonucleotide reductase, designed to inhibit tumor growth. The preliminary study demonstrated that this cyclodextrin polymer-siRNA therapeutic had a better safety profile with low kidney toxicity in humans than was seen in preclinical observations in animals [37]. Apart from the cyclodextrin-polymer nanoparticles, Li *et al.* (2013) investigated the effect of PEI-conjugated cyclodextrin on siRNA delivery [38]. The study used FDA-approved 2-hydroxypropyl- β -cyclodextrin (HP- β -CD) and low molecular weight PEI to synthesize an HP- β -CD /PEI siRNA nanocomplex for targeted cancer therapy. The nanoparticle demonstrated good *in vivo* stability and efficient gene knockdown, leading to tumor growth inhibition in a mouse model [38].

7.3.4. Dendrimers

Dendrimers form another class of polymer-based siRNA nanoparticle system of synthetic origin [39]. Typically, a dendrimer molecule consists of a central core from which repetitive branch units arise to a predetermined branch number, known as generations. The internal cavities thus formed can be utilized for encapsulation of small molecules or drugs, and the end functional groups of branchlets determine whether they can interact with nucleic acids. Thus, a dendrimer can be precisely designed for siRNA delivery by making them cationic for efficient condensation and protection of siRNA. Different kinds of dendrimers, including poly(amidoamine) (PAMAM), poly(L-lysine), poly(propyleneimine), carbosilane, triazine, and poly(glycerol)-based dendrimers, have been explored for siRNA delivery [40]. Among these, PAMAM is the best studied, since it is relatively easy to synthesize and is available as a fully characterized commercial product. However, the use of cationic dendrimers is limited due to toxicity, which greatly depends on their surface chemistry [41]. Surface modification with PEG has shown to be effective in

reducing PAMAM dendrimer toxicity and improving gene silencing efficiency [42]. Another study demonstrated that arginine modification of low generation dendrimers resulted in effective gene delivery vectors both *in vitro* and *in vivo* [43]. Further, arginine modification also reduced the toxicity associated with PAMAM dendrimers, demonstrating their potential as a safe nanovector for siRNA delivery.

7.3.5. Chitosan-based nanocarriers

Chitosan, a natural polysaccharide polymer, has been extensively studied in the field of gene delivery research. The strong positive charge of chitosan allows its electrostatic interaction with siRNA to form self-assembled nanocomplexes. These nanocomplexes are able to protect siRNA from degradation and act as carriers. The important advantages of chitosan are its biocompatibility, biodegradability, and strong mucoadhesive nature. Chitosan has recently gained immense interest, as it is generally recognized as safe (GRAS) by the FDA and is a cost-effective delivery system for RNAi candidates. Another unique advantage of chitosan, apart from its ability to form nanocomplexes, is that it forms stable nanoparticles in the presence of strong crosslinkers or counter ions, such as tripolyphosphate and sodium sulfate. Crosslinked nanoparticles provide significant protection and enhanced loading of siRNA, possibly through the combined mechanisms of electrostatic interaction and entrapment in the entangled mass of chitosan-TPP [44]. Despite these advantages, limitations do exist, including insolubility in neutral and physiological pH and a slow endosomal escape rate [45]. However, chemical modifications and improvements in formulation have been developed to overcome the limitations of native chitosan in siRNA delivery applications. The solubility of chitosan siRNA carriers can be increased by PEG modification or by using chitosan derivatives, such as glycol-chitosan. However, siRNA loading was significantly impaired with such systems, as many of the available amine groups are utilized in the bioconjugation process, and the presence of free hydroxyl groups has hindered the electrostatic interaction between siRNA and chitosan. The endosomal escape mechanism is highly influenced by protonation of amines in cationic polymers by acidic conditions in endosomes. Thus, a low charge density of chitosan polymer allows only moderate efficiency in endosomal escape. Modifications of chitosan polymer with suitable polymers or molecules to bypass the endosomal barrier have resulted in enhanced siRNA delivery and transfection efficiency in cancer cells. Grafting of PEI with chitosan polymers was shown to be a successful strategy to increase the endosomal escape and transfection efficiency [46]. Similarly, conjugation of histidine molecules to chitosan polymer enhanced the gene transfection efficiency of the chitosan carrier, perhaps by aggravating the endosomal escape mechanism [45].

7.4. INORGANIC NANOPARTICLES AS siRNA CARRIERS

In recent years, inorganic nanoparticles have emerged as potential siRNA delivery systems. QDots, iron oxide, gold, carbon nanotubes (CNTs), and mesoporous silica nanoparticle are all inorganic nanoparticles that are commonly used for gene/drug delivery applications. Inorganic nanoparticles can also be used in image-guided therapy. For example, gold, iron oxide, or QDot nanoparticles possess unique electronic, optical, and magnetic properties that enable real-time imaging of siRNA/drug delivery. The dual-purpose characteristics of inorganic particles, citing suitable examples, are discussed below.

7.4.1. Quantum dots

Semiconductor QDots, which are light-emitting nanoparticles, have been increasingly used as biological imaging and labeling probes due to their high quantum yield. Researchers recently discovered that QDots can also be used as efficient siRNA delivery material for tumor cells [47]. Thus, QDots act as a dual-purpose platform for the delivery and localization of siRNA inside tumors. This strategy allows for the monitoring of successful transfection in real time. In a typical study, QDots were decorated with siRNA and tumor-homing peptides were targeted to xenograft tumors in mice [48]. This study employed siRNA conjugation with the PEGylated QDot scaffold through a chemical cross linker, rather than using electrostatic interaction, as reported in many other schemes. Escaping the endosomal barrier is a challenge for QDot-delivered siRNA. In order to facilitate proper intracellular transport and endosomal escape of siRNA, QDots were coated with a proton-absorbing polymer in a different strategy [49]. It was noted that the creation of this proton-sponge coating enhanced the gene silencing efficiency of the QDot-siRNA complex 10–20 folds, with a simultaneous reduction in cytotoxicity, compared with commercial transfection reagents.

7.4.2. Iron oxide nanoparticles

Iron oxide nanoparticles were initially developed as feasible imaging agents due to their intrinsic magnetic properties. The superparamagnetic nature of iron oxide nanoparticles was explored for use with T₂ MRI imaging. Later, magnetic iron oxide nanoparticles demonstrated their potential in drug and gene delivery applications. The large surface area of these nanoparticles facilitates multiple functional modifications, such as enabling the conjugation of targeting molecules, drugs, siRNA, and DNA. Thus, the versatile nature of iron oxide nanoparticle delivery systems offers the potential for magnetically guided targeting coupled with gene therapy [50], drug delivery, MRI imaging, and magnetic hyperthermia. Boyer *et al.* (2010) synthesized iron oxide nanoparticles (IONPs) surface-coated with two different kinds of polymers [51]. The inner layer coating for IONP was poly(dimethylaminoethyl acrylate)

(P(DMAEA)), and the outer layer was coated with poly(oligoethylene glycol) methyl ether acrylate (P(OEG-A)). The hybrid nanoparticles thus formed were used to create an IONP-siRNA nanoparticle complex. These IONP-siRNA nanocarriers demonstrated good transfection efficiency in a substrate adherent s(type) clone of SKNSH (SHEP) human neuroblastoma cells under an external magnetic field. After attaching polymers and siRNA to the IONPs, studies showed high proton transverse relaxation enhancement (T_2) (160 s^{-1} per mM of Fe) [51]. Apart from magnetic-guided targeting, iron oxide nanoparticles have also shown promise in the peptide-targeted delivery of siRNA. Lee *et al.* (2009) synthesized manganese-doped magnetic iron oxide (MnMEIO) nanoparticles, and then conjugated these with cancer-specific targeting moieties: the Arg-Gly-Asp (RGD) peptide, which targets $\alpha\beta3$ receptors, and Cy5-dye-labeled siGFP, which inhibits the expression of green fluorescence protein (GFP) [52]. The nanoparticle formulation (MnMEIO-siGFP-Cy5/PEG-RGD) showed specific cell uptake and targeted gene silencing in MDA-MB-435 breast cancer cells expressing $\alpha\beta3$ receptors [52].

7.4.3. Silica nanoparticles

Another promising material for siRNA delivery for cancer therapy is mesoporous silica. Mesoporous silica nanoparticles (MSNs) can be used for multiple therapies simultaneously, which allows for combinations of drug/DNA and drug/siRNA, since the silica nanoparticles have high drug-loading capacity [53,54]. The cationic functionalization of MSNs allows for the binding of nucleic acids on the particles' surface, and the porous structure permits the encapsulation of large amounts of drug molecules within the particles. In a feasibility study for co-delivery of nucleic acid(s) and drug, Bhattarai *et al.* 2010 developed a polycation and PEG-modified mesoporous silica nanoparticle [55]. The MSNs were complexed with plasmid DNA (luciferase) or siRNA (luciferase targeting) through electrostatic interaction, and were then loaded with lysosomotropic agent chloroquine (CQ) to treat B16F10 murine melanoma cells. The co-delivery of CQ and the siRNA significantly improved the transfection efficiency and silencing activity of the complexes compared with CQ-free MSNs. This study thus demonstrates an important strategy for combination (siRNA/DNA-Drug) cancer therapy using a single nanoparticle platform [55]. In the same year, another study reported the co-delivery of P-Glycoprotein (Pgp)-targeted siRNA and anti-cancer drug doxorubicin using a cationic MSN [53]. The cationic modification of MSN was performed using a PEI coating for efficient siRNA complexation and delivery. The effective delivery of Pgp-targeted siRNA along with controlled release of doxorubicin mediated by MSNs improved the chemosensitivity of the drug-resistant KB-V1 cancer cell line towards doxorubicin. A new strategy of packaging siRNA into MSN's mesopores has been recently developed by Li *et al.* (2011) [56]. The siRNA was packed under strong dehydrated solution

conditions, followed by capping the siRNA-MSN using a cationic PEI polymer. The siRNA-MSN-PEI nanoparticle demonstrated good siRNA protection efficiency and efficient cytoplasmic delivery of siRNA. High-efficiency knockdown of enhanced green fluorescent protein (EGFP) gene and the Bcl2 gene were achieved using the siRNA-MSN-PEI nanoparticle [56]. A further enhancement in transfection was obtained when they conjugated a fusogenic peptide, KALA, to the siRNA-MSN-PEI nanoparticle to treat lung cancer and human cervical cancer cell lines [57].

7.4.4 Carbon nanotubes

Carbon nanotubes (CNTs) are novel nanomaterials, with unique physical and chemical properties compared with other carbon materials. Recent research has demonstrated that, due to their nano-needle structure, CNTs easily cross the plasma membrane and translocate into the cytoplasm of target cells, using an endocytosis-independent mechanism without inducing cell death. Interestingly, several functionalized CNTs have already been generated and tested for siRNA delivery. Nucleic acids can be conjugated to phospholipid-functionalized carbon nanotubes *via* disulfide bonds, which are cleavable at the cell cytoplasm [58]. This strategy allows the single-walled carbon nanotube (SWCNT) to successfully deliver DNA oligonucleotides across the cell membrane and permits efficient nuclear translocation of DNA. Notably, DNA-phospholipid modified SWCNT demonstrated high RNAi efficiency compared with a commercial transfection agent, Lipofectamine®. The same strategy of siRNA conjugation and delivery was again found successful when another research group transfected human T cells with CXCR4 siRNA using CNTs [59]. In a different strategy, alkylated dendron-functionalized multi-walled (MW) CNTs were used for siRNA delivery [60]. Dendrons with terminal tetraalkylammonium salts imparted a positive charge to MWCNTs, which allowed for efficient siRNA loading combined with the intrinsic cell penetration properties of CNT; the cellular transport of siRNA was remarkably increased. Cationic functionalization also allowed the topical delivery of CNTs. For example, a recent study demonstrated that succinated poly(ethyleneimine) (PEI-SA) functionalized SWCNTs were able to penetrate mouse skin to deliver a siRNA therapeutic to melanoma cells in a C57BL/6 mouse model [61]. Such novel strategies have provided new possibilities for future siRNA delivery and cancer therapy using functionalized CNTs.

7.4.5. Gold nanoparticles

Gold nanoparticles are attractive carriers for drug or gene therapeutics, due to their excellent biocompatibility, chemically inert nature, tunable size and shape, large surface area for functionalization, and controllable surface plasmon resonance property. They are usually hybrid systems with a central gold core surrounded by a layer of organic polymer(s) or biomolecules with desirable functionalities for use as nanocarriers. The plasmon resonance

property of gold nanoparticles offers the possibility of 'on-demand' release of their cargo in response to optical-laser excitation. This property of plasmon resonance has been widely explored in the context of siRNA delivery [62,63]. In one study, the nanoparticle was designed to release its cargo nucleic acid by irradiation with near-infrared (NIR) wavelength light [62,64]. The surface linker bond between the siRNA gold nanoshell was cleaved by femtosecond pulses of NIR radiation, releasing the siRNA therapeutics [62]. Another approach involved the use of light irradiation in the controlled release of siRNA from surface-coated layer(s) or from covalently attached biomolecules on gold nanoparticle surfaces. The energy absorbed by nanoparticles upon light irradiation increased the temperature on the surface of the gold nanoparticles; this broke the surface layer linkages or the linkage (Au-S) between nucleic acids and gold, and ultimately released the therapeutic nucleic acid from the nanoparticles [65,66]. While both strategies show promise in successful gene delivery, the latter may be more advantageous, since the required power of light (laser) is relatively low and the irradiation time required to release the payload is short.

pH-triggered charge-reversal is a novel technique applied to the controlled release of siRNA from gold nanoparticle-polymer hybrid systems. In this technique, the nanoparticle possesses a negative charge while in the blood stream. The charge reverses to positive following entry into the cell endosome, causing endosomal disruption and siRNA release. Gold nanoparticles are functionalized with specific charge-reversal polymers to render them pH sensitive. Guo *et al.* (2010) showed that cy5-siRNA complexed with gold nanoparticles functionalized with charge-reversal polymer poly(allylamine)/poly(ethyleneimine)/11-mercaptopundecanoic acid (cy5-siRNA/PEI/PAH-Cit/MUA-AuNP) successfully delivered pH-triggered siRNA to HeLa cells. The transfection efficiency of these charge-reversal functional gold nanoparticles was better than commercial transfection agent Lipofectamine 2000, with much lower toxicity to cells [67]. Another strategy utilized glutathione-cleavable disulfide linkages to create siRNA-gold nanoparticles that were sensitive to tumor-relevant glutathione levels [68]. In this study, siRNA was first conjugated to PEG-modified gold nanoparticles *via* glutathione-cleavable disulfide linkages. Then, nanoparticles were end-modified with poly(aminoester)s (PBAEs) to facilitate efficient nucleic acid delivery. The PBAE-siRNA-AuNPs were able to knockdown the target gene with efficiency comparable to the commercial transfection reagent Lipofectamine 2000.

7.5. NANOPARTICLE-BASED HUR siRNA DELIVERY

The current efforts in our laboratory are focused on developing powerful siRNA delivery systems for the targeted knockdown of HuR (Figure 1). HuR is a nucleocytoplasmic shuttling protein, is overexpressed in several cancers

including lung cancer, and its overexpression was shown to be a poor prognostic marker in patients diagnosed with lung cancer. Studies from our laboratory demonstrated that HuR knockdown using HuR-siRNA in several lung cancer cell lines significantly reduced cell growth and had a global effect on tumor cell growth inhibition. With enormous interest in the unique characteristics of each of the lipid, polymer, and metal-based nanoparticles, we tried to explore various siRNA delivery systems targeting HuR in cancer cells. This section of the chapter will discuss the characteristics and challenges in the development of HuR siRNA delivery using three different classes of nanocarriers: liposomes, chitosan nanoparticles, and gold nanoclusters.

Figure 1. HuR siRNA delivery strategies using nanoparticles. (A) Liposome-siRNA complex. siRNA is encapsulated *via* charge-charge interactions between cationic lipid and negatively charged siRNA, (B) Gold nanocluster-siRNA complex. siRNA is conjugated *via* glutathione-cleavable disulfide (S-S) bonds, (C) Chitosan nanoparticles encapsulating siRNA. siRNA is entrapped within an ionically crosslinked chitosan-tripolyphosphate nanoparticle. All of the nanoparticle systems are modified with specific ligands for targeted siRNA delivery.

7.5.1 DOTAP:Chol liposomes

Since our previous studies with DOTAP:Chol liposomal carriers were successful delivering genes [12-15], we chose this system to deliver siRNA targeting the RNA binding protein HuR *in vitro* and *in vivo*. *In vitro* studies

were conducted in many cancer cell lines that overexpress HuR protein. Our results demonstrated successful transfection and knockdown of HuR using siHuR-DOTAP:Chol nanocarriers, followed by an enhanced cell killing effect. Intra-tumoral injection of HuR-siRNA using non-targeted lipid nanoparticles showed promising anti-tumor effects in a mouse xenograft model. We later explored the use of these DOTAP:Chol nanoparticles for the targeted delivery of HuR siRNA, in order to enhance its treatment efficiency. We have introduced folic acid or transferrin molecules as targeting ligands in the DOTAP:Chol liposomes for targeted siRNA delivery. Our first step in the exploration of cationic lipid (DOTAP:Chol) tethered to folate *via* PEGylated phospholipid linker was tested in folate receptor (FR)-expressing lung cancer cells. We synthesized the FR-targeting cationic liposomes encapsulated with HuR-siRNA (HuR-FNP). After the preparation of HuR-FNP, characterization, FR dependent cellular uptake, cytotoxicity, and gene silencing were investigated to determine the therapeutic efficacy of FNP. Our *in vivo* pilot studies showed that the FNPs were selectively taken up by the tumor compared with the surrounding organs, when FNPs were administered intravenously into the lung xenograft model.

In the second strategy, TfR-targeted lipid nanoparticles were synthesized in our laboratory. DSPE-PEG-Tf was synthesized by chemical conjugation of thiol-modified transferrin into DSPE-PEG, which is post inserted into DOTAP:Chol lipid nanoparticle to form TfR-NP. The resulting nanoparticle was confirmed for conjugation and subjected to characterization and functional studies. The TEM analysis showed that the TfR-NP were of uniform size, and characterization studies showed sizes of 200–300 nm and a +4.0 mV charge [unpublished data]. Our results revealed the efficient and specific delivery of siRNA to the TfR-overexpressing A549 lung cancer cells, with reduced or silent gene expression of HuR at the protein and mRNA levels. This, in turn, showed a global effect on the other HuR-regulated oncoproteins in A549 cells. The specificity of TfR-mediated gene silencing was illustrated using excess human transferrin as a competing agent in the transfection medium. Transferrin blocked the uptake of TfR-NP. Pre-incubation of cells with desferrioxamine enhanced the expression of TfR over the cells which increased the uptake of TfR-NP. We further confirmed these findings by comparing the gene silencing activity in cells expressing different levels of transferrin receptor. TfR-NP exhibited no cytotoxic effect on the normal lung fibroblast line, MRC-9. *In vivo* studies are currently underway to demonstrate the therapeutic efficacy of the TfR-NP in a lung tumor xenograft model.

7.5.2. Chitosan nanoparticles

As an alternative siRNA delivery system, we investigated chitosan nanoparticles for HuR siRNA delivery. Preliminary studies were carried out to prepare chitosan nanoparticles by mixing cationic chitosan polymer with anionic siRNA specific to HuR mRNA to form polyplexes. The complexation

between chitosan and siRNA was achieved in acidic pH (5.5) by protonating the free amine groups in chitosan polymer for efficient electrostatic interaction with siRNA. It is important to maintain a proper ratio of siRNA to chitosan (N/P or wt/wt) for efficient complex formation between anionic siRNA and cationic chitosan polymer. We have used medium molecular weight chitosan (Sigma-Aldrich) with a 75–85 % deacetylation degree for the preparation of HuR siRNA nanoparticles. The self-assembled nanoparticles allow for siRNA loading of above 90 % and protect HuR siRNA, as demonstrated by siRNA loading and gel retardation analysis, respectively. The nanocomplex retained the positive charge of chitosan even after siRNA complexation (+20 mv), with particle sizes in the range of 200–300 nm [unpublished data]. To study the efficiency of siRNA delivery using the chitosan-siHuR complex, we transfected H1299 lung cancer cells that overexpress HuR. At 1:60 wt/wt ratios of siRNA to chitosan, the siHuR-chitosan nanocomplex was able to knockdown HuR with moderate efficiency. Studies have shown that efficiency of transfection with chitosan nanocarrier can be improved if siRNA is encapsulated within ionically crosslinked chitosan nanoparticles [69]. Since the ultimate goal is to improve the transfection efficiency using chitosan nanoparticles targeting HuR, we propose to investigate ionically crosslinked chitosan as siRNA delivery vehicle. HuR siRNA will be incorporated into chitosan-tripolyphosphate (TPP) nanoparticles during the ionic gelation procedure. The anionic counter ion sodium tripolyphosphate will be mixed with the siRNA solution and carefully added to the chitosan solution (pH 5.5) under mild stirring. The siRNA to chitosan ratio may need to be re-evaluated for use with chitosan-TPP/siRNA nanoparticles, as the ratio may vary with the change with the introduction of TPP. A fully characterized chitosan-TPP/siRNA system is expected to show enhanced siRNA loading and thereby improve HuR knockdown efficiency. The numerous functional groups available within the chitosan polymer will be utilized to conjugate targeting ligands and/or small molecule therapeutics for cancer treatment. The successful formulation will be used as an alternative, safe, biocompatible and targeted nanocarrier system for HuR siRNA delivery in cancer cells and mouse models, as preliminary steps for their translation.

7.5.3. Gold nanoparticles

We are also currently working with HuR-siRNA delivery using gold nanoparticles or nanoclusters. Among different modifications of gold nanoparticles, gold nanoclusters have unique characteristics, such as sub-nanometer size (<2 nm in diameter) and prominent photoluminescent properties. Gold nanoclusters modified with polycations or proteins are currently being explored as capping/stabilizing agents for the incorporation of drug molecules or siRNA therapeutics [70,71]. In our laboratory, we have synthesized bovine serum albumin-capped gold nanoclusters intended for HuR siRNA delivery in cancer cells. The siRNA can be either electrostatically linked or physically conjugated to the surface coating of the gold nanoclusters.

Our strategy is to use a thiol-modified HuR siRNA to physically link to the bovine serum albumin (BSA) coating of gold nanoclusters through a glutathione-cleavable disulfide bond. The presence of glutathione in cancer cells allows for disulfide bond breakage and the release of HuR siRNA into the cell cytoplasm. Targeting ligands (transferrin or folic acid) will be conjugated to the surface functional groups of BSA-gold nanoclusters for targeted siRNA delivery. The ultrasmall structure of nanoclusters coupled with the targeting properties will result in enhanced siRNA delivery in cancer cells that overexpress specific receptors. Step-by-step screening of the transfection and gene silencing efficiency of HuR siRNA-gold nanoclusters in cancer cells and tumor models will allow us to develop a novel potential platform for simultaneous siRNA delivery and imaging.

7.6. CONCLUSIONS

siRNA, the prominent gene therapy tool for the treatment of cancer, requires safe and efficient delivery vehicles to overcome the barriers to systemic exposure. To date, numerous nanoparticle-based siRNA delivery systems have been developed. Many of these have shown promise in the successful delivery of gene therapeutics intended for cancer therapy. The present chapter discusses the recent progress made in siRNA delivery systems in each major category: liposomes/liposomal variants, polymers, and inorganic nanoparticles. Liposomal systems are highly sought-after nanocarriers for siRNA delivery due their biocompatibility and their clinically proven efficiency of drug delivery. Despite their toxic nature, cationic liposomes are still the best choice, because of their unparalleled capacity for siRNA delivery. Modifications with PEG, the insertion of neutral lipids, and the addition of targeting ligands are cationic liposome modifications that can allow for safe and efficient gene delivery. A recent advancement and liposome variant, the lipid nanoparticle, allows for enhanced protection of siRNA and provides robust gene silencing in tumors. Polymer nanoparticles have been investigated as alternative gene delivery systems because of their versatility and tunable controlled-release. siRNA can be either encapsulated in polymer matrix or electrostatically coupled to cationic polymers. Modification with stimuli-responsive polymers adds additional advantages to polymer nanoparticles for the precise and controlled release of siRNA into the cell cytoplasm. The availability of numerous functional groups in polymers allows for the conjugation of targeting ligands and antibodies for enhanced specificity in siRNA delivery towards cancer cells that overexpress specific receptors. Though toxicity of highly cationic synthetic polymers, such as PEI, restricts their *in vivo* use, biocompatible polymers, such as chitosan or PLGA, hold promise for safe and efficient nucleic acid delivery. Inorganic nanoparticles have the advantages of multifunctionality and good safety profiles make these nanoparticles attractive as siRNA delivery systems. Along with siRNA delivery, the imaging capabilities

can be coupled to localize the nanoparticles during the process of siRNA delivery and transfection. Inorganic nanoparticles are smaller in size and can be functionalized to add intracellular cleavable bonds for stimuli-responsive release of siRNA. Our studies demonstrated the advantages of using nanoparticles representing all three categories in delivering HuR siRNA for the treatment of lung and ovarian cancer cells. DOTAP-Chol liposomes demonstrated safe delivery and efficient gene silencing in many types of cancer cells. Ligand-coupled DOTAP:Chol liposomes carrying HuR siRNA enhanced the specificity of gene delivery, producing efficient antitumor effects. Similarly, we expect that our current work investigating chitosan nanoparticles and gold nanoclusters will also strengthen our siRNA therapeutic arsenal for the fight against cancer. Moving forward, we expect major advancements in these gene delivery systems, which will make them strong enough to be tested in clinical settings for cancer gene therapy.

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APOFERRITIN: PROTEIN NANOCARRIER FOR TARGETED DELIVERY

**Simona Dostalova^{1,2}, Zbynek Heger^{1,2}, Jiri Kudr^{1,2},
Marketa Vaculovicova^{1,2}, Vojtech Adam^{1,2}, Marie Stiborova³,
Tomas Eckschlager⁴, and Rene Kizek^{1,2*}**

¹ Department of Chemistry and Biochemistry, Laboratory of Metallomics and Nanotechnology, Mendel University in Brno, Zemedelska 1, CZ-613 00 Brno, Czech Republic, European Union

² Central European Institute of Technology, Brno University of Technology, Technicka 3058/10, CZ-616 00 Brno, Czech Republic, European Union

³ Department of Biochemistry, Faculty of Science, Charles University, Albertov 2030, Prague 2 CZ-128 40, Czech Republic, European Union

⁴ Department of Paediatric Haematology and Oncology, 2nd Faculty of Medicine and University Hospital Motol, Charles University, V Uvalu 84, Prague 5 CZ-150 06, Czech Republic, European Union

*Corresponding author: kizek@sci.muni.cz

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8.1. INTRODUCTION

The delivery of drugs using nanoparticles is an important and relatively new branch of nanotechnology. In the past, the development of a new drug concentrated mainly on its effectiveness with low or even no regard to the negative side effects that the drug can have a negative impact on the patient's body. Nowadays, considerable attention is paid towards the elimination of these negative side effects as much as possible [1].

Nano-based drug delivery carriers, or nanocarriers, can consist of wide variety of materials, both organic (polymeric, lipid, protein, or viral) and inorganic [2]. The largest nanocarriers are liposomes, which are 80–200 nm in diameter [3], polymeric nanoparticles (40–100 nm) [4] or micelles (20–60 nm) [5] and the smallest ones are dendrimers with less than 10 nm in diameter [6]. The use of suitable nanocarriers can significantly reduce negative side effects, as well as increase the biocompatibility, specificity, shelf-life and water solubility of the drug [7]. A diagnostic marker can also be encapsulated within the nanocarrier for theranostic approach – combining diagnostics and therapy [8].

The hunt for the perfect nanocarrier is still on. The most important property of a perfect nanocarrier is its size. The nanocarrier needs to be small enough to make use of the unique characteristics of tumour tissue and be able to enter cells, but large enough that it is not removed from the body by renal clearance [9]. It also needs to be easily produced, encapsulate large quantities of drug and have reliable mechanism of drug release to prevent premature release of the drug but ensure its release in target cells [10]. The size of the nanocarrier should be uniform and enable surface modification with targeting molecules [11].

Inorganic nanocarriers are easier to produce; however, they are often immunogenic or even toxic to human cells. Natural nanocarriers that can be found in the human body are therefore more suitable [12]. One of these natural nanocarriers is the protein ferritin, responsible for the storage and transfer of iron in most organisms [13]. When the iron is removed, it creates a hollow cage called apoferritin [14].

Apo ferritin has been known since 1942, but the increased study came about it in the last 15 years (Figure 1A). It is mostly studied in relation to the nanoparticle formation in its cavity; however, in recent years, it has also been studied as a possible drug delivery vehicle. Figure 1B shows the results from the Web of Science database with the keyword “apoferritin” in relation to research areas.

The first mention of apoferritin as a cage for anticancer drug encapsulation was published by Sismek and Kilic in 2005 [15]. The encapsulation of drugs in apoferritin does not require any modification of either drug or carrier

molecules because it exploits apoferritin behaviour in the surrounding environment [16]. Entry into cells is possible through specific receptors, which are found on most cells in the body. This natural ability of cells to incorporate ferritin can be more enhanced by targeting moieties [17].

Figure 1. The results from the Web of Science database using the keyword “apoferritin”. (1A) Number of published papers *per year* since 1942. (1B) Number of published papers in different research areas.

8.2. APOFERRITIN – BASIC INFORMATION

Apoferritin is a protein cage, which forms the basis for ferritin [18]. Ferritin is responsible for the storage and transfer of ferric ions, that can be toxic to the organism in higher doses [19]. The apoferritin protein shell is in the form of a hollow rhombic dodecahedron, created by 24 roughly cylindrical subunits of two types [20]. Its most important property is its responsiveness to the surrounding proton concentration, which allows for a very simple encapsulation protocol [21]. The different channels in its shell allow for the entry of small molecules [22].

8.2.1. Functions of ferritin in organisms

Iron, in the form of ferrous or ferric ions, is fundamental for the life functions of all eukaryotes and most prokaryotes; however, it is toxic when in excess of that required for cellular homeostasis [23]. Water-soluble ferrous ions (Fe^{2+}) can react with metabolically generated hydroxyl radicals and create dangerous reactive oxygen species (ROS) [24]. Many organisms, from humans to microorganisms, possess defensive mechanisms that transform ferrous ions into the insoluble ferric form (Fe^{3+}). One of these detoxifying factors is ferritin – major iron-binding protein in non-erythroid cells [13,25].

The phenomenon of ferritin reversible iron storage is supported by the mechanisms involved in the control of their expression, mostly connected to iron and stress, both at the transcriptional and the translational levels (Figure 2) [26,27]. The ferritin-H gene (FTH) is regulated by iron regulatory proteins (IRPs) and contains one iron responsive element (IRE) recognised under a low cellular Fe content by IRP1/2 repressing FTH translation [28]. As a response to high cellular Fe content, IRP1/2 is degraded *via* the 26S proteasome pathway, which promotes FTH and ferritin-L gene (FTL) translation [29,30]. The regulated expression of FTH appears to be of particular importance for cellular adaptation to Fe overload [26,31]. Transcriptional mechanisms are strictly involved in determining the tissue-specific H to L ratio [32]. In the case of oxidative stress, a significant elevation in the expression of ferritin-coding mRNA can be achieved [33]. In many respects, ferritin-H can also be viewed as a factor responding to inflammation. Inflammatory cytokines, particularly $\text{TNF}\alpha$ and IL-2, up-regulate ferritin biosynthesis [34]. The ferritin shell is able to store 4500 iron atoms in its structure in the form of ferric oxide phosphate, which is structurally similar to ferrihydrite [21], although it generally contains less than 2000 iron atoms in the inner core [35]. The iron stored in the ferritin cavity is usually in the form of ferric hydroxyphosphate micelles [18] and it is readily available to cellular demand [36]. The major pathway of iron release in cells is *via* the degradation of the ferritin cage [37]. In this degradation, lysosomes play the pivotal role [38]. Thus, lysosomal inhibitors can block ferritin degradation, with simultaneous accumulation of protein cages with iron in organelles. The intra-

lysosomal degradation of ferritin shields the other cellular components from the potential toxicity of iron, allowing the controlled release of the metal into the cytosol, where it increases the labile iron pool level [19] and induces ferritin expression.

Although ferritin is considered to represent cytoplasmic iron storage, novel functions for ferritin have recently been discovered, including ability to deliver iron into the brain across the blood brain barrier *via* clathrin mediated endocytosis, using recently described receptors [transferrin receptor 1 (TfR1), T cell immunoglobulin and mucin domain-containing protein-2 (TIM2), scavenger receptor class A, member 5 (SCARA5)] [39,40]. Ferritin can also be an important factor in angiogenesis, where the binding of ferritin to the anti-angiogenic cleaved form of kininogen abrogates its anti-angiogenic function, thus mediating pro-angiogenic processes [41].

Figure 2. Iron-dependent expression of ferritin on the translational level. IRPs exhibit high affinity towards Fe ions, however with low intracellular amounts of iron, IRPs preferably bind to the iron responsive element (IRE), and thus translation of ferritin is stopped. On the other hand, with high levels of intracellular iron, IRP-Fe complexes are formed and ferritin subunits are translated to form the assembled protein, which can be further used for safe iron storage. Furthermore, possible ways of controlling ferritin expression, which can be involved both at the transcriptional and post-transcriptional levels, are listed.

8.2.2. Apoferritin structure

The ferritin protein can be expressed in iron-free conditions, thus creating a hollow nanocage without iron in its structure called apoferritin [42]. Another approach is the reduction of the iron from ferritin with sodium dithionite with subsequent removal by dialysis, which also produces apoferritin [14].

The apoferritin cage is 450–475 kDa in size and it forms a shell with an outer diameter of 12–13 nm and an inner diameter of 7–8 nm [43]. The protein shell is in the shape of a hollow rhombic dodecahedron with 4-3-2 symmetry that forms intersubunit channels along the three- and four-fold axes to accommodate iron [21]. The faces of the dodecahedron are formed by two subunits related by a two-fold symmetry axis at the centre [18]. Each axis is connected to subunits by electrostatic interactions and hydrogen bonding. The two-fold axis is the most stable one, since the greatest number of interaction sites are there. The fewest interaction sites are found in the three-fold axis, the least stable one [44]. The crystal structure of apoferritin contains the ferritin core [42].

The structure of animal apoferritin protein shell is composed of two types of 24 subunits – heavy (H) and light (L) – differing by the molecular weight – 21 and 19 kDa, respectively [20], with 53% sequence identity [45]. The H and L sequences are very heterogeneous between (apo)ferritins from different tissues within the same animal species with only 40–50% homology. However, sequences from the same tissue from different species are very homologous (85–90%) [20].

The apoferritin subunit is almost cylindrical and it is formed by four α -helices with parallel and anti-parallel orientation (H1-H4), showing a left-handed twist [18]. The length of the H1, H2 and H3 helices is about 4.3 nm and H4 is about 5.2 nm long. The α -helices are connected *via* loops and a short fifth α -helix (H5, 1.6 nm) is located on the C-terminus, oriented almost at a 60° angle to the major helices [20]. Almost 75% of the 174 residues of the subunit are within the α -helices, corresponding to residues 14–40, 49–76, 96–123, 127–161 and 163–173 [18]. The short non-helical regions are located on both the N- and C-termini with two right-handed turns between the H1-H2 and H4-H5 helices [18]. There are abundant side chain interactions within each subunit. Many side chains interact to form hydrophobic cores at the two ends of the helical bundle. A large number of polar and hydrophilic residues, forming a network of hydrogen bonds, are located in the centre of subunit. This part is the most differentiated between the H and L subunits. The orientation of the helix bundle and the H5 helix is similar in both subunits, although they differ in the H4-H5 turn – in H subunits, it is formed by three overlapping turns, but in L subunits it is rather tight with only one amino acid residue between the end of H4 and the start of H5 [18]. The length of the apoferritin subunit is 5 nm with a width of 2.5 nm [18].

The tissue specificity of subunits is mediated by the different functions of each subunit. The mammalian H subunit contains a ferroxidase centre in the major helix bundles with a bridging ligand (numbered as E62) between two iron atoms. The L subunit, however, contains a lysine at E59 instead of this residue, blocking the ferroxidase site by forming a salt bridge with glutamic acid at E104 of the same subunit. In addition, strong hydrogen bonding is present among the inter- and intra- L subunits [46]. The H subunits are thus mainly

responsible for iron oxidation from Fe^{2+} to Fe^{3+} and the L subunits are responsible for nucleation and mineralisation of iron [47] – only the H subunit is catalytically active [48], which is in contrast to prokaryotic apoferritin, where each of the subunits possess catalytic activity [49] and is more similar to the animal H subunit [18]. Due to the structural change, L-subunit-rich apoferritin is much more stable than H-subunit-rich apoferritin [20]. Some apoferritins also contain additional catalytically active subunits called M [48]. Plant apoferritin subunits are considered to be H/L hybrids, since they contain both a ferroxidase centre and a nucleation centre [50].

Apoferritin possesses channels situated at subunit intersections, which connect the apoferritin cavity with the outside environment. Six channels at the four-fold axis are lined with hydrophobic leucine residues [51], representing an energy barrier for ion uptake [52]. These channels are 1.2 nm long and 0.3–0.4 nm wide [44] and they are formed by contact between the H5 helices of four subunits from this axis, oriented from the outer to the inner part of the protein shell [18]. In contrast, eight three-fold channels are hydrophilic, 0.3–0.4 nm long and wide [44] and consist of negatively charged glutamic and aspartic acid residues [53,54]. They are formed by a symmetrical convergence of three subunits at this axis which are in contact through the N-terminal ends of helices H1 and H4 and the C-terminal ends of H2 and H3 [55]. They attract Fe^{2+} ions and are the location of iron entry [56].

There are extended contacts between pairs of subunits with antiparallel orientation along the two-fold axes (the most stable one), involving helices H1 and H2 on each monomer. The loops of these paired subunits are oriented parallel to the two-fold axes [55]. These interactions are essential for the formation of the apoferritin 24-mer [57].

The most characterised apoferritin is from the horse spleen and has been studied since 1937 [20]. Horse spleen apoferritin has an outer diameter of 12 nm and an inner diameter of 8 nm [58]. The thickness of the horse spleen apoferritin shell is 2.3 nm and the volume of its hollow core is 233.2 nm^3 with a ferrihydrite core volume of 142.0 nm^3 [21]. The ratio between the heavy and light subunits in horse spleen apoferritin is 85–90% for light subunits and 10–15% for heavy subunits, which ensures its high stability [20].

8.2.3. (Bio)chemical properties

The quarternary structure of the apoferritin cage ensures its high solubility and thermostability [55]. It can withstand a wide range of pH (3.4–10.0) and still retain its hollow spherical structure of 12.3 nm in diameter. The denaturation of apoferritin occurs at pH levels below 1.0 or above 13.0. Between pH 1.0 and 3.4, apoferritin undergoes some structural changes. It can also sustain high temperature; denaturation occurs when horse spleen apoferritin is heated above 80 °C for 10 min [21].

At neutral pH, the external surface of apoferritin has a net negative charge. Both the external and internal surface of apoferritin can be modified with different moieties [48] and through genetic and chemical modifications [59]. Some substances can bind to the apoferritin surface through hydrogen bonding (non-ionic molecules) or electrostatic interactions (ionic molecules) [60]. Small molecules (less than 2 nm) can enter the apoferritin cavity through channels in its structure *via* diffusion [22]. Electron transfer through the apoferritin shell is also possible [61]. Furthermore, apoferritin metal derivatives can coprecipitate with specific polycations and can thus be used for protein sensing [62].

The important properties of apoferritin for its use as a nanocarrier for drug delivery are its self-assembling ability and the fact that its structure is dependent on the surrounding environment. It can reversibly dissociate and associate based on the pH [21].

At pH levels above 3.4, apoferritin has spherical structure. Below pH 3.4, it becomes unstable, collapses and disassembles. The disassembly of apoferritin occurs stepwise through structural intermediates [21]. It starts at the weakest site, the three-fold axis, in the vicinity of the three-fold channels in the opposite direction [44]. First of all, at pH in the range of 3.4–2.4, apoferritin has an overall hollow spherical shape with changes in the structural details. At pH 3.24, a pair of subunits is disassembled from each of the north and south poles of apoferritin, creating two holes with a diameter of 6.5 nm. One more subunit is disassembled from each pole at pH 3.08, creating holes 6.7×9.2 nm in size. Another subunit (a total of eight) is dissociated from each pole at pH 2.85, still retaining the overall spherical structure. At pH 2.66, due to the loss of subunits, apoferritin becomes a headset-shaped structure and below pH 2.4 apoferritin becomes even more opened. Ultimately, at pH 1.96, there are significant changes to apoferritin, which starts to form a rodlike structure consisting of oligomers, mostly trimers [21]. Moreover, below pH 0.8, the disassembled subunits are aggregated, which is attributed to denaturation and driven by van der Waals and non-specific hydrogen bonding. Apoferritin in such a highly acidic environment is inhomogenous, polydisperse, and it has no definite size [21].

The reassembly process of apoferritin is dependent on the disassembly process. It can never recover to the intact spherical shape. Furthermore, if disassembly was conducted at pH 1.96 or below (to a rodlike structure), the structural recovery is limited to the headset-shape structure formed by 12 subunits. If the disassembly was limited to the headset-shape structure (between pH 3.4 and 2.0), the resulting apoferritin has an overall spherical structure with two hole defects 6.5 nm in size at the north and south poles, and is formed by 20 subunits. The difference in reassembly recovery is probably due to the improper contact angles between the dimer and monomer components of the rodlike structure, thus the resulting headset structure

cannot adopt more trimers or monomers to complete the reassembly process [21].

The reassembled spherical structure with two hole defects can be useful for nanoparticle fabrication since the holes can serve as channels for mass transport [21].

8.3. APOFERRITIN AS A NANOREACTOR

Apo ferritin's cage is most often used as a template in nanoparticle or quantum dot (QD) synthesis [63]. It can also be used as a scaffold for supramolecular wrapping, nanoscale incarceration and nanostructure templating [64]. The entry of ions for nanoparticle formation is possible through the many channels in the shell of apoferritin [22]. Apoferritin used as a nanoreactor was found to be an effective tool for the synthesis of various well-defined nanoparticles.

8.3.1. Synthesis of nanoparticles within the apoferritin cage

Ferritins are known as iron storage proteins and similar biomimetic strategies as in case of iron can be used to synthesise other inorganic nanoparticles (NPs) (oxide, metallic and semiconductor) within the apoferritin cavity. It is able to accommodate several inorganic molecules and metal ions. Positively charged ions can enter the apoferritin cavity in the same way as iron. However, it has not yet been elucidated how anions enter ferritins. Recently, it was shown that highly conserved glutamic acid residues mediate iron transfer from channels to the ferritin enzyme site and with aspartate-promoted nanoparticle formation [65,66].

Oxide nanoparticles and semiconductor nanocrystals (QD's) with biocompatible and bioactive properties can be synthesised inside apoferritin [67,68]. Ferritin with iron oxide cores can be sulphidised with $\text{H}_2\text{S}/\text{Na}_2\text{S}$ within the cavity and used to produce FeS NPs. Moreover, redox-active metal ions can be synthesised in apoferritin with the most satisfying results found for Mn(II), which can create amorphous manganese oxide cores [68]. Other bivalent metal ions like Cd^{2+} bind to the apoferritin cavity and thus can be used for the reconstruction of semiconductor NPs inside apoferritin [67,69]. QDs and nucleated nanoscale CdS crystallites within the apoferritin cavity, created by the reaction of Cd^{2+} and HS^- , can offer unique photoresponsive and photoredox properties. These CdS NPs are preferentially developed within the cavity rather than on the surface and their size can be tuned. The presence of native iron-containing cores reduces CdS NP growth. In this process, the charged lining of the protein channels and cavity are important [67].

In a case of creating semiconductor NPs inside apoferritin, successful synthesis depends on the suppression of chemical reactions outside of the cavity. Firstly, positive metal ions need to be introduced to the demetallated protein cavity

(apoferritin) and condensed at nucleation sites, which after the addition of anions encourages nucleation. Anion induction in the cavity should also be faster than chemical reactions outside [69].

In the case of II–VI semiconductor nanocrystals, it must be pointed out that they are usually prepared in non-polar organic solvents with phosphine ligands. It means that their aqueous dissolution requires surface modification with more polar or hydrophilic molecules. Bio-templated synthesis of such particles benefits not only from the water solubility of the protein shell but also from its biocompatibility and broadens their application possibilities. These properties can be achieved not only by using the nanoreactor route but also by using apoferritin shell reassembly in the presence of target NPs [63].

The synthesis of ZnO NPs within the apoferritin cavity can be very difficult. Since a concentration of OH⁻ anions around 10 mM (~ pH 11) is required for ZnO formation, most apoferritin molecules are denatured during the procedure. However, ZnO nanoparticles can be synthesised within the apoferritin cavity if Zn²⁺ is stabilised in water against reaction with OH⁻ by the addition of ammonium water [Zn(NH₃)₄²⁺] [54]. When this complex is situated near the threefold channel, Zn²⁺ decouples from the complex due to chemical equilibrium and flows inside. Zinc inside the cavity is hydroxylated to Zn(OH)₂ due to OH⁻ diffusion inside and subsequently becomes ZnO by a dehydration reaction. Also, other nanomaterials such as Zn₃(PO₄)₂, Cd₃(PO₄)₂, Pb₃(PO₄)₂, CO₃O₄, ZnSe, Pd, Ni, Cr, and several other nanoparticles can be synthesised using apoferritin [53,65,70-73]. The scheme of NP synthesis using apoferritin is presented in Figure 3.

Figure 3. A scheme of the individual steps of NP synthesis within ferritin. The removal of the ferrihydrite core of ferritin (1). The addition of cations and anions to apoferritin (2). Cations condense at nucleation sites and anions remain outside the cavity because of a large energy barrier. NPs are created inside the cavity after reduction (3).

An attractive apoferritin nanoreactor application is the reconstruction of ferromagnetic iron oxide magnetite or maghemite (Fe₃O₄/Fe₂O₃) within the

apoferritin cavity. This artificial magnetic protein is called magnetoferritin and it can be used as a dispersed bioorganic and magnetic material in various medical applications [74]. A solution of $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ is slowly and in small increments added to a buffered solution of apoferritin (pH 8.5) along with small amounts of air to produce slow oxidation. Subsequently, anaerobic synthesis with the addition of a stoichiometric amount of oxidant probably reduces the creation of non-magnetic ferric oxides [75]. The creation of ferric/ferromagnetic nanoparticles is important in several technological applications like high-density magnetic data storage [76].

8.4. APOFERRITIN AS A DRUG / GENE NANOCARRIER

The spherical cage in apoferritin can be used for the encapsulation of fluorescent dyes [77], drugs [78-85], magnetic resonance imaging contrast agents [86,87], genes [88] or other compounds [89]. Due to the self-assembly activity of apoferritin and its response to the surrounding environment, it has several advantages for use as a nanocarrier. The formed cavities have uniform size, ensuring the high reproducibility of cargo encapsulation. In addition, the encapsulation protocol is very simple, based only on pH changes in the presence of molecules that are to be encapsulated. It shows very low cytotoxicity and high biocompatibility [90].

Figure 4. A scheme of the drug encapsulation protocol in apoferritin and its release in cancer cells. The lower pH disassociates the subunits that are mixed with the drug (1). After increasing the pH, the drug is encapsulated (2). The acidification of the endosome disassociates apoferritin and the drug is released (3).

Apoferritin is mostly discussed in relation to cancer treatment. In patients, when apoferritin is administered intravenously, there is close to no undesired release of cargo due to the near-to-neutral pH. However, the low endosomal pH in cancer cells allows the release of the cargo [88]. Figure 4 shows the encapsulation protocol and the release of apoferritin cargo in cancer cells.

8.4.1. Targeting of apoferritin

Apoferritin can enter target cancer cells through both passive and active targeting. Passive targeting is enabled by the enhanced permeability and retention (EPR) effect [91]. To ensure the EPR effect, the spherical nanocarriers should be less than 100 nm in size [92-94]. Healthy blood vessels have pores of 10 nm, so the nanocarrier needs to be larger [10]. Apoferritin with its diameter of 12.3 nm has just the right size. However, nanoparticles smaller than 20 nm, including apoferritin, can undesirably be removed from the body *via* renal clearance [9]. It is therefore suitable to increase the size of apoferritin slightly *via* surface modifications. Moreover, to prolong the blood clearance time of apoferritin, its surface can be modified with dextran [95].

Apoferritin can enter cells through receptor-mediated endocytosis [17] as well as clathrin-mediated endocytosis by its specific receptors, TfR1 [96] and SCARA5 [97]. However, these receptors can be found not only on cancer cell membranes but also on the surface of many healthy cells [98], such as TfR1 in kidney cells. Therefore, it is better to modify the surface of apoferritin with actively targeting moieties, which can also increase its size. This way, the apoferritin gets to the vicinity of the tumour by passive targeting (the EPR effect) and can enter cells by active targeting.

As a target, tumour cells or tumour endothelial cells are employed [99]. Using cancer cells as the target, apoferritin not only accumulates at the tumour site, but its uptake by cancer cells is enhanced. The targeting of cancer endothelial cells can even inhibit the growth of tumour blood vessels, leaving the tumour without an oxygen supply. A suitable targeting moiety should have its receptor homogeneously overexpressed on the cancer cell membrane or endothelium [100].

The apoferritin surface can be modified with antibodies [101], antibody fragments [89], proteins [102], peptides [103], vitamins [104], DNA or RNA aptamers [105] or small molecules such as folic acid [106]. Covalent bonding of surface lysine with targeting moieties can be used for the surface modification of apoferritin [107].

The simplest targeting molecule is folic acid or folate. Folate receptors α and β are overexpressed by many types of cancer cells due to their greatly enhanced metabolism [108]. However, folic acid is a very small molecule and even with this modification, apoferritin is still small enough to be removed from blood *via* renal clearance.

Aptamers, *i.e.* DNA or RNA oligonucleotides, can bind to a variety of targets, including prostate-specific membrane antigen (PSMA) [109] or nucleonin [110]. They can be effectively used with other nanocarriers, but apoferritin modified with these aptamers may still be too small and thus removed from the organism through renal clearance. However, surface modification of

apoferritin with aptamers and horseradish peroxidase can serve as a biosensor [105]. Its pH responsiveness can also be employed in biosensor design [111].

Among the possible targeting moieties are cyclic or linear derivatives of arginine-glycine-aspartic acid (RGD) peptides. The introduction of this peptide onto the apoferritin surface can be performed by genetic modification [112]. RGD is a short amino acid sequence, which has high affinity to integrin $\alpha_v\beta_3$. The $\alpha_v\beta_3$ integrin is found in tumour angiogenic blood-vessel endothelial cells and cancer cells [103]. Apoferritin modification with these RGD derivatives leads to higher tumour uptake and better growth inhibition when compared to the free drug [113].

The largest apoferritin particles are achieved by modification with antibodies. When using native antibodies, they can be immunogenic by binding of their Fc domain to the Fc receptors on healthy cells. To eliminate this problem, it is possible to use antibody fragments, containing only the Fab region [114] or inactivate the Fc region by binding to a peptide protein G derivative which can be attached to the apoferritin surface [115]. Moreover, using this approach ensures the correct orientation of antibodies with the antigen-binding site facing outwards from the nanocarrier.

8.4.2. Apoferritin in gene delivery

Recently, considerable attention has been focused on the delivery of genes or siRNAs to target cells for gene therapy. *Via* this process, it is possible to insert [116], remove [117], modify [118] or silence [119] defective genes, thus treating even genetically inherited disease. The most studied nanocarriers for the gene delivery include viral particles [120], liposomes [121] and polymers [122]. Many artificial nanoparticles, however, can have negative side effects once administered to the patient. These include immunogenicity [123] or even embolisation because of nanocarrier aggregation [124]. Nanocarriers naturally found in the human body are therefore more suitable for these applications [12].

A suitable nanocarrier for gene delivery should be able to protect the genetic cargo from degradation by nucleases. Moreover, it should not allow premature release of the cargo but should be able to release the cargo in target cells, thus delivering it to the nucleus [125].

Since apoferritin can be loaded with a wide spectrum of molecules *via* the disassembly/reassembly process [79], it is also possible to use apoferritin as a carrier for the delivery of genes. It is also a molecule naturally found in the human body and it possesses many of the abovementioned criteria making it suitable as a gene nanocarrier [18]. Also, its surface can be modified with moieties that are useful for gene delivery, such as poly(ethylenimine) [126,127].

8.4.3. Delivery of anticancer drugs

The delivery of anticancer drugs using apoferritin as a nanocarrier can lower the negative side effects of these drugs, such as the cardiotoxicity of doxorubicin [79] or seizures caused by cisplatin administration [128]. With water-soluble apoferritin, it is possible to dissolve drugs that are normally insoluble in water [7]. Due to the passive and active targeting of apoferritin, the drug concentration in the vicinity of the tumour is increased by 100–400% [91], but the total administered dose can be significantly lowered [129]. The encapsulation of drugs in apoferritin increases their specificity and biocompatibility [130], as well as their shelf-life [131]. Furthermore, since apoferritin is a major iron storage protein in humans, it has excellent biocompatibility and low immunogenicity [113].

After encapsulation, apoferritin retains its structure and the encapsulation does not influence any groups on the apoferritin surface [85]. In addition, the molecules encapsulated in the apoferritin cavity can retain their properties, such as fluorescence [90], which is very convenient for theranostic uses as a diagnostic marker. The encapsulated drug also retains its therapeutic properties [132]. The very first therapeutic molecule encapsulated in apoferritin was doxorubicin, with 28 doxorubicin molecules encapsulated within one apoferritin molecule [16]. Other cytotoxic drugs, such as daunomycin [133], cisplatin [101], carboplatin [128], and oxaliplatin can easily be encapsulated in apoferritin using the same procedure; these complexes are able to enter tumour cells [85]. When a drug is encapsulated within apoferritin, its toxicity to healthy cells is significantly lowered, while it is still toxic to cancer cells [81]. The maximum tolerated dose of apoferritin with an encapsulated drug is four times higher than for the free drug. Moreover, no additional administration of drug is required [134].

During the acidification of the endosome, the drug is gradually released [135]. The release can also be controlled by external stimuli. For this purpose, the apoferritin surface can be modified with thermoresponsive polymers [136]. Furthermore, it is possible to produce thin apoferritin films with controllable release by assembling negatively charged apoferritin on highly positively charged nanostrands [137].

It is possible to create chimeras by fusing apoferritin with other proteins, such as hemagglutinin. This can create particles with immunisation properties to use as more gentle vaccination agents [138].

The delivery of anticancer drugs can be combined with gene therapy to achieve even higher treatment efficiency [139]. The surface of apoferritin can also be modified with biotin and connected to streptavidin-coated magnetic nano- or microparticles to help guide the movement of apoferritin through the patient's body using an external magnetic field and to combine the targeted drug delivery with hyperthermic therapy [79]. Photosensitisers can be encapsulated

within apoferritin for the combination of photodynamic therapy and chemotherapy [140].

Due to the stability of apoferritin, there is almost no undesired drug leakage with prolonged storage [16]. Its stability and bioavailability can be further increased by the encapsulation of antioxidant molecules, such as curcumin [132]. The only undesired drug release occurs through the two hole defects left after the reassembly of apoferritin at low pH. When drugs containing metals are used, the association with metal binding sites on the interior of apoferritin eliminates this problem [141]. Non-metal-containing drugs can be precomplexed with a metal such as iron or better yet copper for such internalisation. This procedure can be applied to a wide variety of drugs, including doxorubicin, and the encapsulation efficiency is higher than with the simple disassembly/reassembly protocol [113].

Apoferritin can be used not only as a nanocarrier for anticancer drugs, but also for other drugs, including antimicrobial molecules, such as silver ions and silver nanoparticles that can be used in the treatment of methicillin-resistant *Staphylococcus aureus* [80].

8.5. CONCLUSION

The apoferritin protein nanocage is a very suitable molecule for use as a drug delivery vehicle. Its properties, such as size, excellent biocompatibility, easy modification and simple encapsulation protocol based on structural changes in different pH, present a large advantage when compared to the other nanocarriers. It contains multiple channels in its structure, employable for small nanoparticle synthesis.

Due to the encapsulation procedure, a wide variety of different molecules can be encapsulated within its cavity. It can therefore be used for simple chemotherapy, but also the combination of therapy with diagnostics, chemotherapy and photodynamic therapy, gene therapy or hyperthermia therapy. It can be used in not only the treatment of cancer but also other diseases, including bacterial infections.

The size of apoferritin is suitable for passive targeting to tumours and its surface can be modified with a variety of targeting moieties for specific use. Due to all these advantages, apoferritin might very well be the perfect molecule for nanomedicine.

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SILICA AND ORMOSIL NANOPARTICLES FOR GENE DELIVERY

Joana C. Matos¹, Gabriel A. Monteiro^{2,3}, and M. Clara Gonçalves^{1,4*}

¹ Departamento de Engenharia Química, Instituto Superior Técnico,
Universidade de Lisboa, Av. Rovisco Pais, 1049-001 Lisboa, Portugal

² Departamento de Bioengenharia, Instituto Superior Técnico,
Universidade de Lisboa, Av. Rovisco Pais, 1049-001 Lisboa, Portugal

³ IBB – Institute for Bioengineering and Biosciences, Instituto Superior
Técnico

⁴ Centro de Química Estrutural, Instituto Superior Técnico, Universidade
de Lisboa, Av. Rovisco Pais, 1049-001 Lisboa, Portugal

*Corresponding author: clara.goncalves@ist.utl.pt

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9.1. INTRODUCTION

Nanomedicine is an emerging area, combining nanotechnology and medicine. One of nanomedicine's main goals is the design, development and application of delivery systems for gene delivery and therapy. Selective gene transfer to cells, tissues or organs, for clinical treatment, has been discussed throughout the last half century. Recent breakthroughs in the technology required to modify genetic material brought this goal closer to a foreseeable future.

Plasmids (pDNA) are widely used in clinical and non-clinical research in gene delivery, gene therapy and DNA vaccination, however, they are quite inefficient as mediators of gene expression, as these molecules are easily degraded when administered unprotected. To overcome this difficulty, gene delivery systems have been designed and developed to efficiently carry, target and protect the plasmid DNA.

Silica and organically modified silica (ORMOSIL) nanoparticles (NPs) are promising candidates as non-viral delivery vectors. Silica presents several attractive characteristics: high biocompatibility, nontoxicity, non-immunogenicity, biodegradability, and bioconjugation ability. The silica functionalization versatility allows hybrid NPs, with tunable hydrophilic/hydrophobic surface character, high cargo capacity, and a prolonged period of blood circulation, making them even more interesting as gene carriers.

This chapter is a brief introduction to the topic of nanomaterials/NPs and their significance in nanomedicine, followed by a description of the bottom-up sol-gel method of NPs synthesis.

The main concepts of gene therapy will also be addressed. The advantages and disadvantages of different methods will be discussed, with emphasis on the use of synthetic NPs to improve the gene delivery efficiency of non-viral vectors.

9.2. NANOTECHNOLOGY AND NANOMEDICINE

Nanotechnology involves the manipulation of matter on a nanometric scale, with the aim of developing new functional material or systems, whose structures exhibit novel and/or improved properties. Due to their size, the materials developed often have very specific physical, chemical and/or biological properties. The nano prefix refers to the multiplication of a given unit by 10^{-9} . In the international units system (SI), a nanometre denotes one billionth of a metre, corresponding approximately to the length of 10 hydrogen atoms or 5 silicon atoms aligned.

Nanoscale materials may be classified in terms of the number of dimensions not confined to the nanometric scale. Nanoparticles are considered zero dimensional (0D) (zero dimensions not confined to the nanometric scale); nanotubes, nanofilaments and nanofibres extend across one dimension (1D); nanofilms and nanocoatings can be characterised by a width and a length, while height is negligible, and may be regarded as two dimensional (2D) nanomaterials; finally, bulky materials, such as photonic crystals or metamaterials, are classified as three dimensional (3D). Considering the inherent nanoscale functional components of living cells, it was inevitable that nanotechnology would meet biotechnology, giving rise to nanobiotechnology.

Nanobiotechnology is a new and important field of research due to the many applications in areas such as drug discovery and delivery, and pharmaceutical manufacturing. These classes of applications are often included under the term nanopharmaceuticals.

Nanomedicine is a closely related field, resulting from the application of nanotechnology to medicine. There is an increasing optimism about its applications, where significant advances in the diagnosis and treatment of diseases are expected. In the last decade, a deep understanding of the molecular processes underlying mechanisms of disease has been realised. This knowledge changed some of the paradigms of clinical diagnosis methods, supported by detection and monitoring at the molecular level, such as cells, DNA, messenger RNA (mRNA), peptides and proteins. Furthermore, as targets exist on a nanoscale, probes with equivalent size have been developed allowing the integration of nanotechnology and biology/medicine, responding to the increasing demand for gene profiling, and high-throughput drug and disease screening without complex instrumentation or processing steps [1]. Drug delivery and gene therapy become one of the most promising applications of nanomedicine, by making use of NPs as carriers [2]. Figure 1 illustrates the relationship between nanobiotechnology and nanomedicine.

Figure 1. Nanobiotechnology and nanomedicine relationship (adapted from [2])

9.3. NANOPARTICLES

Due to a wide range of attractive characteristics, NPs are of high significance in material science research, to both the academic and industrial communities.

In addition to their outstanding surface-to-volume ratio, NPs possess unique thermal, mechanic, electrochemical, catalytic, optical, electronic and magnetic properties, depending on their size, shape and composition. Instead of changing the NPs composition, one may play with the size and/or shape, to fine tune a desired property or set of properties.

NPs may exhibit a wide range of geometries – from spherical to tubular, through centric, eccentric and star like – may be plain or nanostructured – core-shell or porous structure – exhibit different sizes and shell thicknesses, may be hollow, may differ in crystallinity and surface morphology, and finally may be fine-tuned relative to one or more properties.

NPs can be functionalized and eventually bioconjugated with a wide range of small ligands and/or large biomolecules. NPs enable the encapsulation and controlled release of various substances (*e.g.* drugs, biomolecules, cosmetics, and dyes), and may play a singular role in certain biomedical devices.

Finally, NPs can be capable of accomplishing multiple objectives such as imaging and therapy (theranostics), or performing a single advanced function through the incorporation of multiple functional units [3]. For biomedical

applications, an appropriate size range ($\sim 10\text{--}200\text{ nm}$) and a monosized distribution are normally required for effective operation.

Due to the nanoscale associated with these materials, classical physics are often unable to explain their properties, which are easier to understand in the domain of quantum mechanics. While the use of quantum mechanics enables many interesting hypothesis, it also increases the degree of difficulty in the complete understanding of nanomaterials.

9.3.1. Silica nanoparticles

NPs may comprise material of biological origin, such as phospholipids, lipids, lactic acid, dextran, chitosan, or have inorganic characteristics, such as polymeric, carbonic, silica- or metallic-based materials [4].

Silica is the most abundant mineral on the Earth's crust, and over time it was naturally introduced to human life and later to engineering. It is most commonly found in nature as sand or quartz, as well as in the cell walls of diatoms [5]. With chemistry very similar to C, Si plays a central role in inorganic natural materials. The structural unit of silica is a tetrahedron (with sp^3 hybridization), whose base and sides have a triangular shape with an oxygen atom at each vertex and a silicon atom at the centre (SiO_4)⁻⁴. These tetrahedral units are interconnected by sharing their vertices, which may occur regularly or randomly. In the first case the 3D organisational diversity leads to different polymorphic forms of silica (quartz or tridymite for example). Occasionally an aperiodic 3D network forms, leading to glass formation. Silica NPs may be crystalline or amorphous.

SiO_2 NPs present several advantages over organic polymers. During the SiO_2 NPs synthesis, they may be easily separated by centrifugation; they are more hydrophilic, making the re-suspension of NPs easier, and not subject to microbial attack. Recently, different fluorescent SiO_2 NPs have emerged as a particularly fascinating fluorescent probe which has aroused great interest in biology and medicine [1,6]. Highly luminescent SiO_2 NPs have been prepared for the selective targeting of a wide range of important cells, organs or tissues (in particular when diseased, with cancer, specific infectious, *etc.*) and they have demonstrated advantages in terms of multiplexing capabilities, ease of functionalization and also greater sensitivity and photostability.

Magnetic silica NPs, as an example, can be used for separation and targeting techniques of trace amounts of bioanalytes in biological complex matrices. Their magnetic properties can also be linked to the fluorescence, making multifunctional silica NPs, which can be useful for imaging cells, tissues and other organs as well as for the delivery of therapeutic agents to specific targets. These silica NPs are of great interest from a technological point of view, especially for the diagnosis and treatment of diseases in nanomedicine.

SiO₂ NPs have shown great versatility due to chemical and/or physical surface modification, which may increase their biocompatibility. Extensive studies about SiO₂ NPs biodistribution [7,8] and toxicology [3,9,10] have shown that these NPs are well tolerated and in some cases biodegradable or excreted after performing their function.

SiO₂ NPs present high and controllable mechanical and chemical stability. Their porosity can also be easily modulated in terms of the pore size and structure [11].

Due to a wide range of advantages, SiO₂ NPs are considered to be of great promise in nanomedicine [11], specifically in drug delivery systems and gene therapy. In drug delivery systems, drug molecules are loaded into SiO₂ NPs, where surfaces have been previously changed by the introduction of biorecognition entities, allowing the delivery system to target specific cells or receptors in the body [12]. In gene therapy, in a pursuit of more efficient DNA delivery vectors for both basic research and clinical trials, ultrafine SiO₂ NPs functionalized with amino groups, can bind efficiently to pDNA, protecting it from enzymatic attack while still transfecting *in vitro* cells [13,14].

9.3.2. Silica nanoparticles synthesis and *in situ* functionalization synthesis techniques

Several methods to accurately produce silica NPs with a narrow distribution of sizes, controlled shapes and morphology, surface chemistry, porosity, and homogeneity, have been developed. Most use the bottom-up approach of sol-gel methodology.

The sol-gel process has many advantages, which led to its use even before the underlying scientific principles were understood. These advantages include a lower processing temperature (close to room temperature), allowing minimal thermal degradation and low energy processing costs, high homogeneity, directly obtained in solution on a molecular scale, and a high purity which depends only on the precursor's purity. Furthermore, sol-gel methodology may be used in any basic laboratory (no sophisticated equipment or class room conditions are needed).

The sol-gel chemistry comprises chemical reactions involving colloidal particles in a sol, or between alkoxide-precursors and water, in a solution, leading to a highly porous amorphous gel product, where a liquid phase (solvent, catalyst and eventually excess reactants) may be retained. 0D, 1D, 2D, or 3D products can be produced, depending on the experimental conditions. Figure 2 illustrates the sol-gel versatility.

Figure 2. Sol-gel versatility

9.3.2.1. The sol route

In the sol route, colloidal particles are formed in an aqueous medium from ionic species, following colloidal chemistry principles. In the case of silica, for example, diluted silicic acid sols, containing ~ 1 nm sized NPs, will undergo rapid growth to 2–4 nm, at pH 2–3. Above pH 7, silica solubility increases, enhancing NPs growth, which can reach up to 4–6 μm , by coalescence and Ostwald ripening (Figure 3).

Sodium silicate solution is another sol starter for the synthesis of SiO_2 NPs. Here, monomers, dimers, trimers, *etc.* will hydrolyse to form the amorphous Si–O–Si network.

Figure 3. Transmission electron microscopy (TEM) images evidencing Ostwald ripening in SiO₂ functionalized NPs

9.3.2.2. The solution route

The solution route is the most common synthesis process. Here metallic salts, metal alkoxides, or other organometallic precursors undergo hydrolysis and condensation to form a wide range of sol-gel products. Because of the hydrophobic nature of the alkyl groups, organometallic precursors and water are not miscible and the addition of a common solvent (usually an alcohol) becomes necessary to promote miscibility between reactants.

The pH plays a critical role in all sol-gel processes (Figure 2). At low pH, particularly below 3 (the isoelectric point of silica is 2), complete hydrolysis produces linear or highly branched polymeric species giving rise to 3D structures, with nanopore diameters < 2 nm. As the pH increases towards 7, dissolution and condensation reactions take place and the gel structure coarsens to some extent. Above pH 7, there is maximum NPs growth, due to an increase in silica solubility, promoting depolymerisation of siloxane bonds, and producing the monomeric silica necessary for the aging process (Ostwald ripening coarsening mechanism, Figure 3).

In sol-gel methodology, NPs synthesis is always performed under basic catalysed conditions. Water dissociates immediately at pH over 7, and a hydrolysis reaction take place by hydroxyl attack to the silicon atom, according to Equation 1:

Then, a second nucleophilic attack allows the formation of the Si-O-Si network, according to Equation 2:

Hydrolysis and condensation occur simultaneously rather than sequentially; the initial condensation is quite fast, although it slows down as polymerization progresses.

The SiO₂ NPs solution route comprises two common *via*: the microemulsion process (or reverse emulsion) and the classical Stöber method.

In the first process a reverse-micelle or water-in-oil (w/o) microemulsion system is formed by adding water, oil and surfactant. The hydrolysis and condensation reactions will take place in confined nanoreaction vessels, formed by the dispersed aqueous phase in the continuous oil matrix.

The nucleation and growth kinetics of silica are highly regulated in the size and size distribution of water droplets in the microemulsion system. In the last few years, several dye-doped SiO₂ NPs have been synthesised *via* the w/o microemulsion technique. In this case, to increase the electrostatic attraction of the dye molecules to the negatively charged silica matrix, polar dye molecules are used to ensure successfully encapsulation into SiO₂ NPs [11].

The classical Stöber method was introduced in 1968, by Stöber and co-workers, and allows the synthesis of fairly monodisperse SiO₂ NPs, with diameters between 50 nm and 2 μm [15]. The Stöber method has been extensively investigated and optimised in order to synthesise dye-doped SiO₂ NPs by covalent bonded to organic fluorescent dye molecules [11,16,17]. Through the procedure described by Stöber, an alkoxide precursor such as tetraethyl orthosilicate (TEOS) is hydrolysed in an ethanol mixture, under basic catalysis. The hydrolysis and condensation reactions take place in a plain reactional vessel, allowing the formation of silica NPs.

The Stöber method allows an eco-friendly synthesis of NPs, does not use surfactants, and the synthesis reactions occur at room temperature [16].

Arkhireeva and Hay [17] obtained sub-200 nm NPs by slightly modifying the Stöber method. However, synthesised SiO₂ NPs (in sub-100 nm size range) present high polydispersity and irregular shape [18-20], and therefore, in order to obtain monosized and nanoscale particles, the classical Stöber method is slightly modified [16].

In the methods described above, the concentration of the reactants (TEOS and water), the EtOH to water ratio, and pH, strongly affects the NPs size, size distribution and morphology. This feature is also affected by the nature of the surfactant, the molar ratios of water to surfactant, in the microemulsion process.

NPs prepared through the microemulsion method exhibit smooth surfaces and low polydispersity. Through the Stöber method, NPs smaller than 100 nm radius present less monodispersity, may lose spherical shape and present rough surfaces. For use in biomedicine, however, the microemulsion method is not as safe as the Stöber method. The use of surfactants in the NPs synthesis carries a higher risk of cytotoxicity. A procedure based on the Stöber method is described by Gonçalves and co-workers [21] (Figure 4 illustrates the flowchart).

Figure 4. Stöber sol-gel method flowchart for the synthesis of SiO₂ NPs

In the Stöber method aqueous sodium silicate solution is used as a nucleating agent (Solution 1), and is added to the reaction vessel prior to the silica precursors. Sequentially, aqueous sodium silicate solution (2.2 wt % SiO_2) is homogenised and re-suspended in water through ultrasonication, for 15 min. An aliquot of Solution 1 is diluted in ethanol, under magnetic stirring (15 min.), then, a mixture of ethanol (co-solvent) and ammonia solution (catalyser) is added and magnetically stirred, for an extra 15 min. In a second step, the silica precursors are added drop by drop to Solution 2, under vigorous stirring. A sol forms and it will be stirred for half an hour before centrifuge and washing, with ethanol and bi-distilled water. Spherical, monosized, smooth surface silica NPs are obtained (Figure 5).

Figure 5. TEM images of SiO_2 NPs synthesised through the Stöber method

Based on the classical 2D model described by LaMer *et al.* [22], which was used as a model for qualitative interpretation in monodisperse NPs synthesis, Huang *et al.* [23] proposed a procedure to produce monodisperse spherical SiO_2 NPs with sizes ranging between 30–100 nm. The strategy is based on an effective selection of reaction conditions for the Stöber method, and relies on a modification of the conceptual classical LaMer model of nucleation and particle growth.

Based on these principles, Gonçalves and co-workers used the procedure shown in Figure 6 to obtain monodisperse SiO_2 NPs.

Figure 6. LaMer method flowchart for the synthesis of SiO₂ NPs

In the LaMer procedure no nucleating agent is used. A first solution of ethanol, ammonia and bi-distilled water is formed and heated to 55 °C, under vigorous magnetic stirring. After temperature stabilisation, the silica precursors are injected quickly (into the solution), and left under magnetic stirring for two hours. A sol forms and silica NPs will be separated from the reactional bath through centrifugation. The LaMer procedure allows the synthesis of uniform, monosized, smooth surface, silica NPs under 100 nm (Figure 7).

Figure 7. TEM images of silica NPs synthesised by the LaMer method

9.3.2.3. In situ functionalization

Several attempts have been made to modify the NPs surface chemistry incorporating a variety of functional (usually organic) groups within the inorganic silica matrix (functionalization). This is important for applications such as gene therapy, since the NPs functionalization will increase their biocompatibility, improve the pDNA resistance to enzymatic action and allow a more efficient cellular internalization. [16,24]. The sol-gel route has great potential for *in situ* functionalization. The miscibility of alkoxide and functionalized silane compounds allows a complete polymerisation of all the precursors, yielding highly homogeneous hybrid products. Nonetheless, the specific precursor's hydrolysis rates may cause inhomogeneities or even phase separation at nanoscale. A sequential addition process, in which the least reactive precursor is pre-hydrolysed to some extent before more reactive is added, or the presence of complexing chelating agents, aimed at retarding the hydrolysis kinetics of the most reactive precursor, allow the organic functional group to be placed randomly along the silica chain. More than one functionalized silane compound can be introduced in the sol-gel synthesis process, offering multiple functionalities to the same synthesised NPs. Figure 8 shows TEM images of *in situ* amine- and 3-glycidoxypyrtrimethoxysilane (GPTMS) – functionalized SiO_2 NPs, along with the respective Fourier transform infrared spectroscopy (FTIR) spectra, where amine and GPTMS finger prints are highlighted (Figure 9).

Figure 8. TEM images of *in situ* amine-functionalized silica NPs (a) and GPTMS functionalized SiO_2 NPs (b)

Figure 9. FTIR spectra of inorganic and functionalized SiO₂ NPs, where functional groups finger prints are highlighted

9.3.3. Mesoporous silica nanoparticles

Mesoporous SiO₂ NPs present a very large surface area with controllable pore size, volume and architecture. This characteristic acts as an important advantage when NPs loading is the main purpose.

The synthesis of mesoporous silica NPs is done by modifying the Stöber method, adding surfactants that behave like templates, and will later be chemically or thermally removed [25-27] (Figure 10). Pores size of 10 and 300 Å have been reported along with different porous structured nanophases, using a replica of the surfactant template (Figure 11). During the synthesis of mesoporous SiO₂ NPs, fine synthetic control will result in high surface areas with well controlled particle sizes and shapes that render these NPs very interesting [25-29].

Figure 10. Surfactant as template in nanostructured mesoporous silica NPs

Figure 11. TEM images of nanostructured mesoporous silica NPs: (a) cubic domain, (b) biphasic I1+H1, (c) *La*

9.3.4. Hollow-sphere silica nanoparticles

Hollow-sphere NPs can be created through the condensation of alkoxysilanes (or mixtures of alkoxysilanes and functionalized silanes) onto polymer-based templates, metal organic frameworks or other nanomaterials. Later the template will be removed by chemical etching or thermal degradation (Figure 12).

Figure 12. Scheme of hollow-sphere synthesis

Hollow-sphere NPs are capable of carrying large amounts of payload or filling their cores with other desirable materials such as polymers, gold or silver [30-35] along with the gene delivery performance. Figure 13 shows TEM images of hollow SiO₂ NPs and illustrates the FTIR of all stages of the synthesis process.

Figure 13. Hollow SiO_2 NPs: (a) TEM image, (b) FTIR of NPs in all stages of the synthesis process

9.3.5. Core-shell silica nanoparticles

Another possible architecture is core-shell structure. Here a great diversity of materials can also be used as the core template or shell structure. Gonçalves and co-workers described the synthesis of ORMOSIL core-shell NPs for biomedical purposes [36]. They used two different materials as core – iron oxide and SiO_2 – and amino-, methyl-, vinyl- and phenyl-functionalized ORMOSIL as shell.

Core-shell NPs have great potential in the future of biomedical applications, since these NPs constitute a scaffold to create multi-functional NPs, applicable to several fields; theranosis (therapeutics and diagnostics) [36] and gene delivery performance are some of the possibilities.

Figure 14 illustrate TEM images of iron oxide (~ 6 nm diameter) used as core, its isothermal hysteresis behaviour and the final core-shell nanoarchitecture with multiple iron oxide particles in the core and ORMOSIL shell.

Figure 14. Core-shell NPs: (a) Fe_2O_3 isothermal hysteresis behaviour; (b) TEM images of Fe_2O_3 core; and (c) core-shell NPs

This core-shell nanoarchitecture proved to be an interesting contrast agent for magnetic resonance imaging (MRI), as illustrated in Figure 15, where a MRI image of non-contrasted (blank) zebrafish and MRI image of contrasted zebrafish with iron oxide-coke ORMOSIL-shell NPs are shown.

(a)

(b)

Figure 15. MRI image of non-contrasted (blank) zebrafish (a) and MRI image of contrasted zebrafish with core-shell NPs (b)

9.4. GENE THERAPY

Gene therapy is based on a process, conceptually simple and attractive, which consists of the transfer of one or more functional genes into a cell, tissue or organ (*in vivo*, *in situ* or *ex vivo*) for the treatment of genetic or acquired diseases (infections, cancer and degenerative diseases among others).

The first gene therapy clinical trial took place in the early 1970s, where DNA and RNA of the tumour viruses were successfully delivered as genetic information to the genomes of mammalian cells [37]. Despite many failures throughout history, in 2006 confidence in gene therapy increased when a melanoma treatment using a retroviral formulation was successful [37]. In early 2015, approximately 2100 clinical trials had been conducted or had been approved, the majority based on viral vectors for gene delivery [38].

Over recent decades, new methods aimed at the delivery of genes to mammalian cells have been developed, focussing attention on gene therapy and DNA vaccination. The success of gene therapy is strongly dependent on the efficacy of the transfection processes, that is, the ability of DNA to reach the nucleus of the target cell in sufficient amounts to be effective; able to properly express the gene of interest. It is thus necessary to identify the target cell types and the appropriate DNA sequences in order to use and adopt suitable methods to reach these objectives. Efficient transportation of the therapeutic gene(s) to the nucleus of the target cells can then be carried out by either viral or non-viral vectors. Examples of viral vectors include the modified retrovirus and adenovirus, and are generally more efficient than the non-viral vectors, however, these viral systems raise several safety concerns which are absent in non-viral systems such as plasmids. Plasmids have great potential as safe vectors, can be produced on large scale with high reproducibility, and low costs, and may be stored at room temperature [39]. Plasmid systems have the potential to provide nucleic acid-based therapeutic where the products should have the ability to be easily and repeatedly administered to patients with a reduced immune response, making them similar to the traditional pharmaceuticals. Non-viral methods typically produce low levels of transfection and expression of the gene, however, when compared with viral methods. Nevertheless, some recent developments in vector technology have yielded molecules and techniques with efficiencies that approach those of viruses [40].

The injection of naked DNA is the easiest method of non-viral transfection although is many times inefficient. The main physical methods used to enhance gene delivery are electroporation and the gene gun or particle bombardment. The electroporation method consists of short high voltage pulses that create transient pores in the plasma membranes allowing DNA entrance into cells. In the gene gun method, DNA is coated onto gold NPs and loaded into a particle delivery system which generates a force to achieve penetration of the DNA into the cells.

Non-viral gene carriers normally include organic polymers and liposomes [41,42]. The former have more potential for gene delivery, as they are easily prepared and present a reduced risk of immune response. Lipoplexes (lipid nucleic acid complexes), cationic polymers and solid lipid NPs, have shown promise as a delivery vector in gene therapy [43-45], however, cationic molecule-based systems present low gene transfer efficacy and toxicity

associated with inflammation. Several attempts have been made to decrease the cytotoxicity of cationic non-viral systems [46], however, an ideal method with high transfection efficiency and relatively safety *in vitro* and *in vivo* has not yet been found. Inorganic NPs with high biocompatibility, lack of toxicity and stable chemical and physical properties, are promising as gene carriers [47]. Roy *et al.* [48] used SiO₂ NPs as gene carriers in pDNA transfection studies. Since then several attempts have been made to develop procedures for modifying the carrier surface (functionalization procedures), in order to increase their biocompatibility and resistance to enzymatic action and internalization efficiency [16,24].

The NPs should be stable enough to interact with the cell membrane while still allowing the release of the DNA from the complex. The size of the nanocarrier is an important parameter for consideration. Some processes will influence the diameter of the NPs/complexes and their net charge stability – sonication, filtration and dialysis are some of the examples. The nanometre or colloidal size ranges, around the 10–1000 nm, are an important feature, since their sub-micron size (and also sub-cellular size) allows deep penetration into the tissues by fine capillaries, being normally taken up efficiently by the cells.

Notwithstanding, NPs are metastable materials, with a huge tendency to agglomerate or growth. Prior to any complexation to genetic material NPs need to be stabilised. Another practical problem involves NPs suspension's kinetic stability which may be achieved through the use of buffer solutions, pH, and temperature control, or by steric hindrance.

There are several physiological barriers that must be overcome to achieve the desired gene expression. For efficient gene delivery and transfection, internalisation must take place without disruption of the gene intended to be expressed. Internalisation through the cell membrane must occur, followed by endosomal escape, going through the cytoplasm until entering the nucleus. Bottlenecks may be formed during this process, however, which will limit the transfection efficiency. The internalisation mechanisms are also largely dependent on the cell type, the vector used and the physical-chemical parameters of the complex determined by the synthesis or production process. Another physiological constraint that can influence the expressed protein levels is the nucleases role in pDNA degradation after administration and during the traffic to the cell nucleus [48,49]. It has been shown that the majority of pDNA administered in animal models is degraded by a population of exo/endonucleases that are constitutively present in plasma and cytosol of the receptor cells [50,51]. The use of vectors that encapsulate the DNA is thus essential to avoid its degradation.

9.4.1. Silica and ORMOSIL nanoparticles for gene therapy

Inorganic NPs such as those made of silica are a promising solution for gene delivery systems, since they present some of the desired characteristics such as biocompatibility, lack of toxicity and stability as already described [47]. Several studies have been successful in using silica NPs as a system for gene delivery [52,53]. Several efforts have also been made to modify the surface of various polymer vectors in order to improve their biocompatibility, increasing their circulation time, internalisation efficiency and improving the link to the genes. A common functionalization procedure uses silane compounds with terminal functional groups that will interact electrostatically or covalently with the molecule to be delivered. One common chemical used for *in situ* surface functionalization of polymeric and silica NPs is the (3-aminopropyl) triethoxysilane (APTES). The amino group will electrostatically interact with proteins, enhancing their adsorption [54-56]. Studies carried out recently have shown that, among the commonly used alkoxide precursors, APTES is the one promoting plasmid DNA interactions more efficiently [16,52,57,58]. It has been shown that SiO₂ NPs, functionalized with amino groups, bind and protect pDNA from enzymatic digestion allowing cell transfection *in vitro* [13,14,21,59]. APTES can also dissolve in both polar and non-polar solvents, and has high solubility in cell membranes [60,61].

Gonçalves and co-workers have shown that amino ORMOSIL NPs efficiently bind to the pDNA forming complexes that have been successful internalized by Chinese hamster ovary cells [21]. SiO₂ NPs functionalized with APTES are promising candidates for use as carriers in gene therapy.

Figure 16 shows the high efficiency in pDNA complexation of NH₂-ORMOSIL NPs, by the common agarose gel electrophoresis, and the internalization in both Chinese hamster ovary cells and zebrafish larvae.

Figure 16. pDNA and ORMOSIL NPs agarose gel electrophoresis (a); fluorescence-labelled ORMOSIL NPs internalised and transfected by Chinese hamster ovary cells (b); fluorescence-labelled ORMOSIL NPs internalised by zebrafish *larvae* (c)

9.5. CONCLUSIONS

Over recent decades a large number of viral and non-viral systems for gene delivery have been developed and studied. Both types present advantages but also still have drawbacks. Gene delivery through viral-based systems present long-term expression, effectiveness and expression of the therapeutic genes, with a high transfection rate, however, they also have some limiting disadvantages: immunogenicity, toxicity, the risk associated with virus handling and limitations on the production of large batches. The non-viral methods are supported by higher biosafety, simpler techniques, and can effectively target cells and tissues, however, their biggest disadvantage for clinical use is their low transfection efficiency.

Silica and ORMOSIL NPs are considered a good scaffold for multi-functional biomedical applications. They present a large surface area which could allow them to be loaded with one or more types of drugs to be used in drug delivery, or even by a fluorophore, for real time imaging and diagnosis. Through surface functionalization with specific groups, SiO₂ NPs can be conjugated with several types of biomolecules, such as specific linkers, fluorescent labels, antibodies or DNA, giving them simultaneous therapeutic and diagnostic (theranosis) capabilities. Many studies have shown that SiO₂ NPs or ORMOSIL NPs are promising candidates for use as non-viral carriers in gene therapy. These NPs have many unique properties, such as a high biocompatibility, nontoxicity and good potential to control their size, shape and cargo. It has also already been shown that these NPs bind efficiently to plasmid DNA and are able to protect it from enzymatic degradation. These are all promising characteristics for non-viral vectors, which are likely to be used in gene therapy.

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**INTRACELLULAR DELIVERY AND SENSING
BASED ON POLYELECTROLYTE
MULTILAYER CAPSULES**

Moritz Nazarenus*, Pilar Rivera Gil, and Wolfgang J. Parak

Fachbereich Physik, Philipps Universität Marburg, Germany

* Corresponding author: moritz.nazarenus@googlemail.com

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10.1. INTRODUCTION

10.1.1. Polyelectrolyte multilayer capsules

Polyelectrolyte multilayer capsules were first introduced in 1998 by the group of Helmuth Möhwald and coworkers, and since then have gained increasing interest over the years, especially in biomedical fields [1,2]. These capsules are typically fabricated using template particles, onto which polymers of alternating charge are deposited using layer-by-layer (LbL) assembly [2]. After dissolution of the template, stable hollow capsules remain. The size and the shape of the capsules are thereby dependent on the choice of templates, whereas the physico-chemical properties are determined by the polymers. Yashchenok *et al.* produced anisotropic, *i.e.* non-spherical calcium carbonate cores, and showed that the resulting capsules kept the form of the templates after their removal [3]. Different materials, such as polystyrene, silica or calcium carbonate have been used to fabricate capsule cores [4], but even red blood cells or *Escherichia coli* can be used [5,6]. With the right choice of template the capsules can be tuned in size from 30 nm up to several microns, opening the way for customization of the capsules according to the problem of interest [4]. Very small capsules of tens of nanometers can be produced with silica templates, whereas capsules assembled on calcium carbonate cores typically reach sizes of 2–10 microns. Capsules can be produced with a great variety of different polymers. The most widespread and best understood pair of polymers is the system of poly(styrene sulfonate) (PSS) and poly(allyamine hydrochloride) (PAH). However, there are many other polymers in use and it is also possible to produce biodegradable capsules made of polymers that can be digested/metabolized inside living cells, for example by proteases present in the lysosome [7,8]. There are two ways to load the capsules, the co-precipitation method, where the cargo is entrapped in the template and remains inside the capsules after removal of the template material, and the postloading method, where the permeability of the capsules can be controlled externally by variation of pH, salt concentration or temperature [9], and the cargo can be loaded into the empty capsules after the synthesis [10]. During the removal of the template core the capsules become much more porous due to the osmotic pressure that arises by dissolving the core. This is the main reason why cargo is lost during the fabrication of capsules using the co-precipitation method. There are also other ways of synthesizing capsules, apart from the LbL method and the reader is referred to recent reviews on capsules as biological carriers for further information [4,11,12]. The capsule wall represents a semi-permeable membrane, as small molecules can diffuse almost freely through the pores [13], whereas large molecules, for example dextran molecules or proteins, cannot pass the capsule walls. The molecular weight cut-off (MWCO) for PSS/PAH is around 200 Da [14]. The MWCO depends on the polymers, and

Miller and Bruening showed that, for example, the combination of hyaluronic acid (HA) and chitosan had a MWCO of $\approx 17,000$ Da [14,15]. Due to the semi-permeability of the capsule wall a Donnan equilibrium occurs [16], which means that the concentration of ions and molecules inside of the capsules is different of the concentrations outside, also in case of molecules or ions, for which the membrane is permeable. Sukhorukov *et al.* showed, for example, in a theoretical model that the pH inside capsules is different from that in the solution [17]. The permeability of capsules reduces as the pores of the capsules close over time. This effect can also be achieved by enhancing the temperature to anneal the pores [18,19]. The permeability of the capsules walls is furthermore influenced by the hydration of the polymer multilayers and the charge densities of the polymers. With higher charge densities the permeability of the capsule walls decreases and the hydration is reduced [15]. Hydration of the polyelectrolytes has not only an effect on permeability but also on the thickness of the layers as well as the interaction of the polymers, and the elasticity of the capsule shells [20]. In case of PSS/PAH, the amount of water associated with PSS is roughly double as much as the amount associated with PAH [21]. It has to be noted that the water content of planar polyelectrolyte multilayer films is smaller than that of capsules. This means that the planar films are packed more densely than the capsule walls. The difference is about 45–55 % of water [20]. Furthermore, the water content of the multilayers is dependent on the outermost layer. Wong *et al.* showed that water is pressed out of the multilayer when PAH is adsorbed as top layer and that more water is absorbed by the shell when PSS is the top layer [22]. The outer layers are thereby more sensitive to the environmental humidity than the inner layers. This means that with increasing numbers of layers the inner layers are more and more unaffected by hydration [23]. Concerning applications of capsules in biomedical fields, they can generally be divided into delivery and sensing applications [24,25]. A large size of the capsules can be of advantage in cell culture models, as large capsules are easily traceable with optical techniques. In contrast, small capsules in the size of nanometers are of interest for *in vivo* applications, where large capsules cannot be used as they may block capillaries. The cellular uptake is a statistical process that *in vitro* occurs in almost all mammalian cells, both immortal cell lines and primary cells [26–28]. It is triggered by phagocytosis and lipid-rafts-mediated macropinocytosis and the final location of the capsules is in the heterophagolysosomes of the cells [27]. One should note that the uptake is dependent on the shape of the capsules. Spherical capsules are internalized faster and in higher quantities compared to anisotropic capsules, yet the final location is not affected [29]. After cell division, the capsules are passed to the daughter cells [8]. The distribution, however, is asymmetric, *i.e.* they are not passed on in a fifty-fifty manner [30,31]. Capsules can be filled with various cargos for delivery, among them drugs, genetic material like desoxyribonucleic acid (DNA) or ribonucleic acid (RNA), or proteins [12,32–37]. The experimental challenges lie in the difficulties to encapsulate certain cargos in a sufficient

way, but also in problems to release the cargo at the desired target site. Concerning release, one major challenge is to free the capsule content from the organelle, *i.e.* the lysosome where the capsules are located after incorporation into cells, so that the cargo becomes available to reach targets outside this vesicle. For sensing applications the capsules can be filled with a sensor that does not necessarily need to be released. Often, this is a dye sensitive to a specific ion or other analyte, respectively [38-41]. These dyes are widespread as they are easy to handle and there is a large variety of dyes for many different analytes commercially available. Moreover, the biocompatibility of organic fluorophores usually is not an issue as they typically show very low or no toxicity [42]. Dyes need to fulfill certain requirements to be suitable for the use in sensor capsules for intracellular sensing. Fluorophores with the absorption maximum in the UV to blue range, for example, may be problematic as autofluorescence can occur in the cells. Moreover, light of short wavelength has high energies and may thus be harmful to the cells. To entrap the dye molecules inside the capsule cavity, they need to contain functional groups, with which they can be conjugated to dextrans or other large molecules so they cannot diffuse through the capsule walls. These conjugations must be possible without the dye molecules losing their fluorescent properties and analyte sensitivity. Depending on the application, the photostability of the dye may be an important parameter. Many fluorophores bleach fast in biological media, which can be problematic, for instance in long-term experiments. The spectra of fluorescent dyes often are quite broad and therefore multiplexed measurement with dyes, *i.e.* the sensing of two or more analytes simultaneously, is limited due to spectral overlap of the dyes [42]. Additionally one has to keep in mind when working with dyes, that crosstalk can occur from different ions or pH [39,43]. As will be highlighted further in the section on sensing, many organic fluorophores are sensitive to more than one ion and the fluorescence may also be dependent on the local pH. Besides dyes, also more complex sensor systems have been described, such as enzymes that convert a metabolite and a component that gives a signal quantifying the conversion [44-46].

10.2 APPLICATION I: DELIVERY

Delivery and release of carried compounds from the carrier (capsules) is still a challenge that affects the performance of the compound and of the carrier itself. Release of the cargo must be ensured not only from the carrier but also from the vesicle in which the carrier is entrapped. There are different strategies to ensure this. With help of triggers (internal or external) opening of the carrier can be performed. Internal triggers include responsive polymers that are sensitive to their environment, whereas external triggers can be electromagnetic waves, magnetic fields, or heat, for example. Release from the vesicle can be achieved by changing the osmotic pressure of the organelle. All this strategies will be described in the following.

10.2.1. Responsive polymers as internal triggers to mediate cargo release

In this case, polymers which are sensitive to degradation/metabolization by lysosomal enzymes are used as constituents of the capsule wall. When capsules of this nature enter the lysosomes, wall degradation occurs and cargo release follows [8,47,48]. An example of success of this strategy has been described by demonstrating the release of genetic material (DNA and silencing RNA (siRNA)) [36] and will be described in more detail in the following. Indirectly, the delivery of cargo to the cytosol of the cell (out of the organelle) was demonstrated in this study by measuring the activity of siRNA after reaching its target messenger RNA (mRNA) located in the cytosol. Cytosolic release was hereby not performed by the polymers of the capsules but by encapsulated polymers capable of disrupting the lysosomes of the cells. The lysosomal escape in this case was ascribed to the so-called proton sponge effect [49-51], although this effect is still under debate. The proton sponge effect is stated to take place when polycations with buffering capacity, such as poly(ethylenimine) (PEI), which at slightly acidic pH contains many deprotonated amine groups, buffer the pH in the lysosomes. The lysosomal pH hence does not decrease when proton pumps transfer protons from the outside into the lysosomes and thus influx of further protons takes place [52]. To ensure the charge and ion activity equilibrium across the lysosomal membrane, also chloride and water enter the lysosomes. This leads to swelling of the lysosomes. When the pressure increase is high enough, the vesicles finally burst releasing their content to the cytosol [53].

10.2.1.1. Biodegradable capsules as non-viral vectors

Despite the established methods, there is need for other or improved means of delivery of genetic material to cells. Viral vectors usually show a high transfection rate and are therefore the preferred vectors. They, however, raise concerns because of mutagenesis, *i.e.* the possibility that the viral vectors trigger mutations of the genome, and immunogenicity, *i.e.* the possibility that

viral vectors induce an unwanted immune response [54-56]. Non-viral vectors such as lipoplexes and polyplexes, in contrast, show only small transfection rates. Microinjection does not allow for high numbers of transfected cells, as the transfection is very time-consuming and in general not well automatable. Furthermore, it generally does not allow for *in vivo* transfection, which is also true for electroporation. Thus, there is need for new carriers for transfection both *in vitro* and *in vivo*. A bio-compatible carrier is desired, which protects the genetic material against degradation, before it reaches its target, facilitates cell internalization, and enables controlled release of the genetic material [57]. Biocompatible capsules have been synthesized by many groups and subsequent release into the cytosol has been achieved for different cargos, such as proteins, DNA, or RNA [8,26,33,34,58,59].

10.2.1.2. Delivery of DNA

Ganas *et al.* performed a comparative study where the delivered amount of DNA was quantified for different delivery systems [36]. Cells were incubated with plain DNA, PEI/DNA polyplexes, PEI/DNA polyplexes encapsulated in biodegradable capsules, and PEI/DNA polyplexes encapsulated in non-degradable capsules. Standard transfection protocols with polyplexes were performed in serum-free growth medium [60]. Here, a comparison for media with and without serum was performed. The amount of DNA for each of the experiments was equal so that a comparison based on the administered amount of DNA was possible. In Figure 1, images of HeLa cells at different points in time and for the different ways of delivery are shown. The images show cells incubated without serum. The DNA was labeled with the fluorophore Cy5 and is colored in violet and PEI was labeled with fluorescein isothiocyanate (FITC) and is colored in green in the images. The result of the overlay of violet and green is white. In the images, one can see that cells with plain DNA and PEI/DNA polyplexes show no fluorescence signal at any point in time. With both capsule types, the cells show fluorescence signal, but only cells incubated with biodegradable capsules show fluorescence in the cytosol, whereas in non-degradable capsules the signal is confined to the area of the capsules. The PEI, however, is distributed in the cytosol also in case of non-degradable capsules. Note that the amount of PEI/DNA polyplexes is very small compared to what is stated in standard protocols. This was due to the small amounts needed for the delivery by capsules and the fact that the comparison was based on the amount of DNA added to the cells. Furthermore, the cells begin to become spherical at later times as the serum-free medium stresses them.

Figure 1. MDA-MB-231 cells were incubated with free DNA, DNA/PEI polyplexes, biodegradable (DexS/pArg)₅ capsules with walls made out of dextran sulfate (DexS) and poly(L-arginine) (pArg) with embedded PEI/DNA, and non-degradable (PSS/PAH)₅ capsules with walls made out of poly(sodium 4-styrenesulfonate) (PSS) and PAH with embedded PEI/DNA. After different incubation times, t , as indicated in the top panel, cells were imaged. The transmission channel shows the cells, and the green and violet channels show the emission of the FITC labels and Cy5 labels of PEI and DNA, respectively. Images from serum-free experiments are shown. The scale bar corresponds to 20 μm . The figure has been taken from reference [36]. Reprint with permission from Elsevier.

The delivery efficacy was quantified by quantitative image analysis. The area of each cell was determined from the transmission channel and the mean fluorescence intensity in that area was measured. Fluorescence from the area of the capsules was not included. The mean fluorescence of the cells was averaged and plotted as column diagrams in Figure 2. The left graph corresponds to serum-free incubation and the right graph to cells in serum-supplemented medium. The amount of DNA delivered was highest for biodegradable capsules and already took place within less than 24 hours of incubation. DNA transport was not observable for free DNA or PEI/DNA

polyplexes in serum-free medium. However, non-degradable capsules could deliver a small but observable amount in serum-free medium. In serum-supplemented medium, free DNA and PEI/DNA polyplexes show a small effect. Nevertheless, here, as well as in serum-free medium, the biodegradable capsules had a much higher efficacy. Moreover, the amount of delivered DNA with biodegradable capsules was found to be clearly higher in serum-supplemented medium than in serum-free medium. Cells deprived of serum suffer from stress, which in turn influences the metabolism. Therefore, transfection in serum-supplemented medium is clearly preferred. Ganas *et al.* showed that transfection with biodegradable capsules offers a possibility to do that and the capsules provide protection for the DNA in the extracellular medium [36].

Figure 2. From the microscopy data as presented in Figure 1, for each cell in each recorded image the integrated fluorescence intensity, I_{DNA} , was determined. The integrated fluorescence intensity corresponds to the area of the cell (determined from the transmission channel) times the mean fluorescence intensity from the Cy5 channel in that area. Fluorescence originating from the capsules was not considered, but only fluorescence located in the cytosol. The mean fluorescence intensities, as observed for the different ways of delivery, are displayed for incubation in: (a) serum-free, and in (b) serum-supplemented media together with the corresponding standard deviations.

For each data point at least 30 cells were analyzed. The figure has been taken from reference [36]. Reprint with permission from Elsevier.

10.2.1.3. Delivery of siRNA to block the expression of green fluorescence protein (GFP)

In a further step within the same study [36], siRNA was used to transfect cells and to determine the efficacy of transfection with biodegradable capsules. For this purpose, GFP-expressing HeLa cells and siRNA capable of knocking out GFP were used. The cells were kept in serum-free medium for these

experiments. The comparison here was made only between cells incubated with biodegradable siRNA-containing capsules and polyplexes. In Figure 3, images of the cells are shown. In this case, PEI was labeled with Dy-651 and colored in violet, and siRNA was labeled with AF-546 and colored in red. GFP is depicted in green. The left panel shows cells incubated with polyplexes and the right one cells incubated with polyplex-containing biodegradable capsules. The cells incubated with polyplexes kept their green fluorescence over the entire time, while in the images of cells incubated with capsules the fluorescence was lost after 30 h in the cells marked with asterisks (*). After 20 h, the cells with capsules clearly showed distribution of PEI (violet) in the cytosol. The insets labeled with the number sign (#) in the polyplex panel show that there is siRNA present in the cells (red label). These images were enhanced in contrast to make the red fluorescence visible.

Figure 3. GFP-expressing HeLa cells were incubated with free (left column) and encapsulated (right column) PEI/siRNA polyplexes. Live images of the same cells were taken over time, *t*. Besides the transmission channel showing the cells, also GFP (green), AF-546 labeled siRNA (red), and DY-651 (violet) labeled PEI are displayed. The straight arrow indicates a homogenous cytosolic distribution of PEI, whereas the dashed arrow shows a punctuate distribution. Cells marked with asterisks have clearly lost their GFP fluorescence. The insets (#) correspond to images of the polyplexes. For clarity, these insets were enhanced in contrast in order to show the presence of the siRNA inside the cells. The scale bars correspond to 10 μ m. The figure has been taken from reference [36]. Reprint with permission from Elsevier.

In Figure 4, the mean intensity is plotted against time for cells incubated with polyplexes, cells incubated with capsules, and untreated control cells. The amounts of siRNA added to the cells were comparable, $6.4 \cdot 10^{-3} \mu\text{g}$ *via* capsules and $10 \cdot 10^{-3} \mu\text{g}$ *via* polyplexes added to $2 \cdot 10^4$ seeded cells in $300 \mu\text{L}$ of medium. The graph clearly shows that encapsulated polyplexes decrease the GFP fluorescence after 30 h of incubation. On the contrary, both polyplexes and control cells do not show any effect.

Figure 4. GFP-expressing HeLa cells were incubated with free encapsulated PEI/siRNA polyplexes. The total amount of siRNA and PEI was $6.4 \cdot 10^{-3} \mu\text{g}$ and $25 \cdot 10^{-3} \mu\text{g}$ for capsules and $10 \cdot 10^{-3} \mu\text{g}$ and $12.5 \cdot 10^{-3} \mu\text{g}$ for polyplexes, respectively. Images were taken at different points in time, t , and the mean integrated fluorescence density of GFP, *i.e.* the area of each cell times its mean fluorescence intensity, is displayed. The error bars represent standard deviations of the mean. The figure has been taken from reference [36]. Reprint with permission from Elsevier.

These results demonstrate that encapsulated polyplexes can be more efficient for the delivery of siRNA into cells than plain polyplexes. Furthermore, as already mentioned in the beginning of this paragraph, protection of the siRNA against degradation, high biocompatibility, and potentially controlled release of the cargo offer additional advantages of polymer capsules as delivery vehicles.

10.2.1.4. Cytotoxicity of PEI polyplexes and encapsulated PEI

One concern not dealt with yet is the toxicity of PEI for cells, which has been described regularly for PEI polyplexes [50,61,62]. The capsules offer a way to reduce this problem, as they not only facilitate protection of siRNA against degradation but also protection for the cells from PEI. Ganas *et al.* verified this by a resazurin-based toxicity assay [36]. The results are depicted in Figure 5 as a function of added mass of PEI to make the experiments comparable.

Figure 5. Reduction of cell viability caused by encapsulated and non-encapsulated PEI. The normalized cell viability in terms of resorufin fluorescence intensity, I , versus the amount of added PEI, m_{PEI} , is displayed. The average of six (PEI capsules) and three (PEI solution) independent measurements is displayed. Higher amounts of encapsulated PEI could not be measured as there is a threshold at which the capsules themselves can induce toxicity if added in too high quantities to the cells [48,63]. The error bars represent standard deviations of the mean. Note that the value for zero PEI could not be plotted due to the logarithmic scale but lies in the same range as the smallest plotted value. The figure has been taken from reference [36]. Reprint with permission from Elsevier.

For the capsules, larger amounts of PEI than plotted were not added to the cells because at some point the capsules themselves may introduce adverse effects [48,63], and furthermore the cells do not have the capability to take up arbitrarily high numbers of capsules. Nonetheless, the graphs show that the toxicity of encapsulated PEI is much smaller than that of free PEI. The number

of capsules added to the experiments corresponding to Figure 5 was 20 capsules *per* seeded cell, which accords to 25 ng in the whole well. In the toxicity test, this accords to 0.1875 ng, as only $1.5 \cdot 10^4$ instead of $2 \cdot 10^4$ cells were seeded *per* well. At this amount, however, the viability of the cells is not affected. This means that the capsules protect the cells from the PEI of the encapsulated polyplexes [36].

10.2.2. Light acting on Plasmonic nanoparticles as external trigger to mediate cargo release

For light-triggered release, plasmonic nanoparticles can be integrated in the wall of the capsules. These nanoparticles can be excited with light at their plasmon frequency, inducing oscillations of the surface electrons, so-called plasmons. The plasmons subsequently transfer their energy first to the crystal lattice and from there to the environment, *i.e.* they dissipate heat [64-66]. The locally generated heat is sufficient to open the capsules and facilitate cargo release, not only from the capsule but also from the organelle [67-69]. Carregal-Romero *et al.* demonstrated this upon release of a pH sensor, which monitored differences in pH of the lysosomes and the cytosol along its way [68]. Moreover, more complex and labile molecules such as siRNA, mRNA or proteins can be released following this strategy while maintaining their performance [69]. Whereas in the previous paragraphs the release of nucleic acids was achieved making use of biodegradable capsules and the ability of PEI to trigger cytosolic release of the nucleic acids from the lysosomes [36], in this paragraph the release by external laser opening is described. This technique has the advantage that the release can be confined very specifically to a single cell and the capsule of interest. The release of macromolecules such as protein-encoding mRNA, but also proteins themselves is possible with this technique. In Figure 6, the burst of the lysosomal vesicle is shown [68]. Cells were incubated with seminaphtharhodafluor (SNARF)-containing light-responsive capsules. Before opening, the capsules appear in green-yellow due to the acidic lysosomal pH. After irradiation with the laser, the capsules appear in yellow, indicating a neutral or slightly alkaline pH (Figure 6A). The images in A were taken with a low exposure time to image the capsules, whereas the images in B were taken with a high exposure time to detect the fluorescence in the cytosol, and thus the capsules appear overexposed in Figure 6B [68].

Figure 6. A549 cells were incubated with light-responsive SNARF-filled capsules. Before opening of the capsules, they appear in green-yellow indicating the acidic lysosomal pH. After opening, the SNARF-dextran is released and spreads and the cytosol of the irradiated cells appears in red due to the neutral to slightly alkaline pH. Images in A were taken with a small exposure time optimized to image the capsules; images in B were taken with a high exposure time in order to image the cytosol. Capsules in B therefore appear overexposed. The scale bar represents 20 μm . The figure has been taken from reference [68]. Reprint with permission from Elsevier.

10.2.2.1. Delivery of mRNA with light-responsive capsules

Delivery of mRNA often is performed with polyplexes such as mentioned already above for the delivery of siRNA. The uptake and subsequent release of mRNA from the endocytic vesicles to the cytosol is statistically distributed. Determining the kinetics of cytosolic release is therefore difficult, as there is no clear starting point in time. Delivery of mRNA by light-responsive capsules in the contrary offers external control over the point in time of release and thus can circumvent this issue. In Figure 7, the kinetics of delivery of GFP-encoding mRNA is depicted for HeLa cells incubated with mRNA polyplexes on the left and mRNA-containing light-responsive capsules on the right side [69]. The kinetics of the polyplexes is plotted dependent on the incubation time showing saturation after 15 h. The cells show a uniform expression of GFP as visible in the images after 10 h and 25 h. The cells incubated with mRNA-delivering capsules show different kinetics. The expression possesses Gaussian distribution with the maximum at 10 h after the opening of the capsules.

Figure 7. GFP expression of HeLa cells following transfection by polyplexes (left panel) and light-responsive capsules (right panel). (A) represents a sketch of polyplexes, (B) Confocal images of HeLa cells expressing GFP after treatment with polyplexes, (C) mean fluorescence intensity of HeLa cells dependent on the incubation time, (D) a sketch of light-responsive capsules as carriers of mRNA, (E) confocal images of cells expressing GFP after treatment with light-responsive capsules, (F) time-dependent GFP intensity after light-mediated opening. The scale bars represents 20 μm . The figure has been taken from reference [69]. Reprint with permission from Wiley-VCH.

Controlling the beginning of release externally could offer a way to release mRNA of various proteins sequentially and study, for example, the interaction of different mRNAs or other cargos in a time-resolved manner independent of the uptake time and the time needed for cytosolic release. In order to demonstrate sequential release, Ochs *et al.* employed the phosphatase-mediated reaction of ELF97 phosphate to ELF97-alcohol [69]. Cells were co-incubated with light-responsive capsule filled with ELF97 phosphate or phosphatase, and a blue or red marker. The reaction could be taken out in cells containing capsules of both types. In Figure 8, the intracellular reaction is demonstrated once in the way that first phosphatase was released and only afterwards ELF97 phosphate (top panel), and once the other way around [69]. As visible in the images, the reaction product ELF97-alcohol sediments in the capsule opened secondly. The highest benefit from light-triggered release as described here can be taken for reactions of short timescale rather than for

long-lasting systems, in which uptake and cytosolic release is negligible compared to the reaction itself.

Figure 8. HeLa cells were incubated with light-responsive capsules containing non-fluorescent ELF97 phosphate and the red fluorescent AlexaFluor594-dextran (red capsules) and light-responsive capsules containing non-fluorescent phosphatase and the blue fluorescent Cascade Blue-dextran (blue capsules). Phosphatase is capable of converting ELF97 phosphate into fluorescent ELF97-alcohol (A). In a cell containing both, ELF97 phosphate-filled capsule and phosphatase-filled capsule, first the phosphatase capsule is opened (B) and afterwards the ELF97 phosphate capsule (C). The enzymatic reaction takes place and the ELF97-alcohol's fluorescence is observable within the yellow circle (D). When first the ELF97 phosphate containing capsules and subsequently the phosphatase-containing capsules are opened (E-F), the ELF97-alcohol sediments in the area of the phosphatase capsule (G). The scale bar corresponds to 25 μm . The figure has been taken from reference [69]. Reprint with permission from Wiley-VCH.

10.2.2.2. Protein release from light-responsive capsules

Laser opening of capsules can also facilitate the release of the proteins themselves rather than the mRNA. In Figure 9, the release of GFP from capsules by laser irradiation is depicted [68]. After irradiation, the cytosol of the cells shows the green fluorescence of GFP, resulting from homogeneous spreading of the encapsulated GFP. In contrast, the GFP release from biodegradable capsules shows a dotted pattern indicating that GFP is located in small cellular vesicles, most likely exosomes.

Figure 9. Release of GFP from light-responsive capsules in HeLa cells. The capsules were irradiated with a laser at intensity of 3.8 mW cm^{-2} . Before irradiation the green fluorescence is confined to the capsules, afterwards the fluorescence is visible in the cytosol showing the release of GFP. The arrow points at the opened capsule. The scale bar corresponds to $20 \mu\text{m}$. The figure has been taken from reference [68]. Reprint with permission from Elsevier.

10.3 APPLICATION II: SENSING

10.3.1. pH sensing with fluorescent dyes

In the context of medical or pharmaceutical purposes, monitoring of the internal properties of a cell is of highest interest as many diseases are triggered by malfunctions of cells. In many cases, the homeostasis of the cells is influenced and alterations of intracellular properties can occur. One of the most fundamental properties in this manner is pH. The activity of enzymes, for example, is dependent on pH and deviations from the appropriate pH lead to low activity and can in the worst case harm the enzyme irreversibly. Furthermore, pH gradients across cellular membranes play an important role, as they ensure that cells can extract energy from transport processes across membranes. pH monitoring in living cells can help to study proliferation, apoptosis, and endocytosis, among others [70-73]. Moreover, there are many examples of diseases where the pH regulation in the affected cells or tissue is disturbed [74,75]. Monitoring pH can therefore be used to identify affected cells or *vice versa* tell if a certain drug is capable of stopping the pH regulation disturbance. A straightforward way is to use cell-permeant forms of pH-sensitive dyes, *e.g.* acetoxymethyl ester derivatives. These dyes are electrically neutral and are taken up by cells *via* osmosis. Their signal is either a spectral shift upon change of pH or the emission intensity alters. As cellular fluids are very complex, there are many ways in which the dye can be influenced, *e.g.* by binding to proteins [76]. Therefore, to measure accurate pH values, the dye must be calibrated intracellularly. The most common way to do this is the use of nigericin, a K^+/H^+ ionophore [77]. After treatment with nigericin, the intra- and extracellular pH values equilibrate and the intracellular pH can be adjusted by change of the extracellular pH. However, there are some problems associated with dye-based measurements. During long-time measurements, the cells sequester parts of the dye, which leads to lower signal, although no actual change of pH may have happened. Fading, also called photobleaching, is another issue in long-time experiments. In this case, repeated illumination leads to lower signal, due to reaction of the dye with radicals, *e.g.* reactive oxygen species, into non-fluorescent molecules [78,79]. Furthermore, a ratiometric measurement is preferred as it excludes sources of errors such as local gradients of the concentration of the dye, fluctuations of the illumination intensity over time, different exposure times, *etc.* [80,81]. For ratiometric measurements a second dye is needed as a reference, where the ratio of the local concentrations of the two dyes is constant and the ratio of the intensities is pH-dependent, at least in the interesting pH range around 4.5–8 for cells.

10.3.2. pH dependence of organic fluorophores

Apart from protons, the most critical ions in cells are Na^+ , K^+ , Ca^{2+} , Mg^{2+} , and Cl^- . The concentrations of Na^+ , K^+ , and Cl^- are, for example, of special importance for muscle cells and neurons as the formation of action potentials is dependent on them. Ca^{2+} is important for the release of neurotransmitters and muscle contraction and can serve as cofactor for enzymes. Mg^{2+} also is a cofactor for many enzymes. The use of adenosinetriphosphate (ATP) as energy source for the cell is, for example, only possible if magnesium is bound to the ATP. There are many organic fluorophores available for sensing and measuring intracellular concentrations of the mentioned ions. However, many of the dyes available tend to be pH-sensitive in addition to respond to their specific ion and may also be sensitive to other ions [82]. For example, the potassium- and sodium-sensitive dyes, a fluorescent potassium-sensitive indicator (PBFI) and a fluorescent sodium-sensitive indicator (SBFI), respectively, show crosstalk with the correspondent other ion [39,43], and the Mg^{2+} -sensitive dye Mag-Indo-1 can also be used for sensing Ca^{2+} . In Figure 10, the pH dependence of the chloride-sensitive dyes: *N*-(ethoxycarbonylmethyl]-6-methoxy-quinolinium bromide (MQAE) and 10,10'-bis(3-carboxypropyl)-9,9'-acridiniumdinitrate (BAC), the calcium sensor dye Calcium Green-1, and the magnesium sensor dye Mag-Indo-1 are reported by Kantner *et al.* [43].

Figure 10. Response curves of ion-sensitive dyes to the respective ion and pH.

Fluorescence signal of the chloride-sensitive dyes (a) MQAE and (b) BAC, in dependence of Cl^- and pH, (c) calcium-sensitive Calcium-green-1 against Ca^{2+} and pH, and (d) magnesium-sensitive Mag-Indo-1 in dependence of Mg^{2+} and pH. In all cases, the signal intensity is clearly dependent on pH in addition to the respective ion. The image is taken from reference [43]. Reprint with permission from Wiley-VCH.

In all cases, the fluorescence intensity is clearly dependent on pH. In the plots of the chloride-sensitive dyes, intensity values increase especially for low pH values, whereas for higher values, from pH 6–10, the profile only slightly changes for constant chloride concentration. For the other two dyes, the fluorescence intensity is clearly dependent on pH as well. This means in principle that measurements with all these dyes need to be supported also by pH measurements to obtain clear results on changes of concentrations of the desired ions.

10.3.3. Intracellular pH sensing with capsules

As mentioned in the introduction, one application of capsules is their use as intracellular sensors. Thereby, advantage is taken of the porosity of the capsules, as small ions can virtually freely diffuse through the capsule walls [13], whereas the dye can be bound to bigger molecules such as dextran to keep it trapped inside the cavity. The use of capsules or other carriers has some advantages over the free dye. As the probe is confined to a small volume, the local concentration of the dye does not influence the measurement. For the free dye, problems can occur when the concentration of the dye is not evenly distributed as then the signal obtained is dependent on the concentration of the dye and on the concentration of the analyte [83]. Moreover, the capsules protect the dye from unspecific binding, sequestering, and degradation inside the cells [38]. *Vice versa*, the capsule also protects the cell from the dye. This opens the possibility to use molecules, which are toxic for the cells or have other undesired effects. To perform intracellular pH-sensing with capsules, they were filled with the pH-sensitive dye SNARF® bound to dextran. This dye is intrinsically ratiometric, as it has two emission maxima, one at 580 nm, and one at 640 nm. The maximum at 580 nm grows with decreasing pH, whereas the one at 640 nm grows with increasing pH. The ratio of both maxima can be used to obtain a calibration curve of the dye. In Figure 11a, the calibration curve for one batch of SNARF® capsules is depicted [41]. The capsules can be used as sensors roughly from pH 6–9, as above and below the slope vanishes and hence the ratios cannot be related to a specific pH value anymore. In one recent report the SNARF® capsules were used as intracellular reporters for the lysosomal pH of MCF-7 breast cancer cells [41]. Capsules in the extracellular medium served as control capsules to confirm that changes of the color of the capsules did not take place automatically over time. In Figure 11, curves of intra- and extracellular capsules are plotted as function of time. The red line displays extracellular capsules and the green one internalized capsules. The ratio of the red and green intensity is plotted, which means that higher values correspond to higher pH. The capsules were used now to monitor the lysosomal pH under influence of substances that are reported in the literature to influence the lysosomal or generally the cellular pH. The substances tested were amiloride, bafilomycin A₁, chloroquine, and monensin. The mechanisms influencing the pH were of different nature. In this book chapter, we only

describe the example of monensin; details for the other substances can be found in the publication and its supporting information [41]. Monensin is a Na^+ ionophore, which means that it can bind to sodium ions and transport them through cell membranes. It is not specific to sodium ions but can bind also to potassium and other monovalent cations [84]. However, in cells it plays an important role as Na^+/H^+ antiporter. This means that it transports sodium ions through membranes in one direction and protons in the other direction. Thereby, it abolishes sodium and pH gradients in the cells [85]. The capsules were calibrated in buffer solutions using fluorescence microscopy, Figure 11a [41]. The pH profile of untreated cells was obtained as control (Figure 11b) and the pH profile of cells treated with different substances was monitored. In Figure 11c, the plot of one monensin experiment is displayed. The yellow bar indicates the period when monensin was present. The red curves in a) and b) show photo-bleaching in the beginning. The green curve has a baseline slightly above one. The untreated cells do not change the pH over the time monitored. Cells treated with monensin show a rise in pH to that of the extracellular capsules very fast, indicating that the lysosomal and the extracellular pH equilibrate during the presence of monensin. The peak keeps a high level during the presence of monensin. As soon as there is equilibrium of pH and sodium ions across a membrane, a further increase of the pH value is not possible. This means that the equilibrium was reached within one hour. The fast decrease of the green curve after the washing supports this. By rinsing the cells, the effect of monensin is completely diminished within one hour.

Figure 11. Monitoring the long-term behavior of the lysosomal pH of MCF-7 cells upon stimulation with monensin. The calibration curve (a) shows the applicability of the capsules for pH measurements from pH 6–9. In the control cells (b), no change of pH is observable. Cells treated with monensin (c) for 4 h (indicated by yellow bar) show increasing pH during the presence of monensin. The lysosomal pH reaches the level of the extracellular medium and returns to the basic value after removal of monensin. The image is taken from reference [41]. Reprint with permission from Wiley-VCH.

10.4 THERANOSTICS AS BIOMEDICAL APPROACH FOR POLYELECTROLYTE MULTILAYER CAPSULES

One of the future goals in medical treatment is so-called personalized medicine [86]. This term describes the idea to customize treatment of different patients based on their individual condition. The treatment should involve the identification of the molecular factors of the disease and from this the appropriate treatment strategy should be chosen. Moreover, this approach is expected to reduce unnecessary treatment, as ineffective drugs and therapies are to be prevented by the identification of the specific disease markers. In a further step, the outcome of the treatment should be monitored, so to be able to respond to side effects [87]. The major example where personalized medicine is expected to improve treatment is cancer [88,89]. Theranostics is the term to describe the combination of therapy and diagnostics in one treatment [90,91]. Within this section we outline the advantages and possibilities of polymer capsules for use in both diagnosis and therapy. Diagnosis hereby is based on capsules as intracellular optical sensors (for example of pH due to their lysosomal location). There are numerous publications on optical sensors for intracellular ion sensing. However, very often there are also numerous shortcomings included [43,92]. In many articles, sensor systems are presented that show very good behavior in defined environment, for example buffers with varying concentration of the targeted ion. Yet, the claim that these sensors will work the same way intracellularly is often too strong, as the intracellular environment is in many aspects different from a simple buffer solution. Hence, a study presenting a sensor system but no proof that the sensors work inside cells is incomplete and should be interpreted carefully. Studies including cell experiments often focus only on the proof that intracellular measurements are generally possible. Monitoring the behavior of the targeted ion concentration, however, is often excluded or only done over very short times [93,94]. Instead, it is important to also focus on the long-term monitoring of the dynamics of the lysosomal pH upon induced ion deregulation. pH is a preferential choice, as it is a ubiquitous quantity relevant for many cellular processes and because optical sensing of other ions is not as easy and straightforward as might be expected due to the pH dependence of many dyes as shown above [43]. Concerning therapy applications of capsules, integration of genetic material, *i.e.* DNA and RNA, into eukaryotic cells, the so-called gene therapy, was already shown as exemplary application previously in this chapter. Another therapeutic strategy in which capsules can be utilized is enzyme replacement therapy, for example in lysosomal storage diseases, will be discussed in the following.

10.4.1. Capsules for sensing and enzyme delivery in lysosomal storage disease models

10.4.1.1. Lysosomal storage disorders

Polymer capsules are excellent candidates for the design of a theranostic tool to be applied in lysosomal storage disorders (LSD). Those disorders are a large family of rare diseases, which are characterized by defective enzymes in the lysosomes of one or more types of cells in the body [95-97]. So far, round about 50 LSDs are known. Each single disease is very rare in the population, but taken together makes for a rate of 1 in 4000-8000 live births [97]. Approved treatment is only available for six of these diseases so far and relies on so-called enzyme replacement therapy (ERT), in which the patients are administered the defective enzyme *via* intravenous infusion. The unfavorable outcome of these disorders is commonly death during the first years. The effect of the altered metabolization process on the lysosomal pH is not yet understood. As mentioned already, capsules are trafficked to the lysosomes of the cells after uptake. This opens the possibility for applications of capsules as carriers for the introduction of biologically active cargo into the lysosomes and at the same time live-monitoring the dynamics of pH.

10.4.1.2. Fabry disease

One of the six diseases where treatment is available is Fabry disease. Patients with Fabry lack the enzyme α -galactosidase A (GLA). This enzyme is responsible for the degradation of globotriaosylceramide (Gb3). There are two drugs approved for treatment, Replagal® from Shire and Fabrazyme® from Genzyme. However, there is need for improvement of these treatments for several reasons. First, so far the defective enzyme is injected as solution into the blood stream, which means that the enzyme is unprotected against degradation. This degradation already can happen in the body outside the cells, but also inside the cells the enzyme can be degraded before it begins its work in the lysosome [98]. The advantage of capsules is therefore the protection of the drug against degradation in the body. Second, GLA enters the cells through receptor-mediated uptake by mannose-6-phosphate receptors (M6PR). These receptors, however, may not be expressed homogeneously in all relevant organs of the patient, leading to imbalanced distribution of GLA in the body and thus ineffective treatment of the patient [95]. This can be evaded by use of capsules as shell, as capsule uptake is independent of receptors (in case there is no specific ligand attached to the capsule surface). Third, many patients begin to express antibodies against GLA and treatment becomes ineffective or inefficient [95,99]. The mechanism behind the immune response is the production of antibodies binding to GLA which can influence, among others, receptor binding and subcellular trafficking, which means that the enzymes might not reach the lysosomes anymore [99].

10.4.1.2.1. Determination of GLA content of capsules by Western Blot

Determination of capsule content is, in general, a difficult task. Absorption spectroscopy, for example, does not work properly as the capsules themselves usually absorb and scatter light much stronger than the cargo itself. Measuring the content of all washing solutions and subtracting the value from the amount used for synthesis is also highly error-prone. As the cargo in this case is a protein, Western Blot can be used to determine the content of the capsules. The exact mechanism why the capsules break and release their content is not clarified yet and to the best of our knowledge, Western Blots with polyelectrolyte capsules to determine the loading level have not been published so far. As the polyelectrolytes of the capsules are not covalently bound to each other, the electric field might rupture the capsules and subsequently, the content could be released. In so far unpublished work the amount of GLA *per* capsule found was approximately 400 fg [100].

10.4.1.2.2. Intracellular effect of GLA and GLA capsules

GLA capsules were tested by Nazarenus *et al.* for their intracellular effectivity with mouse aorta endothelial cells (MAEC) from GLA knockout mice [100]. To determine the intracellular activity of the GLA capsules, the cells were co-incubated with the capsules and the GLA substrate 7-nitrobenzofurazan (NBD)-labeled Gb3 (NBD-Gb3). The substrate is fluorescent while the product after conversion by GLA is non-fluorescent. Therefore, the intracellular activity of the capsules or free GLA can be obtained from the fluorescence of NBD-Gb3 in the cells. Concerns that the substrate is converted already in the cell medium before uptake by the cells can be ruled out as the enzyme only works well at acidic pH and thus only when it has reached the late endosomes or lysosomes in the cells. The efficiency of the capsules compared with GLA was determined by the fluorescence loss of NBD-Gb3. In Figure 12, the fluorescence loss is plotted against the mass concentration of GLA, which was calculated from the amount of GLA determined by Western Blot [100]. The amount administered with capsules was higher than that of the GLA solution. The curves show that the GLA solution has a higher efficacy, since already with a concentration of 1 mg mL⁻¹ a fluorescence reduction of more than 90 % was obtained, while the capsules do not reach such a high level, but only about 80 %. Furthermore, the GLA concentrations needed in case of capsules are higher than those in case of the GLA solution.

Figure 12. Fluorescence reduction of NBD-Gb3 in MAEC as determined with flow cytometry. The plot depicts the loss of fluorescence, ΔI , as function of the administered concentration of GLA, either as solution (black curve) or as capsule suspension (red curve). Unpublished work [100].

10.4.1.3. Krabbe disease

Krabbe disease, also called globoid cell leukodystrophy, is an inherited neurological disorder. Knowledge about the disease is limited and so far no cure but only palliative and symptom-related treatment is available. Symptoms occur at the age of 3–6 months and involve fevers, limp stiffness, and decelerated mental and motor development among others. Later blindness and deafness can appear. The disease is triggered by a dysfunction of the oligodendrocytes of the patients. Oligodendrocytes are a type of brain cells, which provide the axons of neurons with an electrically insulating myelin sheath that also serves as scaffold. When the oligodendrocytes produce myelin, side products such as psychosine or other sphingolipids, and galactosylceramides are produced, which are metabolized by galactocerebrosidase (GALC). This enzyme is located in the lysosomes of the cells. In Krabbe disease, however, the oligodendrocytes lack this enzyme, and therefore, sphingolipids and galactosylceramides accumulate in the lysosomes. This accumulation leads to cell death, and subsequently, the axons are not provided their myelin sheath. The lack of insulation leads to slower signaling which can be measured in a nerve conduction study [101]. A simple model of Krabbe disease can be obtained from the M03.13 cell line [102,103]. These cells are immortalized oligodendrocytes. When incubated with psychosine

they internalize and store it in lysosomes. In a so far unpublished study, the wild-type (MO3.13 WT) was used as well as cells where the enzyme GALC was knocked out (MO3.13 KO) [100]. The knockout cells (KO) represent dysfunctional oligodendrocytes in the brain of Krabbe patients whereas the wild-type (WT) served as control.

10.4.1.3.1. pH sensing in MO3.13 oligodendrocytes

So far, there is not much knowledge about the influence of psychosine and other sphingolipids on the pH of oligodendrocytic cells and especially on cells lacking GALC. In so far unpublished work, SNARF® capsules were used to monitor the pH to obtain a time-resolved pH profile of the lysosomes during the accumulation of psychosine in both MO3.13 KO and MO3.13 WT cells [100]. In Figure 13, the color change of one capsule in a WT cell is shown. The two images were taken within a period of 20 min. The color changes from green to red, which shows that the local pH rose between those two images.

Figure 13. Example of a capsule where the color changes from green to red in a MO3.13 WT cell. The scale bar corresponds to 10 μm . Unpublished work [100].

In Figure 14, images of cells incubated with 2 μM psychosine are shown (a). The capsules appear in yellow due to the acidic environment of the lysosomes. Images of both WT and KO cells are depicted and “PSY” indicates cells incubated with psychosine whereas “Control” shows cells kept in psychosine- and serum-free media. After 20 h of incubation, the cells in all the four conformations appear healthy and no sign of apoptosis is visible. Images like these were evaluated to obtain the time-resolved pH profile as seen on the right. A low-pass frequency filter was applied to the curves to abolish short-term fluctuations and visualize the long-term trends more clearly. The blue lines represent the cells incubated with psychosine and the red lines the controls without psychosine. The graphs show that both the WT cells in (b) as well as the KO cells in (c) show small variations but no significant changes of pH during the presence of psychosine. Figure 15 contains images of MO3.13

cells incubated with 10 μM of psychosine at different points in time. The marking is similar to Figure 14. Cells incubated with psychosine show round shapes after 600 min, which is a sign of apoptosis. Both WT and KO cells show this behavior. In the KO cells, no significant color change is detectable, which is also true for both WT and KO controls. In the graphs, a significant difference between WT and KO cells is observable. While the KO cells in (b) do not show pH changes, the WT cells in a) show a clear rise in pH during the presence and accumulation of psychosine [100].

Figure 14. MO3.13 cells incubated with psychosine at a concentration of 2 μM . (a) Images of the points in time indicated at the top are shown. “PSY” indicates the panels with cells incubated with psychosine, whereas “Control” indicates the cells in medium not supplemented with psychosine. From the images it can be seen that neither the WT nor the KO cells suffer from 2 μM psychosine. SNARF[®] capsules in all images are yellow indicating the acidic pH environment of the lysosomes. The scale bar represents 10 μm . (b) MO3.13 cells treated with 2 μM psychosine and controls without treatment. MO3.13 WT cells (b) as well as MO3.13 KO (c) cells show only small variations but no eminent change of lysosomal pH during incubation with 2 μM of psychosine. Unpublished work [100].

Figure 15. MO3.13 cells incubated with psychosine at a concentration of 10 μ M. (a) Images of the points in time indicated at the top are shown. “PSY” indicates the panels with cells incubated with psychosine, whereas “Control” indicates the cells in medium not supplemented with psychosine. From the images it can be seen that both the WT and the KO cells suffer from 10 μ M psychosine as cells become round, which is a sign of apoptosis. SNARF® capsules in all images are yellow indicating the acidic pH environment of the lysosomes. The scale bar represents 10 μ m. MO3.13 WT cells show increase of the lysosomal pH during treatment with 10 μ M psychosine, whereas the control cells only show small variations (b). In contrary, MO3.13 KO cells (c) only show small variations of the lysosomal pH whether treated with psychosine or not. Unpublished work [100].

10.4.1.3.2. Delivery of galactocerebrosidase to MO3.13 oligodendrocytes

So far, there is no drug to cure Krabbe disease. Other LSDs like Fabry or Gaucher can be treated by enzyme replacement therapy where the missing enzyme is delivered to the patients by intravenous infusion. Here, the idea was to introduce the lacking enzyme GALC into the cells by biodegradable capsules and scrutinize if the delivered enzyme could reduce the toxic effects of psychosine. As seen from the images in Figure 15, cells of the wild type as well as KO cells treated with 10 μ M of psychosine show signs of apoptosis after longer incubation times, *i.e.* the cells appear spherical [100]. This was

quantified further by MTT cytotoxicity assays [100]. 5 or 10 GALC capsules, and in case of KO cells also 10 empty capsules *per* seeded cell were added to the cells together with the serum-free medium. Control cells without capsules were treated similarly. Afterwards, psychosine was added in serum-free medium at different concentrations from 0 to 20 μM . The results are found in Figure 16. The blue and black curves show the cells treated with capsules, the red curves the cells without capsules. The 100 % value was always assigned to cells without capsules and without psychosine, *i.e.* the 0 μM value of the red curves. In KO cells, the red curves show the same trend in both diagrams, and the black curve in (b), which represents empty capsules, shows the same behavior. KO cells incubated with GALC-containing capsules show high viability both with 5 and with 10 GALC capsules *per* seeded cell. The viability, however, is higher for cells treated with 5 capsules. With 10 capsules, the cells show a small loss in viability with rising psychosine concentration and generally lower viability. In WT cells, a contradictory behavior is observed, as shown in (c). The untreated cells show high viability independent of the concentration of psychosine, whereas cells incubated with capsules show clearly reduced viability, which means that the capsules have an adverse effect on the WT cells.

Figure 16. Viability test based on the chemical MTT (MTT assays) of MO3.13 KO cells. The viability, V , after 24 h is plotted *versus* the concentration of psychosine. The red curves represent cells without capsules whereas the black curves represent cells treated with (a) 5 GALC capsules *per* cell, (b) 10 GALC capsules *per* cell, and (c) 10 empty capsules *per* cell. The error bars represent standard deviations. Unpublished work [100].

10.5 CONCLUSION

Knowledge of intracellular ion distributions and concentrations is of high value for the diagnosis of diseases. Especially pH plays a prominent role. In general, the analysis of the entire intracellular ion milieu is of relevance for the health condition of cells. However, the intracellular measurements of other ions such as sodium, potassium, chloride, calcium, or magnesium, which are the most important ions for the cells, are problematic. The available ion-sensitive dyes usually are not only sensitive to the specific ion of interest but also show sensitivity to other ions and crosstalk with pH. Therefore, pH is a very good parameter for diagnostic monitoring. The capsules thereby are a good choice of diagnostic tool as they are taken up by many different cell types, they are non-toxic, they allow non-invasive readout, and their intracellular stability allows for long-term monitoring. In the cited literature the usefulness of different sorts of capsules for therapeutic purposes was demonstrated. Regarding gene therapy, the delivery of RNA by biodegradable or by light-responsive capsules results in successful gene knockdown or gene expression, respectively. In case of biodegradable capsules, integrated PEI facilitates lysosomal escape due to the proton sponge effect. siRNA-filled biodegradable capsules have been demonstrated to enhance transfection efficacy compared to polyplexes as observable from the shorter transfection time of 20 h compared to 48 h in standard transfection protocols. Furthermore, the delivery can be performed in serum-supplemented medium. This is a big advantage over standard protocols, as deprivation of serum results in stress for the cells. This feature is especially important for *in vivo* experiments, where the serum levels cannot be regulated externally. In case of light-responsive capsules, the advantage of a distinct beginning time of transfection was shown. It is possible to open capsules with different cargos sequentially in the desired order and therefore study the interactions of various cargos in a time-resolved way. Another form of therapy addressable by capsules is enzyme replacement therapy. The introduction of proteins both by biodegradable and by light-responsive capsules is possible. In case of the biodegradable capsules, one can make use of the natural uptake route of capsules terminating in the lysosomes of the cells. Models of lysosomal storage disorders were used to demonstrate the successful delivery of deficient enzymes to the respective cells. The functionality of the delivered enzymes was shown in models for Krabbe and Fabry disease. In case of the light-responsive capsules, the successful introduction into the cells was demonstrated with the phosphatase-mediated reaction of ELF-97 phosphate to ELF-97-alcohol, as well as the release of GFP from the capsules into the cytosol.

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A NEW PERSPECTIVE TO LIPID NANOPARTICLES FOR ORAL DRUG DELIVERY

**Neslihan Üstündağ Okur^{1*}, Mehmet Evren Okur²,
and Evren Gündoğdu³**

¹ Department of Pharmaceutical Technology, School of Pharmacy,
University of Istanbul Medipol, 34083 Istanbul, Turkey

² Department of Pharmacology, School of Pharmacy, University of
Anadolu, 26470, Eskisehir, Turkey

³ Department of Radiopharmacy, School of Pharmacy, University of Ege,
35100 Bornova, Izmir, Turkey

*Corresponding author: neslihanustundag@yahoo.com

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11.1. INTRODUCTION

Oral administration is the most commonly used and readily accepted route when compared the numerous drug delivery routes. Oral administration of drugs still remains the favored route for majority of clinical applications, due to the exceptional accessibility, and patient compliance [1]. In addition, most oral drug delivery systems such as tablets can be manufactured for comparatively and low costs because do not require sterile conditions [2-4]. Despite these advantages, the oral bioavailability of drugs is severely limited and oral formulations face several common problems, especially for peptides and proteins: (i) low stability in the gastric tract, (ii) low solubility and/or bioavailability and (iii) poor permeability across intestinal biological membranes, the mucus barrier can prevent drug penetration and subsequent absorption [5,6].

Nanoparticles, a sole subgroup of wide area of nanotechnology and they are sized between 1 and 200 nanometers. Nanoparticles may or may not display size-related properties that vary significantly from particles or bulk materials and atomic or molecular structures [7]. Lipid nanoparticles, including solid lipid nanoparticles (SLNs) and nanostructured lipid carriers (NLCs), are colloidal carriers with a lipid matrix. They are generally composed of lipids, surfactants and cosurfactant [8]. Lipid nanoparticles have been reported as an alternative drug delivery system to polymeric nanoparticles [9]. The lipid matrix is made from physiologically tolerated lipid components, which decreases the potential for acute and chronic toxicity [10].

Lipid nanoparticles are extensively employed as oral drug delivery systems, are being developed that encapsulate and protect drugs and release them in a temporally or spatially controlled manner. The nanoparticle surface can also be modified to enhance or decrease bioadhesion to target specific cells [6]. Nanoparticles are considered as alternatives to various conventional drug delivery techniques and often used to improve the oral bioavailability of drugs [7]. They can ameliorate the demerit of conventional dosage forms by prolonging the drug release, improving the drug solubility, minimizing side-effects, keeping the drug activity, and targeting [11].

11.1.1. The anatomy of gastrointestinal tract

To understand oral absorption of drugs the anatomy of gastrointestinal tract should be known. The anatomy of the mammalian gastrointestinal tract presents a host of impediments to the uptake of nanoparticles delivered *via* the oral route, including low regional pH, a protective mucous layer, and digestive enzymes specifically designed to break down ingested proteins [12]. The digestive system contains the gastrointestinal tract and the auxiliary organs of digestion including the salivary glands, liver, gall-bladder, and pancreas. The

gastrointestinal tract, which extracts nutrients, electrolytes, minerals, and water, is tend to injury as a result of oral drug administration [13]. The digestive system functions to ingest and digest foods, absorb necessary nutrients (proteins, carbohydrates, vitamins and minerals), and eliminate waste. The gastrointestinal tract also has a critical role in immune observation *via* gut-associated lymphoid tissue found throughout the tract [14]. Stomach serves the most mainly mixing part and a reservoir that secretes pepsinogen, gastric lipase, hydrochloric acid, and the intrinsic factor. This section of the gastrointestinal tract is normally impervious for absorption of most ingredients into the blood except water, ions, alcohol, and certain drugs [15,16]. Gastrointestinal enzymes, which contribute for presystemic metabolism of a drug, are categorized as luminal enzymes, gut wall/mucosal enzymes, bacterial enzymes. Luminal enzymes are the enzymes present in the gut fluids and contain enzymes from pancreatic and intestinal secretions. Gut wall/mucosal enzymes are primarily present in the stomach, intestine and colon [17].

Any foreign molecule that is absorbed from the gastrointestinal lumen goes across gastrointestinal mucosa, capillary beds of small and large intestine, liver *via* portal circulation and is then transported to the rest of the body organs. Only exception to this is the molecules absorbed into the lymphatic system or distal rectum which efficiently bypass the liver [17].

The anatomical and physiological parameters of the gastrointestinal tract strangely affect the rate and concentration of drug absorptions. There are some problems in formulating controlled release systems to be considered for better absorption and enhanced bioavailability of drugs embedded in the orally administered dosage forms. One major obligatory for the successful performance of orally administered drugs is that the drug should have good absorption throughout the gastrointestinal tract, to certify continuous absorption of the released drug [18].

A drug that is administered orally must survive transit through gastrointestinal liquids, cross the mucus layer and the epithelium before being absorbed. If most small molecules are resistant to the environment of the gastrointestinal tract and can be absorbed, the intestinal barrier limits the oral absorption of macromolecules. Hereafter, protective vehicles to avoid destruction in the gastrointestinal tract and potentially enhance oral absorption are preferred [4]. It is clear from the recent scientific literatures that an increased interest among the academic and industrial research groups still exists in formulating novel dosage forms that are retained in the stomach for a prolonged and predictable period of time [1]. For this purpose new perspective is considered for lipid nanoparticles.

11.1.2. Gastrointestinal absorption

Absorption is a vital process bridging digestive system and human life. This process takes place right from the mouth to the stomach, small intestine and lastly colon [19]. Gastrointestinal tract displays site specific absorption based on the nature of drugs and regional differences such as pH, enzyme activity, mucosa thickness, retention time and surface area [20,21]. The pH of gastrointestinal tract varies from 1–7, with stomach pH between 1–3, duodenum pH between 6.0–6.5, and large intestine pH from 5.5–7.0 [20,22].

In general, the gastrointestinal absorption of macromolecules and particulate materials involves either paracellular route or endocytotic pathway [23]. The paracellular route of absorption of nanoparticles employs less than 1 % of mucosal surface area. Endocytotic pathway for absorption of nanoparticles is either by receptor-mediated endocytosis, which is, active targeting, or adsorptive endocytosis that does not need any ligands. This process is initiated by an unspecific physical adsorption of material to the cell surface by electrostatic forces such as hydrogen bonding or hydrophobic interactions [24].

Orally administered drugs must pass through the intestinal wall and then through the portal circulation to the liver; both are common sites of first pass metabolism (metabolism of a drug before it reaches systemic circulation). Thus, many drugs may be metabolized before adequate plasma concentrations are reached resulting in poor bioavailability [25]. Moreover permeability of drugs across the gastrointestinal membrane is one of rate limiting step in the absorption of drugs. The solubility and permeability of drugs together determine extent of oral absorption. The physicochemical factors such as log P, molecular weight, polar surface area, charge/ionization, number of hydrogen bond donors and acceptors determine permeability of molecule [26,27].

Liibenberg *et al.* were developed azithromycin nanoparticles to use perorally for AIDS therapy. The area under the curve (AUC) in the organs that are mainly infected by the human immunodeficiency virus (HIV), namely the reticuloendothelial cell containing organs, the blood, and the brain was increased [28]. In another study, SLNs of cryptotanshinone, a poorly water-soluble drug, showed twofold improved oral bioavailability than free drug due to increased solubilization capacity and change in metabolism behavior [29].

11.1.3. Transport mechanisms in the gastrointestinal tract and targeted drug delivery

There is one or more transport mechanisms of drugs is absorbed from gastrointestinal tract [7,30] (Figure 1). To cross the cell membrane there are four different mechanisms: *via* paracellular, transcellular, carrier-mediated, and receptor-mediated transport [31]. Gastrointestinal absorption is dependent on different physical characteristics, such as molecular weight, hydrophobicity, ionization constants, and pH stability of absorbing molecules

[31]. Due to lipid components and the surfactants containing ester groups from lipid nanoparticles is all the substrates of the hydrolysis reaction by pancreatic lipase, it is rational to deduce that lipid digestion process is inevitable *in vivo*, which may influence drug absorption and bioavailability [32].

Figure 1. A schematic demonstration of absorption pathways 1) transcellular endocytosis; 2) paracellular transport and transcellular transport of drugs across the epithelial cells of the gastrointestinal tract into the systemic circulation; 3) transcellular passive diffusion; 4) carrier-mediated transport processes

To improve the oral bioavailability of nanoparticles has available many advantages. Lymphatic delivery is helpful not only for absorption of poorly soluble drugs but also for targeting drug carriers to the lymphatics. Moreover, lymphatic delivery of nanoparticles avoids the first-pass effect, and increases drug concentration in the plasma [7,33].

Oral nanoparticles were found to be taken up by the gut in a lot of study. There are certain mechanisms suggested to be responsible for the peroral uptake the major site for the uptake of nanoparticles seems to be the M-cells of the Peyer's patches. Up to 60 % of the uptake is accounted by these cells of the gut [28,34].

Li *et al.* showed enhanced oral absorption of SLNs through different segments of the gastrointestinal tract. To confirm mechanistic absorption of SLNs, quarcetin-loaded SLNs were administered by oral route in rats and their pattern of absorption observed in both stomach and intestine. The results indicated that quarcetin-loaded SLNs could be absorbed in all gastrointestinal tract segments with different percentage and pattern of absorption [7,35].

Hydrophilic molecules such as PEGs, carbohydrates are useful for the stabilization and diffusion of the particles in the mucus, whereas a hydrophobic coating enhances cellular and lymphatic uptake. Thiols, ammoniums and lipophilic carbon chains reinforce adhesiveness and thus

residency time by forming disulfide bonds, electrostatic interactions and hydrophobic interactions, respectively. Moreover, the presence of acidic or functional groups leads to pH-dependent activities that are useful for targeting different parts of the gastrointestinal tract [36].

Chitosan is a polymer that is very commonly used in oral nanoparticles. Mechanism of chitosan nanoparticles transport across gastrointestinal tract is most probably through adsorptive endocytosis. Electrostatic interaction between positively charged chitosan and negatively charged sialic acid of mucin causes association of chitosan nanoparticles to the mucus layer and then internalization *via* endocytosis [37-39]. Behrens *et al.* indicated that chitosan nanoparticles were found to be higher in the jejunum and ileum than in duodenum [40].

11.1.4. Lipid nanoparticles

Nanoparticles are smaller than 1 micron and possibly as small as atomic and molecular length scales (~ 0.2 nm) [24]. Nanoparticles can have amorphous or crystalline form and their surfaces can act as carriers for liquid droplets or gases. They are commonly classified based on their dimensionality, morphology, composition, uniformity, and agglomeration [41]. During the last years, different materials have been entrapped into lipid nanoparticles, extending from lipophilic and hydrophilic molecules, including labile compounds, such as proteins and peptides [42-44].

11.1.4.1. Solid lipid nanoparticles (SLNs)

SLNs were developed at the beginning of the 1990s as an alternative carrier system to liposomes, emulsions and polymeric nanoparticles as a colloidal system for controlled drug delivery. SLNs consist of a solid lipid, where the drug is normally incorporated, with an average diameter below 1 μm [7,45]. The common excipients used in SLN formulation are solid lipids, emulsifiers, co-emulsifiers and water [46]. They display major advantages such as controlled release, improved bioavailability, protection of chemically labile molecules, cost effective excipients, enhanced drug incorporation and extensive application range [47]. Per oral administration forms of SLN may include aqueous dispersions or SLN loaded traditional dosage forms, *e.g.* tablets, pellets or capsules [46].

11.1.4.2. Nanostructured lipid carriers (NLCs)

NLCs are drug delivery systems composed of both solid and liquid lipids as a core matrix. NLCs disclose some advantages for drug therapy over conventional carriers, including increased solubility, the ability to enhance storage stability, improved permeability and bioavailability, reduced adverse effect, prolonged half-life, and tissue-targeted delivery [47].

11.1.4.3. Preparation of lipid nanoparticles

SLNs and NLCs can be produced by different formulation techniques providing reasonably high drug encapsulation efficiency. Furthermore, scaling up of most production processes can be easily succeeded [48]. SLNs are produced by replacing the liquid lipid (oil) of an oil/water emulsion by a solid or a blend of solid lipids, *i.e.* the lipid particle matrix being solid at both room and body temperature [49]. In the NLCs, the particles are produced using blends of solid lipids and oils. To obtain the blends for the particle matrix, solid lipids are mixed with liquid lipids. Due to the oil in these mixtures a melting point depression compared to the pure solid lipid observed, but the blends obtained are also solid at body temperature [50]. Selection of suitable lipids is essential prior to their use in preparation of lipid nanoparticles. The lipid is the main ingredient of lipid nanoparticles that influence their drug loading ability, their stability and sustained release performance of the formulations. Lipid nanoparticle dispersions based on a variety of lipid materials including fatty acids, glycerides and waxes have been investigated [8,51].

11.1.4.4. Toxicological effects of lipid nanoparticles

Paralleling the development of nanoparticles, a field known as nanotoxicology has also occurred. Nanotoxicology refers to the study of the potential negative impact of the interactions between nanomaterials and biological systems [52,53].

Nanoparticles can be prepared from a variety of materials such as proteins, polysaccharides and synthetic polymers. The selection of matrix materials is dependent on many factors (a) size of nanoparticles; (b) inherent properties of the drug, *e.g.*, aqueous solubility and stability; (c) surface characteristics such as charge and permeability; (d) degree of biodegradability, biocompatibility and toxicity; (e) drug release profile desired; and (f) antigenicity of the final product [24].

A strong advantage of the use of lipid particles as drug carrier systems is the fact that the matrix is composed of physiological components, that is, excipients with generally recognized as safe (GRAS) status for oral application, which reduce the cytotoxicity [8,42].

In a previous study, SLNs prepared up to concentrations of 2.5 % lipid do not exhibit any cytotoxic effects *in vitro* [54]. Even concentrations higher than 10 % of lipid have been shown a viability of 80 % in culture of human granulocytes [42]. Silva *et al.* evaluated the toxicity of risperidone loaded SLNs with *Caco-2* cells. The results suggest that SLNs evaluated are biocompatible with *Caco-2* cells and well tolerated by the gastrointestinal tract [55].

11.1.5. Effect of characteristics of lipid nanoparticles on oral drug delivery

Nanoparticle characterization is necessary to establish understanding and control of nanoparticle synthesis and applications. It is confirmed that drug encapsulated in nanoparticles have better absorption through gastrointestinal tract as compared to their native counterpart. The factors affecting uptake include the particle size of nanoparticles and their release characteristics [56].

11.1.5.1. Particle size and release characteristics

Particle size and size distribution are the most important characteristics of nanoparticles. They affect the *in vivo* distribution, toxicity, drug loading, drug release and stability of nanoparticles [24]. In addition, particle size plays a key role in particle functions, such as degradation, vascular dynamics, targeting, mechanisms of clearance and uptake [57]. Particles have been shown to have different speeds, diffusion characteristics and adhesion properties, depending on their particle size [58-61].

The poor bioavailability of orally administered drugs can have mainly two reasons: low dissolution speed (i); poor permeability (ii). There is a very simple traditional approach to raise the dissolution speed by enlarging the surface, *i.e.* micronisation. The particle size of normally sized drug powders (in the range 20–100 μm) is reduced to a size in a range of approximately 1–10 μm , the mean diameter being typically in the range somewhere between 2 and 5 μm . Nanoparticles possess sizes of approximately 10–1000 nm; most manufacturing methods yield a main diameter somewhere between 200 and 400 nm [62]. Furthermore limiting nano-sized particles to less than 500 nm in diameter seems to be a key factor in permitting their transport through the intestinal mucosa most probably through an endocytosis mechanism [63]. In addition nanoparticles explain the size-dependent absorption mechanism based on mucoadhesion, *in vivo* drug release, cellular uptake, and transport across the intestinal epithelium involved in the oral absorption progression [64,65].

Lipase/colipase activity is affecting the drug release from lipid nanoparticles in the gastrointestinal tract. In a pre-step of the absorption, food lipids become degraded by the lipase/colipase complex. To evaluate degradation of lipid and surfactant of the lipid nanoparticles degradation study was performed with pancreas lipase/colipase complex [42].

11.1.6. Stability of lipid nanoparticles after oral administration

Matrix encapsulation nature of lipid nanoparticles can protect drugs from adverse conditions encountered in the gastrointestinal tract. Metabolism of drugs especially by hydrolysis in gastrointestinal tract or in plasma can be controlled by encapsulating them in nanoparticles [19]. Nanoparticles promising an intimate contact with the intestinal mucosa are also beneficial in

order to keep incorporated drugs towards a presystemic metabolism. Due to the close contact with the mucosa, drug degradation on the way between the delivery system and the absorption membrane is reduced to a minimum [48].

The microclimate of the stomach favors particle aggregation due to the acidity and high ionic strength. It can be expected, that food will have a large impact on nanoparticle performance [46]. For better stability of nanoparticles must be comprehensively tested due to pH changes and ionic strength as well as the drug release upon enzymatic degradation [42,66]. The idea that nanoparticles might protect labile drugs from enzymatic degradation in the gastrointestinal tract leads to the development of nanoparticles as oral drug delivery systems [67]. In addition protective effect of nanoparticles coupled with their sustained/controlled release properties prevents drugs/macromolecules from premature degradation and improves their stability in gastrointestinal tract [19].

Lipids containing a mixture of wax and glycerides may increase stability of nanoparticles than the isolated constituents due to appropriate amalgamation of different properties. Reports suggest that numerous properties of lipids such as lipid crystallization speed, recrystallization index and self-emulsification affect particle size and stability, which are major worries for the oral administration. A longer chain lipid with charge modification improves absorption and stability of nanoparticles in gastrointestinal tract [19].

11.1.7. Strategies for oral drug delivery with lipid nanoparticles

The lipid nanoparticles undergo digestion similarly to food lipids. Lipid nanoparticles show a high specific surface area for enzymatic attack by intestinal lipases [68]. This enzymatic degradation of the lipids leads to release of loaded drugs. The bile salts help their solubilization in the intestine and successive absorption [50]. Transport of drugs through the intestinal lymphatics *via* the thoracic lymph duct to the systemic circulation, avoids presystemic hepatic metabolism and then improves bioavailability of drugs [25].

Damge *et al.* reported that blood glucose levels were decreased at diabetic rats with oral insulin nanoparticles [69]. In another study, docetaxel loaded SLNs surface-modified with Tween 80 or d-alpha-tocopheryl polyethylene glycol succinate evaluated in terms of their feasibility as oral delivery systems. A sustained-release profile of docetaxel from the SLNs was shown. Tween 80-emulsified SLNs showed enhanced intestinal absorption, lymphatic uptake, and relative oral bioavailability of docetaxel compared with taxotere in rats. These results may be attributable to the absorption-enhancing effects of the tristearin nanoparticle. Moreover, compared with Tween 80-emulsified SLNs, the intestinal absorption and relative oral bioavailability of docetaxel in rats were further improved in d-alpha-tocopheryl polyethylene glycol succinate-

-emulsified SLNs, It is noteworthy that these surface-modified SLNs may help as effective oral delivery systems [70].

Jain *et al.* developed oral insulin nanoparticles, they were able to achieve a bioavailability of up to 20 % relative to injection. This enhanced bioavailability makes the delivery of insulin more possible [71].

Furthermore, when permeation enhancing polymers such as chitosans [72], carbomers [73] or thiomers [74] are used to prepare the lipid nanoparticles, the permeation enhancing property of these polymers seems to be improved. Albrecht *et al.* prepared diethylenetriaminepentaacetic acid gadolinium (III) dihydrogen salt loaded nanoparticles and applied orally to rats without any uptake in the systemic circulation. Due to the application of thiolated polyacrylate the condition did not change. However, when the thiolated polyacrylate was prepared with nanoparticles by the addition of poly(vinylpyrrolidone) a systemic uptake of diethylenetriaminepentaacetic acid gadolinium (III) dihydrogen salt was determined [75].

Chitosan, a cationic polymer, is widely used as a safe and effectual intestinal absorption enhancer, owing to its natural mucoadhesive property and ability to moderate the integrity of epithelial tight junctions reversibly [76]. Mucoadhesion property of chitosan can locally increase the concentration of a drug and thus raise the driving force for drug diffusion into cells, which may be useful even if the nanoparticles themselves stay trapped extracellularly in mucus [77]. In a study incubation of *Caco-2* cells with chitosan-insulin loaded nanoparticles lead to in greater cell binding and uptake compared with a solution of chitosan-insulin [78]. In addition chitosan nanoparticles could also reduce the transendothelial electrical resistance (TEER) of the cell monolayers at pH 5.3 and 6.1 [79]. Moreover *in vitro* studies in *Caco-2* cells have shown that chitosan is able to induce an opening of tight junctions thus increasing membrane permeability especially peptides and proteins [80].

11.1.8. Pharmacokinetic evaluations of oral lipid nanoparticles

Pharmacokinetic behaviour of drug loaded lipid nanoparticles need to distinguish if the drug is present as the released free form or as the related form with lipid nanoparticles [81]. However, it is very difficult to design of pharmaceutical formulations for the poor aqueous solubility of drugs and leads to variable bioavailability [82,42].

Orally administered fenofibrate presented absorption ranging from 30 to 90 % depending on gastrointestinal tract condition however; plasma level of fenofibric acid was considerably low due to rapid excretion and/or little accumulation in fat tissue. Therefore, Hanafy *et al.* developed fenofibrate loaded SLNs, which consequently reduced change of fenofibrate into fenofibric acid. Remarkably, fenofibrate loaded SLNs showed steady oral absorption up to 8 h with a stable release profile of fenofibric acid from SLNs, resulting in twofold around rise in bioavailability [19,83]. In another study, Cyclosporine A

loaded SLN and Cyclosporine A solution were applied orally and were determined the plasma levels and body distribution. The incorporation into SLN protected Cyclosporine A from hydrolysis. It was found that SLN could increase bioavailability and prolonged plasma levels after per oral administration of cyclosporine containing lipid nanodispersions to animals [84]. In another study, relative bioavailability of Lopinavir from nanoparticles was 2 folds higher than the free drug [85].

Tocotrienol loaded NLCs were developed and found to be about 2-fold more effective as compared to free tocotrienol [86]. Additionally, etoposide loaded NLCs have also been applied for oral delivery and approximately 3.5-fold rise in the oral bioavailability was found as compared to free drug [87,88]. Zhang *et al.* were developed tiptolide loaded NLC and SLNs. Tiptolide loaded NLCs showed a better *in vitro* sustained release profile compared to tiptolide loaded SLNs. Moreover, tiptolide loaded NLCs extended mean residence time, delayed Tmax and reduced Cmax compared to free tiptolide and tiptolide loaded SLNs, respectively [89].

11.2 CONCLUSIONS

Effective oral drug administration is desirable but challenging owing to the nature of the gastrointestinal tract. Lipid nanoparticles are widely used to improve oral bioavailability and to achieve sustained release and to overcome hepatic first-pass metabolism effect. Oral bioavailability can be enhanced by incorporating the drugs into lipid nanoparticles possessing a solid matrix (SLN or NLC). In addition lipid nanoparticles are promising for oral and peroral administration route for drugs, proteins, and peptides. Nanoparticles can be extensively used as carriers for oral delivery, particularly for drugs having poor bioavailability. They can effectively overcome the various problems associated with oral delivery of drugs that suffers from low solubility and poor permeability, are unstable in the gastrointestinal tract and undergo extensive first pass metabolism.

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A GENERAL OVERVIEW OF THE NANO-SIZED CARRIERS FOR CANCER TREATMENT

Jeyshka M. Reyes-González^{1,2}, Frances M. Pietri-Vázquez³, and Pablo E. Vivas-Mejía^{1,2*}

¹ Department of Biochemistry, University of Puerto Rico, Medical Sciences Campus, San Juan, Puerto Rico 00935, USA

² Comprehensive Cancer Center, University of Puerto Rico, Medical Sciences Campus, San Juan, Puerto Rico 00935, USA

³ Medical School, University of Puerto Rico, Medical Sciences Campus, San Juan, Puerto Rico 00935, USA

*Corresponding author: pablo.vivas@upr.edu

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12.1. INTRODUCTION

Nanotechnology consists in the manipulation, creation, and use of substances in the nanometer scale for the fabrication of nanomaterials and nanodevices [1]. Nanotechnology has impacted diverse fields of research, including environmental science, cosmetics, the food industry, and medicine [2-5]. The major application of nanotechnology to medicine involves the creation of nanoparticles as delivery carriers for chemotherapeutic agents to prolong drug action and decrease side effects [1,2]. Although several nanomaterials have been proposed as drug carriers, nanoliposome-encapsulated drugs are the most used clinically [6]. In fact, several classic chemotherapeutic agents, including doxorubicin, daunorubicin, cytarabine, and vincristine, are now clinically used as nanoliposomal formulations [7-11]. The use of nanoliposomes for the delivery of emerging cancer therapies such as small interference RNA (siRNA) and microRNA (miRNA)-based molecules is also an exciting research field [6,12-14]. In this chapter, we will discuss the status of the nanoparticles currently used for cancer treatment and diagnosis. We place special emphasis on the use of nanoliposomes as drug carriers for novel cancer therapies. Finally, we discuss the major challenges of using nanoparticles as drug delivery systems for effective cancer treatment.

12.2. NANOTECHNOLOGY AND ITS DIVERSE APPLICATIONS

Nanotechnology is a novel field of research that develops materials and devices in a scale of 1–100 nm. Having access to the nanoscale and the ability to develop nanomaterials has advanced various areas of research, including cosmetics, the food industry, environment-related sciences, and medicine.

In the food industry, nanotechnology has led to several advances in areas such as food packaging and food conservation. One example is the incorporation of silver nanoparticles (AgNPs) into packaging materials such as plastics, taking advantage of the antibacterial properties of silver [15]. AgNPs have also been integrated into a hydroxypropyl methylcellulose (HPMC) matrix, yielding increased tensile strength and barrier properties, and subsequently resulting in prolonged product quality and stability [16]. Nanotechnology has also been applied to assure greater stability, durability, bioavailability, and controlled release of bioactive food ingredients [17]. For example, zein – a storage protein found mainly in maize – is used to entrap, transport, and protect omega-3 polyunsaturated fatty acids such as docosahexaenoic acid (DHA), which has protective cardiovascular properties [17]. Zein has also been used to formulate

curcumin-containing nanoparticles for the potential treatment of several illnesses, including cancer and Alzheimer's [18].

In the cosmetic industry, nanotechnology has been applied to many modern cosmetic products, such as sunscreen. For example, a study showed that using the clay montmorillonite (MMT) as a carrier of titanium-dioxide (TiO_2) – the major component of sunscreen products – yields a nanocomposite that enhances UV protection, and concomitantly increases the photostability and efficacy of sunscreen [19]. Also, nano-delivery systems that allow effective transport of compounds into the epidermis and deeper skin layers while keeping the biological properties of skin (such as moisture and squamous cell integrity) is an important area of research, especially for the ongoing development of topical creams and treatments. A recent study compared the use of three different nanocarriers – liposomes, solid lipid nanoparticles (SLNs), and nanoemulsions (NEs) – for the transport of retinoids (vitamin A derivatives used to treat skin conditions such as acne) [20]. The use of these nanocarriers increased the stability and reduced the irritating secondary effects commonly observed in vitamin A derivatives. Among the three nanocarriers, liposomes and NEs showed the highest skin permeation. Moreover, the biocompatible properties of liposomes resulted in higher retention in the deeper skin layers, no tissue irritation, and increased skin hydration [20].

Nanotechnology extends even further to environmental applications, particularly in water treatment. The application of nanostructure fibers like polyaniline as a coating for solid phase microextraction (SPME) of organochlorine pesticides in contaminated water samples has shown high extraction efficiency [21]. Another example is the use of TiO_2 nanostructures as semiconductor photocatalysts for efficient degradation of organic pollutants (dyes) in contaminated water [22]. The multiple applications of TiO_2 -based nanotechnology have been extensively reviewed elsewhere [23-25].

12.3. NANOPARTICLES AND CANCER MEDICINE

One of the most promising areas of nanotechnology is its application to cancer diagnosis and treatment. Cancer is a group of diseases that figure among the leading causes of death worldwide, with approximately 14 million new cases and 8.2 million deaths per year. Unfortunately, currently available chemotherapeutic agents have the disadvantage of affecting not only tumor cells but also normal cells, and they produce diverse secondary effects such as cardiotoxicity, cytotoxicity, and neurotoxicity [1,26,27]. In the past decade, nanoparticles have been successfully introduced in cancer medicine to overcome the adverse effects associated with the currently used chemotherapeutic agents. The use of nanoparticles as drug carriers has improved treatment efficacy, increased drug stability, and reduced the toxicity

of classic chemotherapeutic drugs [1,7]. In fact, several commonly used anticancer agents are administered now as nanoliposomal formulations [7-11]. Theoretically, nanoparticles are effective as delivery systems because they can be engineered to be more soluble and have reduced renal clearance and a longer half-life ($t_{1/2}$) [28]. Furthermore, nanoparticles have shown improved bioavailability due to their enhanced infiltration, greater retention, and high specificity at target disease tissues, which also serves as a protective mechanism for the surrounding healthy tissue [29].

12.3.1. Common nanoparticles with potential use for cancer treatment and diagnosis

Nanoparticles as drug and imaging systems provide unique approaches for cancer treatment and diagnosis. Different nanoparticle systems like inorganic and organic nanoparticles have been developed as nanocarriers of molecular cargos such as small molecules, oligonucleotides, peptides, proteins, genes, and imaging agents. Inorganic nanoparticles are primarily metal-based and include semiconductors (quantum dots), silica-based nanoparticles, metallic nanoparticles, magnetic nanoparticles, and fullerene nanoparticles (carbon nanotubes). On the other hand, organic nanoparticles, which range in size from 10 nm to 1 μ m, mainly include polymer-based nanoparticles (such as polymeric nanoparticles, micelles, and dendrimers) and liposomes.

12.3.1.1. Inorganic nanoparticles

12.3.1.1.1. Quantum dots

Quantum dots (2–10 nm) are fluorescent spherical nanoparticles composed of an elemental core encompassed by a metal shell [30]. They are mostly used for biomarker screening and medical imaging. Quantum dots have great advantages over organic dyes and allow tracking activities over a period of time [31]. For example, herceptin-conjugated CdSe/ZnS quantum dots (QD-Her) were shown by fluorescence microscopy to have selective internalization into target breast cancer cells through membrane receptors. Following internalization, the release of herceptin to the cytoplasm resulted in the induction of cell death [32]. Unfortunately, quantum dots are synthesized from heavy metals, and therefore their use is limited due to toxicity concerns [33,34].

12.3.1.1.2. Silica-based nanoparticles

Silica-based nanoparticles include mesoporous and core shell silica nanoparticles [35]. Mesoporous silica nanoparticles (MSNs) form a complex network of channels through the interior, whereas core shell silica nanoparticles contain surface pores leading to a central cavity. The distribution and size of the pores determine the kinetics of drug release [36,37]. For

example, Gao *et al.* used doxorubicin-loaded hollow MSNs with three different pore sizes and found that nanoparticles with a larger pore size exhibited higher cellular uptake as well as faster intracellular drug release [38]. A pore size-dependent anticancer activity against multidrug-resistant (MDR) breast cancer cells was observed [38]. Tanaka *et al.* showed that incorporation of siRNA against the EphA2 oncoprotein into multistage MSNs with neutral nanoliposomes resulted in sustained gene silencing in mouse models of ovarian cancer [39]. A reduction in tumor burden and decreased angiogenesis and cell proliferation were observed [39]. No observable toxicity associated with the nanoparticles, nanoliposomes, or siRNA was detected [39].

12.3.1.1.3. Metal-based nanoparticles

Metal-based nanoparticles, particularly gold, silver, and platinum, have also been investigated as drug delivery systems [40,41]. For example, Qian *et al.* used gold nanoparticles (AuNPs) conjugated with cetuximab [a monoclonal antibody that targets the epidermal growth factor receptor (EGFR)] and showed that these nanoparticles enhance the cytotoxicity of cetuximab in EGFR-positive non-small-cell lung cancer (NSCLC) both *in vitro* and *in vivo*. Cetuximab conjugated with AuNPs significantly suppressed the proliferation and migration, and accelerated apoptosis of high EGFR expressing NSCLC cells compared with cetuximab alone. Moreover, a significant reduction in tumor weight and tumor volume with minimal toxicity was observed in a mouse lung cancer model following treatment with cetuximab-conjugated AuNPs [42].

12.3.1.1.4. Magnetic nanoparticles

Magnetic nanoparticles used for drug delivery are made of iron-based crystals, typically magnetite or maghemite. For tissue targeting, a localized magnetic field is generated near the target region using external magnets [43,44]. For example, Wen *et al.* showed that a combination treatment with folic-acid-modified magnetic nanoparticles (FA-MNPs) and a 100 Hz extremely low-frequency electromagnetic field (generated by using a solenoid coil) decreases proliferation and increases apoptosis of liver cancer cells compared to FA-MNPs alone [45].

12.3.1.1.5. Carbon-based nanotubes

Carbon-based nanotubes (1–100 nm) are tubular structures formed by rolling graphene sheets into cylindrical carbon networks [46]. Depending on the number of sheets used, carbon nanotubes can be categorized into single- (SWNT) or multi-walled nanotubes (MWNT) [47]. Despite being used in drug delivery, their insolubility and toxicity limit their application [48,49]. Nevertheless, these limitations can be overcome by surface modifications that increase biocompatibility [47]. For example, Cao *et al.* used MWNTs modified with poly(ethyleneimine) (PEI) and conjugated with fluorescein isothiocyanate (FI) and hyaluronic acid (HA) for targeted delivery of doxorubicin to cancer

cells overexpressing CD44 receptors. Results showed that the carrier material had good biocompatibility at the concentrations tested [50]. In addition, the complexes were able to specifically target cervical carcinoma cells overexpressing CD44, leading to growth inhibition via receptor-mediated binding and intracellular uptake [50].

12.3.1.2. Organic nanoparticles

12.3.1.2.1. Polymer-based nanoparticles

Polymer-based nanoparticles include polymeric nanoparticles, micelles, and dendrimers.

12.3.1.2.1.1. Polymeric nanoparticles

Polymeric nanoparticles are colloidal solid particles (50–300 nm) prepared using synthetic polymers [poly(lactic acid) (PLA), poly(lactic-co-glycolic acid) (PLGA), PEI, and poly(ethylene glycol) (PEG)], synthetic hydrogels [poly(acrylamide)], natural polymers (chitosan), or hydrolytically or enzymatically degradable polymers (collagen) [51]. PLA and PLGA hydrolyze into biologically compatible metabolites, which are eventually eliminated from the body as carbon dioxide and water, and therefore are less cytotoxic [52,53]. The functionality of PLA and PLGA nanoparticles can be improved by surface modifications such as PEGylation, lipid-coating, and cell-targeting ligands [54]. For example, docetaxel-loaded PLA nanoparticles conjugated with a peptide highly specific for binding pulmonary adenocarcinoma tissue resulted in a significant decrease in the metastatic tumor area in the liver of a nude mouse model of lung cancer compared with the absence of the targeting peptide [55].

12.3.1.2.1.2. Micelles

Micelles are amphiphilic shell-core structures able to entrap and carry hydrophobic molecular cargos [56]. Their small size prevents their uptake by the reticulo-endothelial system (RES), increasing circulation time and allowing percutaneous lymphatic transport as well as extravasation from blood vessels to target tumor tissues [30,57,58]. Moreover, micelles can be constructed to respond to external stimuli such as changes in temperature, light, and pH [59]. For example, Sajomsang *et al.* demonstrated that curcumin encapsulated within pH responsive polymeric micelles increased the percentage of apoptotic cervical cancer cells compared to free curcumin [60].

12.3.1.2.1.3. Dendrimers

Dendrimers are synthetic, highly branched oligomers that form three-dimensional molecules. They are synthesized in a stepwise manner resulting in an inner core moiety with radially attached branch layers [30,37,61,62]. Drug loading capacity as well as the kinetics of drug release can be altered by changing the number of layers. Further modulation can be achieved by incorporation of degradable linkages between the dendrimer and molecular cargo, such as amide or ester bonds. For example, Khatri *et al.* used amide-

-bonded methotrexate conjugated dendrimers and found that the viability of uterine sarcoma cells was decreased in a dose-dependent manner compared to methotrexate alone [63].

12.3.1.2.2. Liposomes

Although several nanoparticle systems have been proposed as delivery carriers of therapeutic and diagnostic agents, liposomes – particularly nanoliposomes – are the most frequently used nanoparticles for drug delivery [64]. Liposomes (ranging in size from 30 to 200 nm) are vesicular cell membrane-like structures consisting of a phospholipid bilayer that forms by the self-assembly of dissolved lipid molecules [65]. After assembly, liposomes may contain a mixture of small unilamellar vesicles (SUV), large unilamellar vesicles (LUV), multilamellar vesicles (MLV), or multivesicular vesicles (MVV) [66], as shown in Figure 1. Extrusion or sonication methods are normally used to obtain a homogeneous population of SUV [67].

Figure 1. Cryo-electron microscopy profile of DOPC-PEG nanoliposomes

Liposomes may contain natural or synthetic phospholipids [phosphatidylcholine (PC) and phosphatidylethanolamine (PE)] and cholesterol [7,11,68]. The phospholipid bilayer is capable of storing both hydrophobic and hydrophilic compounds. Therefore, liposomes are often used as delivery systems for a great variety of molecular cargos. The lipids used can be divided into several categories, including cationic, anionic, neutral, or a mixture of lipids, each one with different charges [66]. The overall charge of the liposomes can be manipulated to enhance interaction with molecules of interest.

12.3.1.2.2.1. Cationic lipids

Cationic lipids are often formed with a neutral helper lipid such as dioleoylphosphatidylethanolamine (DOPE), which increases stability and enhances cellular uptake [69-71]. The positive charge in cationic lipids [including *N*-[1-(2,3-dioleyloxy)propyl]-*N,N,N*-trimethylammonium chloride (DOTMA) and [1,2-bis(oleoyloxy)-3-(trimethylammonio) propane] (DOTAP)] allows spontaneous interactions with DNA or RNA as well as binding with negatively charged components of the cell membrane, facilitating the entry of molecular cargos into target tissues [66]. However, although cationic lipids interact easily with negatively charged surfaces, toxicity due to activation of pro-apoptotic and pro-inflammatory cascades is a major concern [72].

12.3.1.2.2.2. Anionic lipids

Anionic lipids such as phosphatidic acid (PA), phosphatidylglycerol (PG), and phosphatidylserine (PS) have been investigated as alternatives to cationic lipids. Unfortunately, the negative charge in anionic lipids results in electrostatic repulsions with molecular cargos, limiting their application as nanocarriers [66]. In addition, negatively charged liposomes are taken up faster by macrophages in the blood circulation. Nevertheless, when the target is the phagocyte monocyte system, the use of these liposomes is an advantage [73].

12.3.1.2.2.3. Neutral lipids

Neutral lipids such as dioleoylphosphatidylcholine (DOPC) have been used as a way to circumvent the problems associated with cationic and anionic nanoliposomes. For example, Tekedereli *et al.* showed that systemic administration of DOPC-nanoliposomes encapsulating Bcl-2-targeted siRNA (NL-Bcl-2-siRNA) leads to anti-tumor activity and growth suppression of estrogen receptor-negative [ER (-)] and ER-positive (+) breast cancer orthotopic xenograft models with no obvious toxicity. Moreover, a combination of NL-Bcl-2-siRNA with doxorubicin increased chemotherapy efficacy in both animal models compared to control groups [74].

12.3.2. Nanoliposomes as delivery carriers of therapeutic and imaging agents

As drug carriers, nanoliposomes can be coated with PEG, which prolongs the circulation half-life of the nanocarrier and improves biodistribution. Therefore, PEGylation may be used to reduce uptake within the reticuloendothelial system (RES) and allow higher drug concentrations to be delivered to target tissues before clearance [9]. Also, PEG provides a linker for attachment of targeting moieties such as small-molecule ligands, peptides, and monoclonal antibodies, improving tissue specificity [75]. Nanoliposomes carrying chemotherapeutic drugs such as PEGylated doxorubicin (Doxil and Lipo-Dox), non-PEGylated doxorubicin (Myocet), daunorubicin (DaunoXome), cytarabine (DepoCyt), and vincristine (Marqibo) have been clinically approved for cancer

therapy [7-11]. Additional liposomal formulations are under clinical investigation [7-11].

Lipid-based nanoparticles have also been combined with imaging agents for medical diagnostics and assessment of treatment efficacy [30,61,76]. Alone, imaging systems such as magnetic resonance imaging (MRI), positron emission tomography (PET), and single photon emission computed tomography (SPECT) suffer from limited resolution or sensitivity [77]. However, nanoliposomes have shown great promise in overcoming these limitations. Imaging agents such as radionuclides can be incorporated into either the bilayer or the interior of the nanoliposome, making it suitable for imaging systems [78-80].

12.3.3. Nanoliposomes as delivery systems of siRNA and miRNAs for cancer treatment

Since the discovery of RNA interference (RNAi), small RNA molecules including siRNAs and miRNAs have become a powerful therapeutic modality for targeting genes of interest, including “undruggable targets” [81]. Therefore, among the drugs that are currently under clinical trials, RNAi molecules have emerged as a treatment modality of exceptional promise for cancer and other diseases. SiRNAs are small non-coding RNAs (21–27 nucleotides in length) that bind complementary target messenger RNAs (mRNAs), preventing translation and selectively silencing gene expression [82]. Synthetic siRNAs are the most commonly used structures in RNAi-based therapeutic formulations and are designed to target a single key gene which is generally overexpressed in cancer cells compared with normal cells [82,83]. In a more recent modality, miRNAs and anti-miRNA agents are used for RNAi-based therapeutics.

MiRNAs are endogenously expressed small non-coding RNAs (21–25 nucleotides in length) that function as post-transcriptional regulators of gene expression [84,85]. Recent evidence indicates that the human genome may encode over 1500 miRNAs, which regulate about 60 % of human genes [7]. Multiple studies involving various types of human cancers have demonstrated that miRNAs have a fundamental role in tumorigenesis, drug resistance, and metastasis [86-94]. While most of these miRNAs are down-regulated and act as tumor suppressor genes, some are up-regulated and may represent novel oncogenes (oncomiRs) [95]. Thus, miRNA-targeted therapies can be used to inhibit the action of oncomiRs by single-stranded RNA molecules complementary to the targeted miRNA (antagomirs) or to activate the function of tumor suppressor genes by partially double-stranded RNA molecules that mimic endogenous precursor miRNAs (miRNA mimics) [7,96].

Despite their potential use in cancer treatment, the major limitations of RNAi-based therapies include off-target effects, poor uptake, degradation, and rapid clearance following administration [6,97-99]. To overcome these barriers, carrier systems such as nanoliposomes have been explored for effective and

safe delivery of RNAi molecules [100,101]. These nanocarriers protect RNAi molecules from degradation, facilitating uptake by target tissues, which is also affected by the charge of the liposome. In 2005, Landen *et al.* used neutral DOPC nanoliposomes in ovarian cancer mouse models and found a 10-fold improvement in the delivery of siRNA compared with cationic DOTAP, and a 30-fold improvement over naked siRNA. Moreover, incorporation of EphA2-siRNA into DOPC nanoliposomes inhibited tumor growth in ovarian cancer mouse models compared with a negative control siRNA. This effect was further enhanced when using DOPC-encapsulated EphA2-siRNA in combination with paclitaxel [102]. Currently, siRNA-EphA2-DOPC is in a phase I clinical trial (not yet recruiting) for patients with advanced solid tumors [103].

Another example of the use of neutral nanoliposomes in siRNA delivery for cancer treatment includes the encapsulation of survivin splice variant 2B-targeted siRNA (2B-siRNA) into DOPC nanoliposomes. Silencing of survivin 2B in taxane-resistant ovarian cancer orthotopic mouse models resulted in a significant reduction in tumor growth, which was further enhanced in combination with docetaxel. Decreased microvessel density (MVD) and cell proliferation was observed, as well as induction of apoptosis in mice treated with 2B-siRNA-DOPC nanoliposomes compared with controls. Silencing of survivin 2B resulted in similar growth inhibitory effects as observed when silencing all survivin splice variants simultaneously, proposing 2B-siRNA-DOPC as a novel and specific anti-survivin therapy in ovarian cancer.

Targeting of the nuclear receptor coregulator PELP1 (proline-, glutamic acid-, leucine-rich protein-1) in ovarian cancer by systemic administration of siRNA-DOPC nanoliposomes has also been shown to effectively reduce PELP1 expression and significantly reduce tumor growth, metastatic tumor nodules, and ascites volume in xenograft models. Results suggest that PELP1-siRNA-DOPC nanoliposomes can be used as a potential therapeutic modality for the prevention or treatment of ovarian cancer metastasis [105]. Several other siRNA-liposomal formulations targeting key genes involved in cancer growth, proliferation, drug resistance, and metastasis are under investigation [106-110].

In addition to siRNA-EphA2-DOPC, a number of other RNAi lipid nanoparticles have entered clinical trials. For example, Alnylam Pharmaceuticals has completed a phase I dose escalation trial for ALN-VSP02 – a dual-targeted lipid particle siRNA drug formulated against vascular endothelial growth factor (VEGF) and kinesin spindle protein (KSP) – in patients with advanced solid tumors with liver involvement [111]. A phase I dose escalation study sponsored by the National Cancer Institute on hepatic intra-arterial administration of TKM-080301 – a lipid nanoparticle formulation of a siRNA targeting the serine/threonine kinase PLK1 – in patients with primary or secondary liver cancer has also been completed [112]. Further phase I/II dose escalation and phase I dose escalation with phase II expansion cohort studies

sponsored by Tekmira Pharmaceutical are currently undergoing trial for the determination of safety, tolerability, and pharmacokinetics of TKM-080301 in patients with advanced solid tumors, and preliminary anti-tumor activity of TKM-080301 in patients with advanced hepatocellular carcinoma (HCC), respectively [113,114]. Silence Therapeutics has also initiated a phase Ib/IIa study for Atu027 – a siRNA-lipoplex directed against protein kinase N3 (PKN3) – in combination with gemcitabine for treatment of locally advanced or metastatic pancreatic cancer. This study aims to evaluate the potential attenuation of further disease progression by the anti-metastatic effect of Atu027 in combination with the anti-neoplastic activity of gemcitabine [115]. Moreover, phase I and phase Ib/II study trials sponsored by Dicerna Pharmaceuticals are evaluating the safety, tolerance, and maximum dose of Dicer substrate short interfering RNAs (DsiRNAs) in lipid nanoparticles targeting the myelocytomatosis (MYC) oncogene (DCR-MYC) in patients with solid tumors, multiple myeloma, or lymphoma, as well as the recommended phase II dose of DCR-MYC in patients with hepatocellular carcinoma [116,117].

Significant advances have also been made in the development of miRNA-based therapies. For example, systemic administration of an miR-34a mimic complexed with an amphoteric liposomal formulation showed significant growth inhibition in orthotopic models of liver cancer, suggesting its potential use for cancer treatment [118]. In fact, a multicenter phase I trial sponsored by Mirna Therapeutics is currently recruiting participants for safety evaluation of MRX34 – a miR-34 mimic liposomal injection – in patients with primary liver cancer (hepatocellular carcinoma), other solid tumors with liver metastasis, and hematologic malignancies [119].

Innovative gene silencing therapies such as the combination of miRNA and siRNA incorporation into nanoparticles have also been evaluated in ovarian cancer *in vitro* and *in vivo* models [120]. In this study, dual inhibition of EphA2 using DOPC-nanoliposomes loaded with miR-520d-3p and EphA2 siRNA resulted in therapeutic synergy and enhanced tumor suppression compared with either therapy alone. Such ‘boosting’ in anti-tumor effects suggests the feasibility of a new concept of RNAi-based therapy for cancer and other diseases [120].

12.3.4. Other nanoparticles as delivery systems for therapeutic and imaging agents in cancer

Another example of a drug delivery system that has shown great promise for tumor targeting and treatment due to its specificity for the affected tissue and low induction of the host’s immune system consists of a RNA-based nanoparticle by itself known as packaging RNA (pRNA) [29]. The pRNA (one of the six pRNA subunits of the DNA packaging motor of bacteriophage phi29) is a 117-nucleotide (nt) RNA molecule that can acquire a stable nanoparticle structure of about 11 nm in size [29]. These RNA-based nanoparticles induce

low toxicity to healthy organs such as the liver, lungs, and kidneys [121]. Therefore, they are attractive systems for tumor targeting and drug delivery due to their biocompatibility and biodegradability [29].

12.4. NANOPARTICLE FORMULATIONS FOR THE TREATMENT OF OTHER HUMAN CONDITIONS

The use of nanoparticles as delivery systems for a variety of drugs and treatments is an emerging area of interest, not only for cancer, but for many other health conditions ranging from bacterial and viral infections to cardiovascular disease. The major concerns of bacterial and fungal infections include the increasing resistance to antibiotics and the systemic side effects associated with the use of antifungal treatments. To overcome these concerns, nanotechnology offers an opportunity to develop drug carrier systems with higher specificity and efficacy. Such nanocarriers offer the possibility of extended release as well as the potential of reducing the efflux of the drug through the transmembrane proteins of the cell wall [122]. An example of a nanocarrier-mediated drug delivery system is the use of liposomal amphotericin B (L-AmB) for the treatment of fungal infections [123].

Liposomal antibiotic formulations are also being developed as aerosols for the treatment of opportunistic lung infections, including infection by *Pseudomonas aeruginosa* in patients with cystic fibrosis (CF). A phase II study of neutral liposomal amikacin (Arikace) administered once daily by a nebulizer to patients with CF resulted in a decrease of the pseudomonal sputum content and an improvement in the pulmonary function and respiratory symptoms (all of which suggest a longer antimicrobial effect), as well as few or no adverse side effects compared to the placebo [124]. The development of liposomal drug formulations as nebulized treatments for lung infections is promising due to the ability to make neutral liposomes containing dipalmitoylphosphatidylcholine (DPPC) and cholesterol, which are two of the main components of the pulmonary surfactant [125].

Nanostructures are also being used to develop carrier systems for the treatment of viral infections. An example is the development of siRNAs carried in stable nucleic acid-lipid particles (SNALPs) for the treatment of ebola-virus infection [126]. Another example includes a recent study on the development of non-viral vectors for chronic hepatitis C virus (HCV) treatment, which showed that a preparation of SLNs coated with hyaluronic acid and transporting the plasmid short hairpin RNA 74 (shRNA74) were effective in silencing the internal ribosome entry site of HCV (HCV-IRES), thus inhibiting its viral replication [127]. Another study showed a nanoliposome formulation with short synthetic RNA (sshRNA) SG220 also targeting the HCV-IRES, which yielded increased gene suppression [128]. In addition to nanostructures as vectors for siRNAs, shRNAs, and sshRNAs in HCV treatment, nanostructures

have been used as delivery systems for antagomirs. For example, anti-miRNA-122 (antagomir-122) complexed with interfering nanoparticles (iNOP-7) resulted in effective delivery into the cytoplasm of hepatocytes and silencing of endogenous miR-122 in HCV-positive mice [129].

Another modality under study includes the use of siRNAs as topical treatments in microbicide formulations to inhibit viral replication of herpes simplex virus type 2 (HSV-2) [130] and human immunodeficiency virus (HIV) type 1 [131] – two of the most common causes of sexually transmitted diseases (STDs) worldwide [132]. In fact, the use of nanostructures in topical microbicides is a promising treatment option for STDs. For example, a recent study showed an enhanced anti-viral effect against HIV as a result of the combination of two anionic carbosilane dendrimers (G2-STE16 and G2-S24P). The combination of both dendrimers resulted in an enhanced inability of the HIV viral envelope protein gp120 to bind to the CD4 receptor. These results suggest the use of such dendrimer combinations as topical microbicide formulations to prevent HIV infection through the vaginal mucosa in high-risk populations [133].

The nanotechnological advances in cardiovascular medicine have opened the door for innovative treatments that can reverse heart disease, the leading cause of death in the United States [134]. For instance, a recent study by Serpooshan *et al.* showed the therapeutic benefit of PEG-based liposome-encapsulated apelin (lipoPEG-PA13) on hypertrophied heart. Apelin – an adipokine that is endogenously found in the human body – is involved in homeostasis regulation of bodily fluids, blood pressure, and heart function. Injection of lipoPEG-PA13 into mice with hypertrophic heart injury led to a reduction in the size of the left ventricle and reduced fibrosis [135]. In addition, lipoPEG-PA13 showed a sustained release in the blood stream of mice compared with mice treated with non-encapsulated apelin [135]. Another therapeutic modality includes the application of siRNAs to target genes involved in lipid metabolism. For example, a phase I study trial of the lipid nanoparticle (LNP)-packaged siRNA drug, ALN-PCS02, proved to be effective in decreasing low-density lipoprotein cholesterol (LDL-C) levels in patients' serum by targeting and inhibiting the enzyme PCSK9 (proprotein convertase subtilisin/kexin type 9). PCSK9 is responsible for the degradation of LDL receptors (LDLR) and subsequent decreased metabolism of LDL-C. Therefore, by inhibiting PCSK9, plasma LDL concentration decreases, thus lowering the risk of cardiovascular disease [136].

12.5. FUTURE DIRECTIONS AND CHALLENGES

The use of nanoparticles as carriers for cancer treatment could drastically improve the stability and therapeutic effectiveness of the currently used and newly discovered anticancer agents. Various nanoparticle systems, including silica-based, liposomes, polymeric, and metal nanoparticles, among many

others, have good biocompatibility, biodegradability, and high encapsulation efficiency. In addition, they are easy to prepare at very low cost, and most of the components in liposomal formulations are already approved by the Food and Drug Administration (FDA). Liposomes have the extra capability to carry both lipophilic and hydrophilic molecules, and they can be functionalized with several ligands, which increase specificity and therapeutic index.

Before proposing nanoparticles as reliable drug delivery systems for cancer treatment and/or imaging, further research should be performed mainly to answer the following important questions:

- 1) How can the circulation time of the nanoparticle formulations be increased to improve their therapeutic efficacy [137]?
- 2) Once the nanoparticles come into the blood circulation, how is the nanoparticle surface altered by blood-associated proteins and how can this affect the interaction between the nanoparticles and the target cells [138,139]?
- 3) What are the major mechanisms by which nanoparticles interact with the plasma membranes to release their cargo [140,141]?
- 4) What amount of nanoparticles and their cargo are truly associated with the tumor tissue relative to other tissues and organs [142]?
- 5) Once in the tumor tissue, what amount of nanoparticles and/or their content remains in the periphery relative to the amount of cargo effectively released inside the tumor cells [143]?
- 6) How can pH-sensitive nanoparticles be created to take advantage of pH differences between normal tissues (pH=7.4) compared with tumor cells (pH= 6.0–6.5) [144]?
- 7) Is the molecular target highly abundant in cancer cells and negligible in normal non-cancerous cells [145–147]?
- 8) If functionalized nanoparticles (targeted nanoparticles) are being proposed, is the target receptor highly abundant in cancer cells as compared with normal tissue cells [148]?
- 9) How can nanoparticles with more than one different drug be designed to target multiple intracellular targets in cancer cells [149]?
- 10) Is the dose/schedule therapy appropriate to avoid the drastic decrease in nanoparticle blood concentration as a result of the accelerated blood clearance phenomena [150]?

The tumor cell heterogeneity is another concern, as the selected molecular target might be expressed only by a small number of cancer cells compared with the enormous genetically different cancer cell populations in the tumor, including cancer stem cell populations [151]. For safety purposes, pharmacokinetics, pharmacodynamics, and tissue distribution studies of the nanoparticle and nanoparticle-cargo formulations are highly desirable [152].

Therapies able to cross the blood-brain barrier (BBB) are also needed for brain tumor treatment and/or imaging [153-156].

The use of nanoparticles – particularly nanoliposomes – for the delivery of RNAi-based therapies is a rapidly growing research area. It would appear that this therapeutic modality has immense potential for the treatment of cancer and many other human conditions.

12.6. CONCLUSIONS

The use of nanoparticles as drug carriers for therapy and diagnostic agents is slowly moving into the clinic, not only for the treatment of cancer but for many other human conditions as well. However, improved nanoparticles able to deliver drugs to desired sites are still urgently needed. Such systems might significantly increase drug safety and effectiveness. Therefore, it is anticipated that in the near future, more nanoparticle formulations, particularly for the delivery of novel therapies including RNAi-based modalities, will be used clinically.

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**TARGETING STRATEGIES FOR THE
TREATMENT OF *HELICOBACTER PYLORI*
INFECTIONS**

Daniela Lopes¹, Cláudia Nunes¹, M. Cristina L. Martins^{2,3}, Bruno Sarmiento^{2,4}, and Salette Reis^{1*}

¹ UCIBIO/REQUIMTE, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Porto, Portugal

² INEB – Instituto de Engenharia Biomédica, Universidade do Porto, Porto, Portugal

³ ICBAS – Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Porto, Portugal

⁴ IINFACTS – Instituto de Investigação e Formação Avançada em Ciências e Tecnologias da Saúde, Instituto Superior de Ciências da Saúde-Norte, Gandra-PRD, Portugal

*Corresponding author: shreis@ff.up.pt

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13.1. INTRODUCTION

Helicobacter pylori (*H. pylori*) are gram-negative bacteria which are able to colonise the gastric mucosa due to several factors [1,2]. These virulent factors are both promoters of cytotoxicity and enhancers of its survival, even in an austere environment such as the acidic pH of the stomach [1,2]. *H. pylori* are therefore able to penetrate within the mucosa and attach themselves to the surface between the mucous layer and epithelial cells [2,3]. They infect a high percentage of the world's population, with infection rates of approximately 50 % [4]. Once colonised, the majority of the population does not manifest symptoms [4], however, 20 % of the infected population evolves from histological signs of chronic gastritis to gastrointestinal symptoms of gastritis and peptic ulcers [5]. Persistent infections may even evolve into cancer, which led to the classification of the bacterium as a human carcinogen (Group 1) by the International Agency for Research on Cancer (IARC) and the World Health Organization (WHO) [4]. In fact, its relationship to gastric cancer and lymphomas of mucosa-associated lymphoid tissue has been proved [6]. Although there is no clear explanation for it, it has also been related to other extradigestive conditions, such as idiopathic thrombocytopenic purpura, iron deficiency anaemia, ischemic heart disease, stroke, Parkinson's disease and Alzheimer's disease [7].

The severity of *H. pylori* is exacerbated by the difficulty of eradication. There is much controversy regarding the development of an effective vaccine, due to the difficulty of achieving a full protective immune response [8,9]. Clinical trials have generally failed and the investment necessary by pharmaceutical companies is high [8,9]. The current approach against *H. pylori* infection is treatment whenever symptoms justify it and includes a drug to reduce the contact between the ulcer and gastric acid, and two or three antibiotics, such as amoxicillin, clarithromycin and metronidazole, for at least 7 days [10,11]. The treatment has evolved over the years, but it still has several problems. In fact, eradication rates of 80 % were achieved by prolonging the first-line treatment for 14 days (14-day triple therapy) and in some countries rates of only 20–45 % have been reported [10,12]. These rates are still far from those desirable for infectious diseases and those proposed by the WHO [11].

The difficulty of eradicating *H. pylori* is a consequence of a sequence of events. Some drugs, viz. amoxicillin and clarithromycin, are degraded by gastric acid [13], and antibiotics do not remain for a sufficient time in the stomach, resulting in increased difficulty in achieving significant concentrations capable of crossing the mucous layer barrier and reaching the surface where *H. pylori* resides [14,15]. These issues both lead to the necessity of higher doses and, consequently, to the exacerbation of gastrointestinal side effects, such as nausea, vomiting and abdominal pain [16]. In fact, the frequency of side effects

combined with the duration of therapy, which varies between 7 and 14 days, and the discomfort resulting from the multiple doses, causes people to give up on the therapy [16,17]. This lack of therapeutic compliance complicates the success of eradication and ultimately it can have a tremendous effect on the development of antibiotics resistance. Actually, the bacterium has developed resistance to several antibiotics, such as metronidazole, with the percentage of resistance at 40 % and 90 % in developed and developing countries, respectively [18].

Different strategies have been attempted in order to overcome these limitations: other approaches to the treatment plan (*e.g.* bismuth-containing quadruple therapy, sequential and concomitant therapy), use of probiotics and phytomedicine [7,19-21]. A nanotechnology approach has also been applied to the development of effective systems to eradicate *H. pylori* [22]. Diverse nano- and microparticles have been tested, such as liposomes, polymeric, magnetic and metallic particles [22]. These systems have strong and specific advantages for each kind of nanoparticle. One approach common to the systems in general is the possibility of using passive and active targeting in order to improve the therapy [22]. Targeting allows the enhancement of the affinity between the nano- or microsystem and the gastric mucosa or the *H. pylori*. In the special case of *H. pylori* infection, vectorization can thus be used to improve the residence time of antibiotics in the stomach and to increase drug concentration at the action site, minimising the side effects. It is also possible to use specific ligands to the bacterium to decrease its adhesion to the gastric mucosa, improving eradication rates [15]. Herein, we will summarise several approaches to targeting both the gastric mucosa and *H. pylori*.

13.2. HOW CAN TARGETING OVERCOME DRUG RESISTANCE?

One of the most worrying problems in the treatment of *H. pylori* infection is the development of resistance to antimicrobial drugs. Some commonly used drugs, such as metronidazole and clarithromycin, are already resisted by *H. pylori*. Rates of 40 % of resistance to metronidazole have already been reported in developed countries [18]. For clarithromycin, rates of 30 % in America and 92 % in Africa have been reported [23]. Some studies also reported resistance to other drugs, such as amoxicillin, tetracycline and levofloxacin [23]. Multidrug resistance achieved rates of around 9 % in Europe and Asia [23]. Antibiotic resistance is disquieting, especially because persistent infections can lead to gastric cancer and it is difficult to use other antimicrobial drugs, as they are degraded by pH [4,13].

Nanoparticles can be used to improve the pharmacokinetic properties of drugs and to protect them from the hostile environment of the stomach.

Improvement of their half-life and a sustained and local release of the drug result in lower doses and a higher efficacy [24]. With a complement of targeting and stimuli-release systems, nanoparticles can be used to achieve higher concentrations of antimicrobial drugs near the bacterium even at low doses [25]. This can minimise side effects and improve the therapeutic efficacy, killing the bacterium and decreasing the potential of developing resistance. This can be improved due to the prospect of using multiple antimicrobial drugs in therapeutic doses [24]. It is unlikely that the bacterium will develop multiple simultaneous gene mutations to resist different mechanisms in the same nanosystem [25]. Given the possibility of using higher doses of the antimicrobial drug, it is possible to saturate transmembrane pumps [25].

Specific mechanisms of the interaction of nanoparticles with bacteria have also proved their usefulness in overcoming bacterial resistance. For instance, the fusion of liposomes with the bacterial membrane and the direct release of its contents inside the bacterium allow the use of sub-minimal inhibitory concentrations (sub-MIC) of antimicrobial drugs [26]. Given the well-known resistance mechanisms related to the membrane, namely the decrease of its permeability and the activation of efflux systems, fusion between liposomes and bacterial membranes can overcome both these mechanisms [26]. Dendrimers have also proved their efficacy since their positive charges promote their link with negative membranes, increasing permeability [25]. Ultimately, they can even destroy the microbial cell membrane [25]. Other kinds of nanoparticles, such as metallic nanoparticles, are unlikely to induce drug resistance for either multiple mechanisms and efficacy even in lower doses [22].

13.3. TARGETING THE GASTRIC MUCOSA

There is a close relationship between the gastric mucosa and *H. pylori* infection. First, these bacteria are able to penetrate within the mucosa and colonise the surface under the mucus layer [1,2]. Their virulence can also damage the gastric mucosa, causing peptic ulcers and ultimately gastric cancer [1,4]. The pharmacokinetic of antimicrobial drugs involves absorption and diffusion across the mucus to reach the bacterium, hence it is significantly affected by the gastric mucosa [14,15]. It is also the first barrier that nanoparticles have to cross to reach their target, and therefore, it is important to know the physicochemical properties of the gastric mucosa in order to take advantage of those characteristics to optimise the nanosystem.

One important feature of the mucosa is its hydrophobicity, which avoids the diffusion of hydrogen ions [27]. Their composition in phospholipids, as well as in other surface groups, viz. sialic acid, carboxyl and sulphate groups, play an important role in the interaction with nanoparticles. These surface groups on the mucosa are negatively charged and can establish several interactions with

nanoparticles, such as electrostatic attraction, hydrogen bond (H-bond) formation and van-der-Waal forces [28-30]. The mucus layer is also characterised by its composition, highly rich in mucins, which are high-molecular-mass oligomeric glycoproteins [31]. Taking this into account, systems with mucoadhesive properties may have an extended residence time at the target and improve contact with biological membranes [30]. The gastric mucosa also has a pH gradient, which is extremely acidic (1 or 2) in the lumen of the stomach and near neutral at the interface between the mucosa and epithelial cells [32,33]. This pH gradient results from the secretion of HCO_3^- molecules and from the restricted diffusion of protons by the mucus layer [32]. The place to release the antimicrobial drug may thus be chosen using different pH-sensitive nanosystems.

A summary of all characteristics which can be used to treat *H. pylori* infections through the targeting of the gastric mucosa is presented in Figure 1.

Figure 1. Scheme of the different strategies that can be used to target the gastric mucosa

13.3.1. Mucoadhesiveness

Mucoadhesiveness is a characteristic of some compounds reflected in their attraction to a mucosal membrane, which results in a temporary retention [34]. This can be very advantageous both for the increment of the amount of drugs locally released and for the improvement of direct contact with the biological membrane involved in the absorption of the drug [30].

Consequently, this strategy has been applied to the treatment of several disorders, through the target of different human mucosa, such as to nasal [35], buccal [36], ocular [37] and vaginal [38] mucosa, among others. Diverse polymers can be used, including natural polymers, such as alginate, chitosan, pectin, xanthan gum, hyaluronic acid and gelatin, synthetic polymers, as poly(ethylene glycol), poly(ethylene oxide), poly(acrylic acid), poly(methacrylic acid) and poly(vinyl amine), and semi-synthetic polymers, namely cellulose derivatives [39].

Due to the complexity of adhesion to the mucosa, several theories have emerged to explain this phenomenon. The electronic theory relies on the existence of electron transference in the origin of electrostatic interactions [34]. In the adsorption theory, other interactions are taken into account, such as H-bonds, van-der-Waals forces, hydrophobic interactions and chemisorption process [34]. Some polymers, namely thiolated polymers or thiomers, can establish disulphide bonds with the mucus gel layer [40]. The wetting theory suggests that the ability of mucoadhesive compounds in the liquid state to extend themselves on the mucosa is due to the surface tension of both mucus and polymers [34]. It is also hypothesised that diffusion events are related to mucoadhesion, which depends on the gradient of concentration, molecular weight, size and mobility of molecules [34]. Adhesion is also related to the force required to detach the material from the mucosa (fracture theory) and with the increased contact area of rough and porous materials (mechanical theory) [34].

Although without certainties concerning the mechanisms involved in mucoadhesion, several bioadhesive polymers have been extensively used. In the special case of *H. pylori* infection, affinity to the gastric mucosa is a useful strategy since the enhancement of the retention time in the stomach is important in overcoming the direct transit through the gastrointestinal tract [41]. Polymeric systems with bioadhesive properties have therefore been used to eradicate *H. pylori*. Polymeric particles have additional advantages, such as their mechanical stability and loading capacity [15]. Several polymers are recognised as biocompatible and some such as gelatin, are classified as GRAS (generally regarded as safe) by the Food and Drug Administration (FDA) [22]. Some mucoadhesive polymers, such as poly(acrylic acid) and poly(allylamine hydrochloride) can decrease the adhesion of *H. pylori* to the gastric mucosa [28].

Table 1 summarises different mucoadhesive polymers reported in nanosystems applied to the treatment of *H. pylori* infection. For instance, poly(acrylic acid) or carbopol was used to develop microspheres. In fact, mucoadhesion properties of carbopol had already been demonstrated by *in vitro* and *in vivo* studies [42]. Proteins such as gliadin and gelatin, can also be used, highlighting their additional advantages, which are their nutritional value and the existence of renewable sources [43]. Polymeric carbohydrate molecules, more specifically chitosan, have emerged as a promising drug

delivery system for *H. pylori*. Due to their positive charge and their mucoadhesiveness, chitosan particles can establish strong electrostatic interactions between their protonated glucosamine residues and the abovementioned negatively charged groups of the gastric mucosa [44,45]. Chitosan was therefore used to develop nano- and microparticles loading different antibiotics. In consequence of its broad-spectrum antimicrobial effect, it has also been used in direct action against the bacteria [46,47]. Another approach is the use of chitosan to plug, seal and remove the bacterium from the body [47]. Other polymers with mucoadhesion properties were also used in microparticles composed of mixture of polymers, such as carboxyvinyl polymers, cholestyramine, glycerol monooleate, hydroxypropyl methylcellulose among others.

The problem of extensive mucoadhesiveness is the lower ability to penetrate within the mucosa [29]. Arora *et al.* (2011) thus modified the charge of chitosan and decreased the particle size to less than the mesh size of mucin fibres [29]. Results revealed the permanency of microparticles in the deepest layers of the mucosa for over 6 h [29].

Table 1. Summary of different mucoadhesive polymers used in nano- and microsystems to eradicate *H. pylori* through distinct mechanisms

Polymer		Mechanism of action/Reference
Poly(acrylic acid) or carbopol		Load amoxicillin [48,49]
Proteins	Gliadin	Load acetohydroxamic acid [50]; amoxicillin [51]; clarithromycin [52]; dual therapy [53] and triple therapy [54]
	Gelatin	Load amoxicillin [55,56]
Chitosan		Deliver dual therapy [57,58]; amoxicillin [17,59]; tetracycline [44]; Antibacterial properties of chitosan [46,60,61]
Copolymers		Deliver antibiotics (several systems with different mixture of polymers, summarised in [22]); probiotics [62]; phytomedicine [63-66] and antacids [67].

13.3.2. Charge

As mentioned above, gastric mucosa is negatively charged, and thus positively charged nanoparticles can be used as means of targeting. Bearing in mind that electrostatic interaction may play an important role in the mucoadhesion process, positively charged nanoparticles can be particularly interesting [55]. Wang *et al.* (2000) demonstrated that positively charged gelatin microspheres

have increased mucoadhesiveness to nasal mucosa compared to negatively charged particles [55]. Applying this strategy to load amoxicillin, they were able to demonstrate through the use of rhodamine isothiocyanate (RITC)-labelled microspheres in a rat stomach, a higher interaction between the mucosa and modified gelatin microspheres than with gelatin microspheres [55]. The adhesion increased with the increase of the number of amino groups [55]. As several facts may affect the results, such as the labelling with RITC, the perfusion speed and time and the number of microspheres, further studies are still necessary [55]. Cholestyramine also has positively charged amino groups, being used by Umamaheshwari *et al.* (2003) [68]. The use of a cellulose acetate butyrate polymer to coat these microparticles masked their surface charge and led to a decrease in their mucoadhesiveness [68].

A different approach was developed by Lin *et al.* (2009), using nanoparticles composed of positively charged chitosan and negatively charged heparin [69]. Once the chitosan:heparin ratio was significantly high, the positive charges of chitosan exceeded the negative charges of heparin [69]. Thus, the final charge of the particle was between +15 mV and +33 mV [69]. They were able to demonstrate the adhesion and uptake of nanoparticles by gastric cells using fluorescence studies [69]. These particles were able to interact with sites of *H. pylori* infection [69].

There are other systems which can be applied to the concept of positively charged nanoparticles. For instance, cationic liposomes have been widely studied for gene therapy and cancer treatment and their application can be extended to target the gastric mucosa [70]. Liposomes in general are recognised for their safety and biocompatibility, however positive charges have been associated with cytotoxicity [70]. In order to overcome this issue, a novel strategy has been adopted, more specifically the cover with biodegradable anionic polymers, which are non-toxic [70]. This would obviously decrease interaction with the gastric mucosa, however, Jain *et al.* (2009) developed positively charged liposomes composed of egg phosphatidylcholine, cholesterol and stearylamine, and coated with poly(acrylic acid) (negative) and then with poly(allylamine hydrochloride) (positive) [28]. This strategy allowed a coating of liposomes, increasing their stability [28]. Simultaneously, it was possible to take advantage of the positive charge of the outer layer to enhance interaction with the mucosa [28]. Their ability to bind both gastric mucosa and the bacteria, which are negatively charged, was proven through agglutination and adherence studies [28]. Nevertheless, it is necessary to take into account toxicity issues concerning the use of cationic polymers [28]. In fact, in general it has been proved that cytotoxicity is related with the surface charge, as cationic nanoparticles are more toxic than anionic and neutral nanoparticles [71].

13.3.3. pH-sensitive nanoparticles

Given the pH gradient of the gastric mucosa, it is possible to develop nanoparticles able to respond to external stimuli. According to the purpose, nanoparticles, either sensitive to acidic pH or sensitive to neutral pH, have been developed. Taking into account the pH of the gastrointestinal tract and the extremely acidic pH of the lumen of the stomach, a local and controlled delivery to acidic pH can be useful to optimise absorption and minimise premature drug degradation [72]. On the other hand, sensitivity to neutral pH will allow the release of the content near the bacterium. Both strategies will be discussed herein.

Concerning the release at acidic pH, it is well known that drugs can be degraded at extremely acidic pH, as in the pH of the lumen of the stomach. For instance, Ramteke *et al.* (2009) reported that amoxicillin, clarithromycin and omeprazole are degraded in acidic pH within 3 h [73]. This degradation can be avoided by encapsulation in a nanoparticle, which can help to conserve an effective concentration at the site of action for long periods of time [73]. Ramteke *et al.* developed a nanoparticle composed of chitosan and glutamic acid conjugates [73]. Glutamate salt dissolves slowly in acidic pH, which allowed sustained release over 5–6 h [73]. The authors also reported that functionalization with fucose, which is a strategy of active targeting that will be discussed later in this chapter (see 13.4.6. Carbohydrate receptors), decreased the exposure to acidic pH and consequently retarded the release [73]. Another strategy was developed by Liu *et al.* (2011), combining buoyancy and mucoadhesion through the use of microspheres of Eudragit® E PO as the modulator of drugs release and glyceryl monooleate as a bioadhesive polymer [74]. The matrix is a polymer soluble at low pH, and thus the drug release rate is higher at acid pH [74]. There are several other examples with a higher release at extremely acidic pH than at neutral pH, which were applied to the eradication of *H. pylori* [22]. This is normally due to the instability of the nanosystem and not necessarily in consequence of an initial plan for the design of pH-sensitive nanoparticles, however, a controlled and sustained release can be achieved with the majority of the systems [22]. Nevertheless, the development of pH-sensitive nanoparticles is a strategy widely applied to other diseases. For instance, both cancer and inflammatory diseases are characterised by an acidic extra-cellular environment [75,76]. Thus, pH-sensitive nanoparticles can be used to a target delivery of drugs, decreasing drugs release at physiologic pH, consequently, decreasing side effects and improving therapy [76]. Their application can be extended to diagnostic and gene therapy [76]. Herein, we will summarise some possible strategies for different nanoparticles.

Liposomes are one of the most studied nanoparticles for delivering antimicrobial drugs [22] and it is possible to develop pH-sensitive liposomes. One approach widely studied is the use of polymorphic lipids, highlighting

unsaturated phosphatidylethanolamine (PE), such as diacetylenic-phosphatidyl-ethanolamine (DAPE), palmitoyl-oleoyl-phosphatidyl-ethanolamine (POPE) and dioleoyl-phosphatidyl-ethanolamine (DOPE) [76]. These phospholipids are usually combined with stabilisers, such as cholesteryl hemisuccinate (CHEMS), which self-assembles into bilayers at neutral pH [76,77]. It was shown by Hafez and Cullis (2000) that CHEMS itself has pH-sensitive properties [77]. When in contact with acidic pH, CHEMS became protonated and, consequently, the liposome reverts from a bilayer to an inverted hexagonal II phase [78]. It is also possible to use liposomes stabilised by external compounds sensitive to pH changes. For instance, carboxyl modified gold nanoparticles were used to stabilise liposomes of 1,2-di-(9Z-octadecenoyl)-3-trimethylammonium-propane (DOTAP) and egg phosphatidylcholine. At pH values below the pK_a of the carboxylic group (pK_a around 5), modified gold nanoparticles are protonated and detach from the liposome, increasing its fusion ability [79]. Solid lipid nanoparticles are a possible alternative for application in the eradication of the bacterium, being useful to protect drugs against hostile environments and to control the release of antimicrobial agents [22]. Kashanian *et al.* (2011) created solid lipid nanoparticles, composed of polysorbate 80, tripalmitin glyceride and *N*-glutaryl PE [80]. Results revealed higher release at acid pH comparatively with physiological pH [80]. Polymeric nanoparticles are also an attractive alternative, using either hydrogels triggered when exposure to a particular pH range or polymeric nano- or microparticles whose physical properties change in response to an external stimuli [81,82]. Different polymeric classes can be used to respond to differences in pH, more specifically poly(methacrylic acid)s, poly(vinylpyridine)s and poly(vinylimidazole)s [82].

Targeting through the release in response to the neutral pH is possible partly due to the abovementioned secretion of HCO_3^- -molecules and the restricted diffusion of protons through the mucus layer, and partly due to a specific survival mechanism of *H. pylori* [1,32]. More specifically, one of the enzymes secreted by the bacterium, urease, is able to produce ammonia [1]. This secretion results on one strategy for its survival, since it results in the conservation of periplasmic and cytoplasmic pH near to neutral [1]. It is therefore possible to benefit from the difference between the pH of the gastric mucosa and the pH of the medium nearby *H. pylori*. For instance, Thamphiwatana *et al.* (2013) used small gold nanoparticles modified with chitosan to stabilise liposomes at acidic pH [83]. Modified gold nanoparticles were charged at acidic pH, being attached to liposomes composed of egg phosphatidylcholine and 1,2-dioleoyl-*sn*-glycerol-3-phosphate (sodium salt) [83]. At neutral pH, however, modified gold nanoparticles became deprotonated and, consequently, detached from liposomes [83]. This detachment allows the destabilisation of the phospholipid bilayer and fusion with the bacterial membrane (Figure 2) [83]. The efficacy of this method was proved by the increased fusion and higher release rates at pH 7.4 [83].

Another approach was used by Silva *et al.* (2009), who used a mixture of pH-sensitive polymers, more specifically, methacrylic acid and methyl methacrylate ester copolymer, known as Eudragit®S100 [84]. This polymer is soluble at pH above 7.0 and, consequently, was used to protect magnetite particles from gastric dissolution and to load amoxicillin [84]. In general, poly(methacrylic acid-*co*-ethylacrylate) copolymers, commercially known as Eudragits, are widely used to develop pH-sensitive nanoparticles and include a large class of different polymers [85]. They are usually mixed with other polymers [85].

Figure 2. Scheme representative of the action mechanism of pH-sensitive liposomes by the stabilisation with modified gold nanoparticles at acidic pH. When the surrounded medium becomes neutral, modified gold nanoparticles become deprotonated and detach from liposomes. The destabilisation allows the fusion of liposomes with the bacterial membrane (adapted from [83]).

A mixture of chitosan and heparin was used to synthesise pH-sensitive nanoparticles by Lin *et al.* (2009), for application in *H. pylori* eradication [86]. At acid pH (4.5–6.5), which simulates the pH gradient of the gastric mucosa, chitosan is positively charged and heparin is negatively charged [86], and thus, polyelectrolyte complexes are formed by electrostatic interactions [86]. At pH 7.0, however, chitosan is deprotonated, which results in the collapse of the nanoparticle [86]. It is also possible to use pH-sensitive hydrogels to incorporate nanoparticles, as was done by Chang *et al.* (2010) through the incorporation of chitosan/poly- γ -glutamic acid nanoparticles in calcium-alginate-gelatin hydrogel [87]. Results showed pH-sensitive properties of hydrogel, which protected amoxicillin from the acidic pH [87].

13.4. TARGETING *HELICOBACTER PYLORI*

H. pylori are gram-negative bacteria, which have a spiral-shaped or a coccoid form depending on the hostility of the environment [1,2]. They can colonise an aggressive environment like the stomach due to virulent factors, such as their ability to adhere to the target cells through adhesins, and four to six flagella, which enhance their mobility [1,2]. They are also able to produce urease, cytotoxins and phospholipases which support their subsistence and their toxicity [1,2].

These virulent factors are related to the toxicity of the bacteria and have a close relationship to gastric cancer. They can however be used to eradicate *H. pylori*. Through several mechanisms discussed in this section, it is possible to take advantage of these virulent factors to release the content of the nanoparticle near the bacterium, improving drugs concentration and decreasing side effects. Figure 3 summarises the strategies discussed in this subchapter.

Figure 3. Summary of different virulent factors, which can be used for actively targeting the bacterium

13.4.1. Phosphatidylethanolamine

The ability of *H. pylori* to colonise the surface between the mucus gel layer and epithelial cells is directly related to their affinity to gastric glycerolipids [88]. In 1989, Langwood *et al.* reported the affinity of this bacterium to a lipid demonstrated through solid-phase thin-layer chromatography (TLC) overlay procedure [88]. This lipid was found in higher amounts in human antral mucosa comparatively with fundal mucosa and in adults comparatively to infants [88]. This explained the tendency of colonisation of the antrum and the higher rate of infection in adults [88]. In this work, the glycerolipid recognised by *H. pylori* was isolated from the antrum of human stomach and from human

erythrocytes [89]. A few years later (1992), Langwood *et al.* analysed high performance liquid chromatography (HPLC) fatty acid profiles and the ability of the bacterium to bind different phospholipids through TLC overlay [89]. The main conclusion of their work was that the glycerolipid receptor for *H. pylori* is PE [89]. Due to the well-characterised PE from erythrocytes, they only analysed the erythrocytes receptor [89], however, they speculated that the receptor was the same in the human mucosa, possibly with changes in the fatty acids [89]. The hypothesis of Langwood *et al.* concerning the importance of PE was supported by Dytoc *et al.* (1993), with the study of adhesion of *H. pylori* to different culture cells [90]. Although other mechanisms are involved, there is an evident correlation between the ability of *H. pylori* to adhere to eukaryotic cells with PE [90].

Taking into account the binding of *H. pylori* to PE in the gastric mucosa, PE can be used as an active targeting strategy to treat *H. pylori* infection. PE constitutes one class of lipids, which assemble into nonbilayer structures under physiological conditions [77]. To overcome this limitation, different approaches have been used, such as a mixture of lipids and phospholipids anchored to polymeric beads (Figure 4). For instance, Bardonnnet *et al.* (2008) used epikuron 170 (phosphatidylcholine > 72 %, PE > 10 %, phosphatidylinositol < 3 %, lyso-phosphatidylcholine < 4 % and free fatty acids 10 %) and cholesterol to produce liposomes [13]. The formulation was however negatively charged and, given the charge of *H. pylori*, electrostatic repulsion occurred and prevented strong interactions [13]. Double liposomes composed of phosphatidylcholine, cholesterol and PE were also evaluated and both growth inhibition percentage and agglutination assays showed a higher efficiency of the system with PE [91]. Lipobeads composed of an acylated poly(vinyl alcohol) (PVA) core surrounded by a PE bilayer were also used to encapsulate acetohydroxamic acid [15]. Using agglutination assays, it was shown that all *H. pylori* strains recognised PE on the lipobeads as well as commercial PE [15]. Interestingly, lipobeads were able to plug and seal the PE receptor and, consequently, block the adhesion of *H. pylori* to KATO-III cells, which was concluded from radiolabelling assays [15]. This may imply that this specific targeting can optimise the treatment of *H. pylori* infections not only by a specific binding and local deliver of antibiotics near the bacterium, but also by precluding the attachment of the bacterium to the gastric mucosa.

Figure 4. Different approaches to using PE in the active targeting of *H. pylori*. These approaches include liposomes, double liposomes and lipobeads, with a phospholipid bilayer anchored to a polymeric core.

13.4.2. Cholesterol

Cholesterol is a steroid with a planar tetracyclic ring system and an extended carbon chain towards the bilayer centre [92]. It is very common in eukaryotic cells, namely in the gastric mucosa [93], and thus, Ansorg *et al.* (1992) evaluated the affinity of *H. pylori* for cholesterol through several studies, such as gas-liquid chromatography, adsorption procedures and agglutination assays [94]. Results showed a high affinity for cholesterol, and, more interestingly, washings were not sufficient to detach the cholesterol from the bacteria, which can indicate a strong binding or an uptake [94]. This feature of *H. pylori* strains (both reference and wild strains) does not extend to other bacteria, such as *Staphylococcus epidermidis* and *Escherichia coli* [93].

The relation between *H. pylori* and cholesterol is not merely affinity. *H. pylori* follows a cholesterol gradient and is able to extract this lipid from gastric epithelial membranes [95]. In fact, excessive cholesterol enhances phagocytosis of *H. pylori* and, consequently, inflammation [95]. Nevertheless, the glucosylation of cholesterol by the bacteria allows escape from the phagocytosis [95]. Cholesterol in combination with sphingolipids and phospholipids may create a rigid domain, called lipid rafts [96]. It is believed that the bacteria use these lipid rafts both to deliver bacterial virulence factors and to invade inside the host cells [96]. The knowledge of these mechanisms may be explored to develop drugs able to inhibit lipid rafts [96] or drugs to inhibit cholesterol glucosylation [95], in order to difficult the long-term persistent infection.

This affinity was also explored to test the potential of steroid hormones as antimicrobial agents against *H. pylori* due to their similarity with cholesterol

[97]. Hosoda *et al.* (2011) reported that progesterone inhibits the absorption of free cholesterol by the bacteria, suggesting that *H. pylori* may have a steroid-binding protein at the cell surface [97]. This may be very useful in order to develop a new strategy to target the bacteria and several nanoparticles were tested using cholesterol in their composition. For instance, Barbonnet *et al.* (2008) tested liposomes composed of epikuron 170 or 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) to load antimicrobial agents (ampicillin and metronidazole) [13]. Using epifluorescence microscopy, the authors visualized liposome-bacteria interactions that could be explained by the presence of cholesterol [13]. Obonyon *et al.* (2012) also evaluated liposomes composed of cholesterol, hydrogenated L- α -phosphatidylcholine and linolenic acid [18]. They observed that the interaction between liposomes and the bacteria was by fusion and not by adsorption or aggregation [18]. Fusion with the bacterial membrane is a significant advantage because it was reported that this mechanism outrides efflux pumps and barely induces drug resistance [18,24]. Both in double liposomes and lipobeads mentioned in the section 13.4.1., they also used cholesterol in the composition of the phospholipid bilayer [15,91]. Thus, positive results in agglutination and adherence studies were possibly enhanced by the presence of cholesterol. As well as the possibility of using cholesterol for specific targeting, it can be useful as a membrane stabiliser, particularly in the case of liposomes [92].

13.4.3. Vacuolating cytotoxin A

The ability of the bacteria to produce a factor that induces vacuolization in cultured cells was reported by Leunk *et al.* in 1988 [98]. In 1992, Cover *et al.* purified and characterised the vacuolating cytotoxin A (VacA) [99] and over the years knowledge concerning its mechanism and its influence on the toxicity of the bacterium has evolved. In fact, several mechanisms have been associated with this toxin, such as alteration of endo-lysosomal function, permeabilisation of the membrane and pore formation in the plasma membrane [100]. It has also been related to the formation of reactive oxygen species and gastric cancer [101].

Given its effect on the membrane, it is possible to use the VacA toxin to release antimicrobial agents, since near the bacterium the toxin will destabilise the phospholipid bilayer of liposomes through the formation of channels (Figure 5) [13]. The content of the liposomes would thus be locally released. This effect was proven using different liposomes. Moll *et al.* (1995) demonstrated that VacA toxin induces efflux of potassium from liposomes of asolectin [102]. In 2000, Pagliaccia *et al.* used liposomes of egg L- α -phosphatidylcholine and asolectin in order to prove the self-induced binding of VacA toxin into membranes and its ability to destabilise the membrane, resulting in the release of a fluorescent probe called calcein [103]. It was thus proposed that VacA toxin would bind to the lipid membrane and through a structural change would induce membrane leakage [103]. In fact, it

is known that VacA toxin binds as a monomer to lipid membrane, with a special affinity to lipid rafts, then oligomerises and penetrates [104]. Consequently, a channel is created, and given this evidence, VacA has been classified as a nonconventional pore-forming toxin with multifunctions [104]. The formation of channels was demonstrated using planar lipid bilayers of egg L- α -phosphatidylcholine, dioleoylphosphatidylserine and cholesterol [105]. Using atomic force microscopy, it was possible to see that the channel is a hexamer composed of assembly of monomers of VacA toxin [105].

Another approach is to use heparin nanoparticles, since a subunit of VacA can bind both heparin and heparin sulphate which would likely improve a local release [63].

Figure 5. Using the ability of VacA toxin to produce pores in the membrane (based on [104]) to local release of the content of a liposome

13.4.4. Enzymes

Similarly to the possibility of use VacA toxin to destabilise the nanoparticle, *H. pylori* is able to secrete different enzymes. For instance, phospholipases secreted by the bacteria are considered pathogenic factors, being related to increased risk of developing ulcers and gastric cancer [106,107]. The bacterium secretes several phospholipases (A1, A2, C and D) which can modify and degrade the gastric mucus [106]. The presence of sphingomyelinases in the bacterium has also been reported [108]. They are also able to induce the production of phospholipases from leukocytes [109], and therefore, liposomes or other type of nanoparticles with a phospholipid bilayer, such as a core-shell nanoparticle, can be used and destabilised by these enzymes. They are able to cleave phospholipids in specific locations, modifying their components and, as

a result, their structure [106]. Consequently, the content will be released near the bacterium and the inflammation. Other enzymes are also secreted by *H. pylori*, such as lipases and proteases [110], which can be used to destabilise lipid and protein nanoparticles.

13.4.5. Charge

In 1990, Smith *et al.* studied the surface hydrophobicity and surface charge of *H. pylori* in order to understand the mechanisms involved in their adhesion to the gastric mucosa [111]. Different studies were performed, namely hydrophobic interaction chromatography and measurement of contact angles with water, among other things [111]. These complementary methods revealed that *H. pylori* have a hydrophilic and negatively charged surface [111]. The negative charge of *H. pylori* was also reported by Pruul *et al.* in the same year [112]. Positively charged nanoparticles, mentioned in subchapter 13.3.2, may therefore be useful, and can simultaneously target the gastric mucosa and the bacterium. The importance of the charge was reported by Bardonnnet *et al.* (2008), since less electronegative liposomes showed the best results due to less electrostatic repulsion between the nanoparticle and *H. pylori* [13]. The charge of the bacterium can be changed as a resistance mechanism. For instance, the majority of gram-negative bacteria are able to mask lipid A negative charges, reducing the affinity of positively charged drugs to the outer membrane [113]. In the special case of *H. pylori*, they are able to modify its majority surface component [lipopolysaccharide (LPS)], through the modification of lipid A [114,115]. The lipid A 1-phosphate group is substituted by a residue of PE in the hydroxyl of the carbon-1 [115]. This results in a reduction of the negative charge [115], which has to be taken into account if the charge only is chosen as a target mechanism.

13.4.6. Carbohydrate receptors

Currently, it is well recognised that *H. pylori* can adhere to the gastric mucosa through specific adhesins. In 1998, Ilver *et al.* identified the adhesin binding fucosylated Lewis b (Leb) histo-blood group antigen (BabA) as a receptor for the adherence of *H. pylori* to the gastric mucosa [116]. A few years later (2002), Mahdavi *et al.* reported sialyl-dimeric-Lewis x glycosphingolipid (SabA) as a receptor for *H. pylori*, relating this fact to chronic inflammation [117]. BabA and SabA adhesins differ from each other in terms of amino acid composition and affinity for specific glycans, as the specific affinity of SabA to sialic acid and BabA to fucose [118,119]. Given the individual glycan profile of human gastric mucosa, *H. pylori* can benefit from having diverse adhesins [118].

Nanotechnology can take advantage of these adhesins on the surface of the bacterium (Figure 6), such as by using lectins, which are a diverse class of carbohydrates with the advantage of being non-immunogenic [120]. They are also bioadhesives, improving their retention time in the stomach [120].

Different lectins, namely *Ulex Europaeus Agglutinin I* (UEA I) and Concanavalin A (Con A) were tested by Umamaheshwari and Jain (2003) [50]. These lectins were covalently bounded to gliadin nanoparticles, which were chosen due to their mucoadhesiveness, and used to encapsulate acetohydroxamic acid [50]. Agglutination assays showed the efficacy of the binding between both lectins and the bacterium, compared with the absence of agglutination with non-conjugated gliadin nanoparticles [50]. The binding was inhibited by fucose and mannose, reflecting the involvement of the receptors [50]. The results were also confirmed using *in situ* adherence assays, where the carbohydrate receptors were completely plugged and sealed by the nanoparticles conjugated with lectins [50]. In the presence of these nanoparticles, the binding between *H. pylori* receptors and gastric mucosa carbohydrates was significantly affected, hindering the adhesion to the gastric mucosa [50]. Applying this strategy, triple drug loaded-nanoparticles were developed by Ramteke *et al.* and the results showed a superior *in vivo* clearance to that of non-conjugated formulations and free drugs [54]. Con A was also conjugated with ethylcellulose microspheres to encapsulate clarithromycin [121] and in conjugation with Eudragit S100 microspheres to encapsulate amoxicillin trihydrate [122].

Figure 6. Different approaches to targeting *H. pylori* using the linkage of fucose and lectins to carbohydrate receptors in the bacterium

Bardonnet *et al.* (2008) developed liposomes of DPPC or epikuron in conjugation with a synthetic glycolipid, which was composed of a cholesterol group, four ethylene glycol units and fucose [13]. The same group had already proved that liposomes with fucosyl neoglycolipids on the surface were stable [123]. Epifluorescence studies showed an enhancement of the interaction of liposomes with the bacterium when the fucosylated neoglypid was present [13]. They were able to bind both spiral and coccoid forms [13]. However, this was not possible in all strains, since some *H. pylori* strains do not express babA2 gene and, consequently, do not have the receptor to bind the fucose [13].

Fucose was also used in other kinds of nanoparticles, such as conjugated with chitosan-glutamate nanoparticles to deliver amoxicillin, clarithromycin and omeprazole [73]. The specific binding was confirmed through agglutination assays [73]. The targeting improved the clearance of the bacteria *in vivo* [73]. Lin *et al.* (2013) developed genipin-cross-linked fucose-chitosan/heparin nanoparticles to encapsulate amoxicillin and demonstrated the targeting through electronic microscopy and fluorescence studies [124].

13.5. STUDIES TO EVALUATE THE EFFICACY OF THE TARGETING

After optimising a nanoparticle for different parameters, such as size, zeta-potential, entrapment efficiency, profile of release at different pH and stability, it is essential to evaluate their efficacy. General studies can be performed, such as *in vitro* *H. pylori* growth inhibition, and specific assays can be chosen to evaluate their interaction with either the gastric mucosa or the bacterium.

13.5.1. Nanoparticle-gastric mucosa interactions

Mucoadhesiveness is one strategy extensively applied in the case of *H. pylori* infection. There are several possibilities for evaluating this property, including indirect and direct methods [39]. Indirect methods include microgravimetric methods, atomic force microscopy and diffusion/particle tracking methods [39]. Direct methods involve cytoadhesion methods and *ex vivo* and *in vivo* administration and imaging [39]. Herein, we will focus on different assays applied directly to the study of nano- and microparticles to be applied in the eradication of *H. pylori*. For instance, a piece of rat stomach, tied onto plastic, can be used to spread nanoparticles [125], then, disintegrating test apparatus is used to promote regular up and down movements of the system with the tissue and distilled water [125]. After 4 h, the number of microspheres still adhered is counted [125]. A more complex assay was performed by Liu *et al.* (2005), using cut stomachs incubated with microspheres in a chamber at 93 % relative humidity and room temperature [126]. After 20 min, tissues were fixed on a polyethylene support at an angle of 45° and washed with pH 1.3 HCl-physiological saline for 5 min (22 ml min⁻¹) [126]. The remaining microspheres were counted [126]. The results can be expressed in different ways, such as by the percentage of the remaining microspheres [126] or by the percentage of mucoadhesion, which can be calculated using the Equation 1 [127].

$$\% \text{ Mucoadhesion} = \frac{W_0 - W_1}{W_0} \times 100 \quad (1)$$

where w_0 and w_1 are the weight of microspheres applied initially and the weight of microspheres rinsed off, respectively.

Scanning electron microscopy was also used to monitor the *in vitro* wash-off test for mucoadhesive microspheres at different time points [128].

Another approach was applied by Wang *et al.* (2000) using male wistar rats to remove stomach under anesthesia [55]. After washing the content of the stomach, RITC-labeled microspheres suspended in simulated gastric fluid were filled into the stomach [55]. After incubation for 30 min, the stomach was washed and perfused with simulated gastric fluid for 30 min (1.0 ml min^{-1}) [55]. Fluorescence spectrophotometry was then used to determine the percentage of microspheres retained in the stomach after degradation of the microspheres with trypsin [55].

Another *in vivo* approach was used by Liu *et al.* (2005), using thirty rats divided into six groups, of which three were administered with microspheres and three with placebo [126]. They were sacrificed at 2, 4 and 7 h and microspheres remained in the gastrointestinal tract were counted [126]. *In vivo* mucoadhesion was evaluated by Ramteke *et al.* (2008) using nanoparticles containing barium sulphate as a contrast agent [54]. The authors used albino rats which were recorded by X-ray photographs at different times [54].

It is also possible to evaluate the ability of the nanoparticle to block the binding of *H. pylori* to mucosa. For this purpose, Umamaheshwari *et al.* (2004) performed adherence assays using labelled *H. pylori* and KATO-III cells (gastric epithelial cells) [15]. *H. pylori* preincubated with lipobeads were washed and added to the suspension of KATO-III cells [15]. Six washes were used to remove non-adherent bacteria [15]. The number of bacteria adherent was counted using disintegrations *per* minute in a scintillation counter after trypsinization to remove adherent bacteria and KATO-III cells from the wells [15]. *In situ* adherence assays can also be used for the same purpose [15]. Fluorescein isothiocyanate-labeled (FITC-labeled) bacteria preincubated with lipobeads for 2 h were washed and then added to tissue sections of samples of oesophagus, stomach, duodenum and colon [15]. *In situ* binding was demonstrated through fluorescence microscopy [15].

13.5.2. Nanoparticle-*H. pylori* interactions

Agglutination assays can be performed by mixing the same amount of bacterial suspension and the nanoparticle formulation [50]. The agglutination reaction can then be scored according to the size of clumps [50]. This assay can also be used to confirm whether a specific ligand is involved, pre-incubating with specific inhibitors of agglutination [50]. The agglutination can be however affected by the self-aggregation of some strains [13]. Bardonnnet *et al.* (2008) used epifluorescence studies, with the bacteria stained with DAPI by fluorescent *in situ* hybridization technique and liposomes marked with NBD-PC

[13]. The mixture was observed by epifluorescence microscopy and the superimposition of the two dyes suggested that liposomes were aggregated around *H. pylori* [13].

Another labelling technique was used by Gonçalves *et al.* (2013), using *H. pylori* previously marked with FITC [61]. The interaction with auto-fluorescent chitosan microspheres was demonstrated through confocal laser scanning microscopy (Figure 7) [61].

Figure 7. Confocal microscopy image of chitosan microspheres (in red) with adherent FITC-labelled *H. pylori* strain (17875/Leb) (in green) under pH 6.0 (adapted from [61]). The scale-line is representative of the whole system.

Quantification of adhesion of ^{35}S -labeled *H. pylori* can be achieved using a luminescence counter through the determination of the radioactivity of each well of polyethylene terephthalate plates [61]. Knowing the concentration in colony-forming unit (CFU).ml⁻¹ of the initial *H. pylori* inoculum (C_{Hp}^i), the activity of *H. pylori* adherent in c.p.m. ($A_{\text{Hp}}^{\text{mic}}$), the activity of the initial *H. pylori* inoculum in c.p.m.ml⁻¹ (A_{Hp}^i) and knowing the number of microspheres of each well (N_{mic}), which can be counted using a camera coupled to a stereomicroscope, it is possible to determine the number of adherent bacteria per microsphere (Equation 2) [61].

$$\frac{\text{Number of adherent bacteria}}{\text{microsp here}} (\text{CFU per microsphere}) = \frac{(C_{\text{Hp}}^i \times A_{\text{Hp}}^{\text{mic}} \div A_{\text{Hp}}^i)}{N_{\text{mic}}} \quad (2)$$

Other techniques of microscopy can be used, such as transmission electron microscopy (TEM) to demonstrate both the distribution of the nanoparticle around the bacteria and their effect on the morphology of the bacteria [124].

13.6. CONCLUSION

Nanotechnology is a growing and promising field, which has been extensively studied in order to overcome limitations of current therapies in several diseases. In the special case of *H. pylori* infection, nanoparticles have been proving their utility in improving the efficacy of antibiotics, by protecting them from the environment and by promoting a controlled, sustained and local delivery of drugs. The local delivery can be achieved by targeting gastric mucosa or the bacterium.

In the development of a targeting nanoparticle is important to initially choose the nanoparticle and the strategy most suitable for the purpose in mind. In this process, it is essential to take into account the balance between various approaches. Extreme mucoadhesiveness may be prejudicial to penetration within the mucosa due to the decrease of mobility and it is affected by the high turnover of the gastric mucosa [29,129]. The pH gradient is also a key point, since the gastric medium is around pH 1.2 whereas the surrounding medium of the bacterium is at neutral pH. This large pH gradient can also affect the charge of the nanoparticles if it includes the pK_a of the nanoparticles components. The charge of the bacterium can be changed by resistance mechanisms, and therefore it is important to take these possible changes into account. Other mechanisms, such as translocation, coordinated response of tissues, enzymes and toxicokinetics, may affect the specificity of the targeting and even be involved in toxicity mechanisms [130].

Above all, the possibility of combining different strategies in order to increase the local release is very attractive, and advantageous to decreasing the development of antibacterial resistance.

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Chapter

14

POLYMERIC MICELLES FOR CUTANEOUS DRUG DELIVERY

Sevgi Güngör*, Emine Kahraman, and Yıldız Özsoy

Department of Pharmaceutical Technology, Faculty of Pharmacy,
University of Istanbul, 34116 Istanbul, Turkey

*Corresponding author: sgungor@istanbul.edu.tr

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14.1. INTRODUCTION

Polymeric micelles are colloidal carriers which are nano-sized assemblies. They are composed of amphiphilic block polymers and characterised by core-shell morphology formed through self-association of hydrophilic and hydrophobic block copolymers in water. Micelles can increase the aqueous solubility of hydrophobic compounds in their inner cores. They have also other advantageous including improvement of the chemical stability of drugs, and easy scale-up procedure for industrial production. Micelles have therefore been widely investigated for use in the nasal, ocular, and skin delivery of drugs to overcome the natural transport barrier of biological membranes [1-3].

In recent years, different types of nano-sized carriers have been widely investigated for the cutaneous delivery of drugs. Micellar carriers have also been explored for the topical delivery of drugs via skin. The cutaneous delivery of drugs, particularly for the treatment of skin diseases, would be beneficial in terms of improving the bioavailability of hydrophobic drugs, the targeting of drugs into skin layers, controlling the release rate of drugs, decreasing side-effects such as irritation, and protecting the drugs from physicochemical conditions such as light, oxidation, *etc.* Nano-sized polymeric micelles have thus been considered a promising drug carrier for the effective treatment of various skin diseases [4-6].

This chapter is an overview of polymeric micelles as nano-sized carriers for the skin delivery of drugs, with an emphasis on micelle-forming copolymers, types of polymeric micelles, the preparation of polymeric micelles, the factors affecting the drug loading capacity of the micelles and the characterisation of micelles. Skin structure and penetration pathways are also briefly reviewed for background information. Finally, recent studies in which micelles have been developed for the cutaneous delivery of drugs.

14.2. MICELLES

Micelles, created from two different regions with opposite affinities towards a particular solvent, are "aggregated colloids" between 5 and 200 nm in size, which are spontaneously formed from amphiphilic or surfactant agents at a defined concentration and temperature. Amphiphilic molecules, while found separately at low concentrations in an aqueous solutions, create aggregation of micelles as the concentration is increased. The monomeric amphiphilic concentration at which micelles are observed is called the critical micelle concentration (CMC) [1]. Below the CMC, the concentration of amphiphiles adsorbed at the water/air interface increases as the total system amphiphile concentration increases. After a period of time the interface and system

monomers become saturated and CMC is obtained. Any amphiphiles added after this concentration has been reached assemble to form micelles in the system, and thus free energy reduces in the system.

Micelles are nano-sized colloidal carriers with a hydrophobic core and hydrophilic shell (Figure 1). While the core acts as a reservoir for hydrophobic drugs, the shell of micelles provides hydrophilic properties for the system [2,3].

Figure 1. The formation of drug loaded micelles

Depending on the weight of the molecule, these systems can be divided into two groups; low molecular weight surfactant micelles, and polymeric micelles [7]. Polymeric micelles formed through the use of amphiphilic copolymers have a lower CMC compared with surfactant micelles, and they are therefore more stable under *in vivo* conditions [8,9]. Another advantage of polymeric micelles is their lack of serious side effects [10].

14.3. POLYMERIC MICELLES

Polymeric micelles are composed of block copolymers consisting of hydrophilic and hydrophobic monomer units. In some special cases, the components of copolymer may also be two hydrophilic blocks. One of these blocks is modified by coupling it with a hydrophobic agent (such as taxol, cisplatin or hydrophobic diagnostic agents) and an amphiphilic copolymer-formed micelle occurs [11]. By controlling the length of the hydrophilic/hydrophobic blocks, copolymers of differing hydrophilic-lipophilic balance (HLB) and molecular weights can be synthesised. The physicochemical and biological properties of the copolymers can be controlled by the molar ratios of the different blocks inside the copolymers. The aggregation number of

polymeric micelles is approximately a few hundred amphiphiles, dependent on size, which have diameters within the range of 10–100 nm. The size of the micelle is dependent on the relative proportion of hydrophilic and hydrophobic chains, the molecular weight of the amphiphilic block copolymer and the number of amphiphile aggregations [8,12].

Polymeric micelles provide several advantages, such as their nano-size, ease of scale-up studies, increased drug solubility and chemical stability. Given these advantages, there has been much research into their use as drug and gene delivery systems *via* parenteral [13,14], oral [15,16], nasal [17,18], ocular [19,20], and topical/transdermal [21,22] applications.

14.3.1. Micelle-forming copolymers

Polymeric micelles made from amphiphilic blocks (di- or tri-) or of graft copolymers (Figure 2) have received much attention in recent years [10,23]. For synthesised copolymers to form micelles, there needs to be a balance between the hydrophilic blocks forming the micelle shell and the hydrophobic block forming the core. For this reason, some simple arrangements are made for the amphiphilic unimers. Poly(ethylene glycol) (PEG) blocks with a molecular weight of 1–15 kDa should be used to form the shell, for example, while the length of the hydrophobic block forming the core should be around the same length or slightly shorter than the hydrophilic block [10].

Figure 2. Main structural types of copolymers and micelles formed from amphiphilic copolymers (reprinted with permission from [24], John Wiley and Sons)

The most amphiphilic block copolymers contain polyester, polyether or a poly(amino acid) derivative as hydrophobic block. Generally, the hydrophobic

core comprises a biodegradable polymer such as poly(ϵ -caprolactone) (PCL), poly(D,L-lactic acid) (PDLL) or poly(β -benzoyl-L-aspartate) (PBLA), and acts as a reservoir for drugs with poor water solubility and protects them from contact with the aqueous medium. The block which forms the core can also be a water-soluble polymer rendered hydrophobic by the chemical conjugation of a hydrophobic drug [*e.g.* poly(aspartic acid) (PASP)]; polystyrene with good stability given its glassy properties (PST) a non-biodegradable polymer such as poly(methyl methacrylate) (PMMA); an alkyl chain or diacyl lipid (*e.g.* distearoylphosphatidylethanolamine (DSPE)) [8].

The hydrophilic polymer forming the shell of the polymeric micelle is responsible for the interaction with cell membranes, and for providing effective steric protection for micellar structure. The shell structure determines the hydrophilicity, charge, size of the micelles, the surface density of the hydrophilic block, and the presence of a suitable reactive group for the addition of targeting molecules. These characteristics control the important biological properties of the micellar carriers, such as pharmacokinetics, biodistribution, biocompatibility, circulation time in the blood, the surface adsorption into biomacromolecules, adsorption into bio-surfaces and targeting [9,10]. PEG, with high solubility in water and the ability to easily combine with hydrophobic blocks, is usually used as the hydrophilic block. In addition, poly(acrylamide), poly(hydroxyethyl methacrylate), poly(*N*-vinylpyrrolidone) (PVP) and poly(vinyl alcohol) (PVA) can also be used as the hydrophilic block [8,10,23].

The physicochemical and biological properties of the copolymer can be controlled by the molar ratios of the different blocks inside the copolymer. The proper arrangements for a specific focus can be made by modifying core functions and the surface chemistry [8,10].

14.3.2. Types of polymeric micelles

Polymeric micelles, which resist the various intermolecular forces and keep the core separate from the aqueous medium, can be divided into three categories.

14.3.2.1. Conventional micelles

These micelles are formed by the hydrophobic interaction between the core and the shell in the aqueous medium. One of the simplest amphiphilic block copolymers, poly(ethylene oxide)- β -poly(propylene oxide)-*b*-poly(ethylene oxide) forms micelles as a result of hydrophobic interactions [25].

14.3.2.2. *Polyion complex micelles*

Like polyelectrolytes, the electrostatic interactions between two oppositely charged parts also allow the formation of micelles. Oppositely charged polymers penetrate the shell of the micelles when added to the solution, and polyion micelles are formed. Electrostatic forces and van der Waals interaction forces control the shell structure and size of the charged micelle. These micelles easily and spontaneously occur in aqueous media, are structurally stable and have a high drug loading capacity [25].

14.3.3.3. *Non-covalently bounded polymeric micelles*

Here, polymeric micelles are obtained through the self-assembly of random copolymers, graft copolymers, homopolymers or oligomers and powered by the driving force of interpolymer hydrogen bonding complexation. The core and shell are non-covalently bounded by the ends of their homopolymer chains, either through intermolecular interactions such as H-bonding, or metal-ligand interactions occurring in their structure, and as such are known as non-covalently bounded polymeric micelles [25].

14.3.3. Preparation of polymeric micelles

Simple equilibrium, dialysis, o/w emulsion, solution casting and freeze-drying methods are used in the preparation of polymeric micelles [26] (Figure 3). If the block copolymer is water-soluble, a simple equilibrium method is used; if not, the dialysis method [10]. While the drug is loading into the micelles, its physicochemical properties are crucial. Hydrophobic drugs can be loaded into the micelle by chemical or physical interaction [8,26]. In order to load hydrophilic compounds such as proteins into the micelles, the molecules should be made chemically hydrophobic. In order to load the drugs *via* ionic interactions into the micelles, there must be an opposite charge on the surface of the copolymers' hydrophobic block. In the event that a drug is to be chemically or electrostatically bound to the hydrophobic block, the formation of micelles and their combination with the drug should be carried out simultaneously. Strong polymer-drug interaction increases the load on the micelle core, but reduces the micelles' drug release. For this reason, the loading amount and drug release kinetics must be optimised. Micelles have also been obtainable through microfluidic technology in recent years [27].

14.3.4. Factors affecting the drug loading capacity of the micelles

There are many factors that affect the loading capacity of hydrophobic drugs to the micelle-forming amphiphilic copolymers. These factors are summarised below.

Figure 3. Common drug-loading procedures: (A) simple equilibrium, (B) dialysis, (C) o/w emulsion, (D) solution casting, and (E) freeze-drying

14.3.4.1. Factors belonging to copolymers

The copolymer concentration, the length and hydrophobicity of the blocks forming the core, and the structure and length of the block forming the shell affect the loading capacity of micelles [28]. An increase in copolymer concentration, hydrophobic block length and the hydrophobicity of the core increases the loading capacity. When the length of the hydrophilic block is increased, the CMC also increases and the loading capacity decreases. By affecting the hydrophobic/hydrophilic balance of the drug molecule, where the drug core or shell are placed and the loading capacity are determined. Drug molecules situated on or close to the micelle shell are released quickly. For this reason, in order to delay the release of the drug, the drug molecules should be dissolved in the core of the micelle or be loaded to the core in a separate phase [23].

Interactions between the drug and the micelle core (hydrophobic, hydrogen bonding, ionic interactions, etc.). These interactions depend on properties such as the polarity, hydrophobicity and charge of the drug. Sometimes the interaction between the drug, hydrophilic shell, and soluble drug can also affect the dissolution process either positively or negatively [10,23].

Micellar preparation methods and process-specific parameters (such as the structure of the organic solvent, solvent ratio) affect the drug loading capacity [23].

The polymer-drug miscibility is a crucial parameter for drug loading capacity of the micelles. Hildebrand-Scatchard solubility parameters are mostly used for this [29] and drug loading capacity can be calculated using the following formula, the Flory-Huggins theory. If the Flory-Huggins parameter is a low value (< 0.5), it signifies that the drug is well dissolved in the core of the polymeric micelle.

$$X_{\text{drug-polymer}} = (V_{\text{drug}} / R \cdot T) (\delta_{\text{drug}} - \delta_{\text{polymer}})^2 \quad (1)$$

where; $X_{\text{drug-polymer}}$ is the Flory-Huggins interaction parameter between the drug and the polymer, V_{drug} is the volume of the drug, R is the ideal gas constant, T is the temperature, and δ_{drug} and δ_{polymer} are the Hildebrand-Scatchard solubility parameters of the drug and the polymer, respectively [30,31].

14.3.5. Characterisation of micelles

14.3.5.1. Size and size distribution

One of the most interesting properties of polymeric micelles is their small size. A micelle's size rarely reaches 100 nm. This situation depends on factors including the relative proportions of hydrophobic and hydrophilic chains, the number of amphiphile aggregations, the molecular weight of the amphiphilic copolymer and the micelle preparation method [8]. Kim and colleagues [32], also found that the solvent used to form micelles affected the size and size distribution of micelles. The hydrodynamic size and polydispersity of micelles can be measured by dynamic light scattering (DLS) in an isotonic buffer or in the water. Micelle size can also be calculated using atomic force microscopy (AFM), transmission electron microscopy (TEM) or scanning electron microscopy (SEM) studies. These methods also allow the micelle size distribution and morphology to be characterised [8,33].

14.3.5.2. Morphology

It is generally accepted that there are spherical particles with a clear distinction between the micelle core and shell. In aqueous media, amphiphilic

block copolymers self-aggregate towards spherical, worm-like or cylindrical micelles, polymer vesicles or polymersomes. Here, the main factor controlling micelle morphology is the hydrophilic volume fraction (f) defined by the hydrophilic-hydrophobic balance of the block copolymer [25]. Along with this, the copolymer aggregation, the organic solvent used to prepare the micelles, and the copolymer composition can be considered the main morphogenic factors [10].

14.3.5.3. Zeta potential

The stability of the colloidal particles is the basic parameter with regards to the zeta potential (ζ -potential) of micelles. It is also affected by the interactions of biological elements such as cell membranes (which define cell uptake and the particles' pharmacokinetics) and proteins [34]. Generally, the absolute value of ζ -potential is 20–50 mV. The charge of the colloidal particles is also very important in terms of system stability. In many cases, with a higher ζ -potential value, there is stronger repulsive interaction between the surface charge of the colloidal particles and the dispersed particles, and as such higher stability and a more uniform diameter is observed [35].

14.3.5.4. Stability

The determination of the stability of polymeric micelles is important for their characterisation, and their stabilities can be examined as thermodynamic stability and kinetic stability. Thermodynamic stability is crucial for *in vivo* conditions, and kinetic stability is crucial for *in vitro* conditions.

The thermodynamic tendency which breaks the individual chains of the micelles reflects CMC [23]. When the polymer concentration is above the CMC in the aqueous medium, the polymeric micelles are thermodynamically stable. If below the CMC, the amphiphilic block copolymers in the aqueous medium are found to have single chains in the bulk phase at the air-water interface. When the polymer concentration is increased above the critical micelle concentration, as a result of the hydrophobic interactions between the hydrophobic blocks, amphiphiles self-aggregate and the system's Gibbs energy (ΔG) is reduced to the lowest level [12].

The kinetic stability of a micelle system is related to the single polymer chain exchange rate between the bulk and micelles. If the block forming the micelle core is semi-crystalline, the structure of the micelle is dependent on the glass transition temperature (T_g) and/or the melting temperature (T_m), and even at dilution levels below the CMC, the micelles remain kinetically stable for a long period of time. The dissociation speed of the micelles is related to the strength of interactions in the micelle core. Those interactions are dependent on factors such as the physical structure of the polymer comprising the core (crystalline or amorphous); the presence of the solvent in the micelle core; the length of

the hydrophobic block; the hydrophilic/hydrophobic block ratio; and the encapsulation of hydrophobic compounds [23,26].

To avoid stability problems, polymeric micelles should have a glassy or crystalline core at their preferred body temperature, and should be formed of copolymers with a low CMC [12]. With the end goal of increasing the thermodynamic and kinetic stability of drug-loaded micelles by reducing the CMC, approaches such as increasing physical interaction/covalent cross-linking, modifying the micelle-forming polymers, and enhancing drug-polymer interactions have been discussed [26].

Briefly, stability tests of micellar solutions consist of observing whether or not there is any basic phase dispersion determining particle size in order to ascertain aggregation, and analytically measuring the concentration of drug and block copolymers in the formulation [8,10].

14.4. MICELLES FOR DRUG DELIVERY *via* SKIN

14.4.1. The structure of human skin

Human skin is a unique, well-designed membrane and its fundamental functions are to protect organisms from environmental factors and, to regulate transepidermal water loss from the body. Histologically, it is composed of three main layers, the *epidermis*, the *dermis*, and the *hypodermis* (subcutaneous tissue) [36-38]. The *epidermis* is also divided into two layers, the *stratum corneum* and viable *epidermis*. The *stratum corneum* consists of *corneocytes* which are dead, flattened, keratin-rich cells. The *corneocytes* are embedded in the mixture of intercellular lipids. The *stratum corneum*, the outermost layer of *epidermis*, is an extremely effective barrier for the penetration of most drugs due to its excellent structure. It behaves as a rate-limiting barrier for diffusion for almost all drugs due to its well-ordered structure [38,39]. In topical treatment, in most cases drugs should pass the *stratum corneum* to reach deeper layers of the skin for the efficiency of therapy, but, the main problem in effective skin delivery is the low diffusion rate of drugs across the *stratum corneum*. As well as the physicochemical characteristics of drugs, the features of topical formulation are also effective parameters in dermal drug delivery. Overcoming the barrier characteristics of skin for the improvement of cutaneous drug delivery is thus the major challenge [41-43].

14.4.2. The skin penetration pathways

Drugs pass through the skin barrier *via* three potential pathways: *transcellular*, *intercellular* and/or *transappendageal* (shunt) routes (hair *follicles*, sweat glands, and *sebaceous* glands) [36,37,44]. The contribution of each pathway to the permeation of drugs across the skin is mainly related to the physicochemical properties of the drugs. While the *intercellular* route has been

regarded as the main transport pathway of most drugs and, the *transcellular* route has become more important as a polar route. Since appendages such as hair *follicles* and glands called as shunt pathway comprise only 0.1 % of the human skin surface area, their contribution to skin penetration is considered less significant. It has been suggested that the shunt route may provide advantageous delivery of polar and ionized drugs that would not be easily delivered *via* the lipid domain of the *stratum corneum* [36,37,44,45]. It has also been indicated that transport pathways for the penetration of topically applied drugs could be important in targeting the skin appendages, in particular targeted follicular delivery. Follicular penetration is suggested as a possible pathway for the rigid particulate carriers [46-49]. Prow *et al.* indicated that nanoparticles (> 10 nm) are unlikely to penetrate the *stratum corneum* and the nanoparticles would accumulate in the hair *follicle* openings [50]. They emphasised that the topical delivery of nanoparticles through skin takes place in three major sites, including the *stratum corneum* surface, furrows, and the openings of hair *follicles* (*infundibulum*) (Figure 4). On the other hand, it was demonstrated that polymeric nanoparticles (20–200 nm) penetrate only into the surface layers, based on the confocal microscopy images [51]. The researchers mostly demonstrated that nanoparticles only permeate the superficial layers of the skin [50-54].

Figure 4. Delivery of nanoparticles into possible sites of skin: (a): *stratum corneum* surface; (b): furrows in skin; (c): opening of hair *follicles* (*infundibulum*); *stratum corneum* (SC); *viable epidermis* (E); *dermis* (D) (reprinted with permission from [50], Elsevier)

14.5. APPLICATIONS OF POLYMERIC MICELLES AS DRUG CARRIERS IN TOPICAL TREATMENT

Topical treatment is an attractive option for curing cutaneous diseases, as it has advantages such as targeting drugs to the site of the disease and reducing the systemic side effect risk of drugs. The efficiency of therapy in skin diseases could be enhanced and high patient compliance can be provided. The success of the topical treatment depends on the penetration of drugs into the targeted layers of skin, however, and the achievement of effective drug levels in these skin layers. In this context, the contribution of topical formulation composition on the efficiency of cutaneous drug delivery is considerable high. In most cases, conventional dosage forms may be insufficient to ensure effective drug concentrations in targeted layers of skin due to poor skin penetration of drugs. The delivery of drugs to target regions of the skin is thus a great challenge in terms of improving therapy.

Numerous attempts have been made to develop novel topical drug delivery systems, including different types of nano carriers that can improve partition and/or drug penetration across skin. Micro- and nano-sized particulate drug carriers have been optimised to carry the drugs to the targeted layers of the skin [4,56-63]. Micelles are one of the polymeric nano-sized drug carriers particularly used to improve the aqueous solubility of drugs and to enhance the permeability of drugs across membranes [5,6]. Micellar particle technology for transdermal delivery of estradiol and other steroid compounds were patented for the first time in 1997. Estrasorb® is a lotion type topical product which consists of micelles loaded 17 β -estradiol, which has been commercially available since 2003. It was claimed that the amount of estradiol in the micellar carrier was improved due to increasing the solubility of drug, and it was anticipated that micellar carriers would act as depots for estradiol in *stratum corneum* and *viable epidermis*. It was also indicated that Ostwald ripening observed in micelles had not occurred in that product, and that the product had stabile structures with a three year shelf-life [6].

In recent years, micelles as nano-sized carriers have also been investigated for the delivery of other drugs *via* the skin. In these studies, micellar carriers have been developed for the treatment of *psoriasis* (tacrolimus and cyclosporine) [64,65]; and photo-aging (all-*trans* retinoic acid) [66]; breast cancer (endoxifen) [67]; and skin cancer (oridonin) [68], fungal infections (clotrimazole, econazole nitrate and fluconazole) [21], and acne (benzoyl peroxide) [69], and the skin permeation of drugs from micelles was also examined. The micellar carriers of quercetin and rutin as antioxidant compounds [70], and polymeric micelle loaded sumatriptan for the treatment of pain in migraine [71] have also been optimised, and the efficacy of these formulations have been evaluated with *in vitro/in vivo* studies. In another study, an anti-inflammatory drug was loaded into a core of polymeric micelles, which resulted in delayed drug release and slow drug permeation due to the

core and shell structure. Based on that data, it was reported that these structures could be also considered to delay or inhibit dermal delivery of drugs [72]. In another study, when compared to its ethanolic solution, the structures resembling micelles increased the solubility of a lipophilic compound, resulting in efficient treatment for chronic wounds and burn therapy [73]. None of the micellar carriers of these drugs have been clinically approved yet, however.

Researchers have also focused on the possible pathways for micelles to pass across skin. It has been shown that polymeric micelles provide the localization of drugs in skin layers following hair follicle pathways based on data obtained from confocal laser microscopy. The deposition of micelles into hair *follicles*, and between coenocytes and in the inter-cluster regions have been shown with confocal microscopy studies, and the micelles have been proposed as potential carrier for targeted delivery to hair *follicles* [21,64,65].

The studies performed on the development of polymeric micellar carriers for cutaneous drug delivery have been summarised below, and the polymers used in the formulation of micelles and the features of micelles optimised are given in Table 1.

14.5.1. Cyclosporin

Cyclosporin A is an immunosuppressant and it is currently indicated in the treatment of psoriasis *via* the oral route [74]. Lapteva *et al.* prepared polymeric micelles using biodegradable and biocompatible diblock copolymers to increase skin delivery of cyclosporin A for dermatological use [64]. Micelle loaded cyclosporin was reported as homogeneous and spherical in shape, with a range of 25–52 nm particle size, and the low aqueous solubility of cyclosporin had been increased approximately 500 times using micellar carriers. In this study, the localisation of both drugs and copolymer was also investigated using confocal laser scanning microscopy. Both copolymer and cyclosporin A were chemically bounded to fluorescent dyes. Based on the images obtained from confocal laser scanning microscopy, it was reported that micelles were localised between corneocytes and in the inter-cluster regions. The authors also indicated that inter-cluster penetration was likely the preferred transport route of micelles, and that it provided enhancement of the cutaneous delivery of cyclosporin A. The authors also emphasised that the efficiency of micellar carriers should be validated *in vivo* with diseased skin due to the features of psoriatic skin.

Table 1. Micelles prepared for skin delivery in the literature

Active compounds	Polymers used in the composition of micellar carrier	Method	Size of micelles	Ref.
Clotrimazole Econazole nitrate Fluconazole	Amphiphilic methoxy-PEG-hexyl substituted polylactide (MPEG-hexPLA) block copolymers	Sonication / film hydration method	30–40 nm	[21]
Tacrolimus	Methoxy-PEG-dihexyl substituted polylactide diblock copolymer	Solvent evaporation method	10–50 nm	[64]
Cyclosporine	Methoxy-PEG-dihexyl substituted polylactide diblock copolymer	Solvent evaporation method	25–52 nm	[65]
Retinoic acid	PEG conjugated phosphatidylethanolamine	NG	6–20 nm	[66]
Endoxifen	PCL with multiple hydrophilic PEG chains mediated by a generation 3 (G3) polyester dendron	NG	40–50 nm	[67]
Oridonin	Monomethoxy PEG-PCL	Film hydration method	25 nm	[68]
Benzoyl peroxide	PEG-b-poly(propylene glycol)-PEG	Film method	25–30 nm	[69]
Quercetin and Rutin	PCL-b-PEG	NG	NG	[70]
Sumatriptan	PEG-b-poly(propylene glycol)-PEG	Emulsification method	NG	[71]

NG = not given

14.5.2. Tacrolimus

Tacrolimus is also a potent macrolide immunosuppressant compound [75]. The same researchers prepared the polymeric micelles of tacrolimus and evaluated the possibility of delivering tacrolimus into targeted layers of the skin [65]. They showed that the accumulation of tacrolimus in the *stratum corneum*, *viable epidermis*, and upper *dermis* has been increased. Based on confocal laser scanning microscopy data, it was reported that copolymers used in the composition of micelles would not pass through skin and that micelles were deposited in hair *follicles*. They also emphasised that micellar carriers of tacrolimus could be considered effective due to their superior efficiency in its conventional ointment formulation.

14.5.3. Sumatriptan

Sumatriptan, 5-hydroxytryptamine 1D (5-HT_{1D})-receptor agonist, is used in the treatment of migraine *via* oral and parenteral administration routes [76], but it has problems of low bioavailability due to its pre-systemic metabolism in oral administration. In addition, nausea or vomiting problems have also been seen in migraine attacks, which lead to insufficiency in oral treatment. In order to overcome these disadvantages of the oral route, the efficiency of transdermal delivery of sumatriptan *via* micellar carriers has been assessed [71]. Lesitin organogels of sumatriptan composed of thermoreversible micelles were optimised to improve its stability and the permeation of sumatriptan across the skin. The authors indicated that the optimised formulation was stable, without any significant changes at room temperature. The improved topical delivery of sumatriptan has been attributed to improved drug solubility. It was also proposed that the prepared sumatriptan micelles containing lecithin and pluronic copolymers were considered to be a safe and stable drug delivery system.

14.5.4. Endoxifen

Endoxifen, one of the active metabolites of tamoxifen, was shown to be effective in the prevention and treatment of oestrogen-positive breast cancer [77], however, severe side effects are observed following its oral administration. The dendron micelles with various surface groups ($-NH_2$, $-COOH$, or $-Ac$) of endoxifen were optimised to increase the localisation of endoxifen in the targeted tissue following its topical administration [67]. The modification of end-groups of micelles were reported to affect the drug loading capacity of micelles, and that resulted in the highest encapsulation efficiency, with micelles having $-COOH$ surface end groups. When the skin delivery of endoxifen was examined, it was determined that the dendron micelles increased the permeation of endoxifen across both mouse and human skin, and the dendron micelles with $-COOH$ end groups showed the highest endoxifen flux through skin. Based on these results, the authors claimed that dendron micelles could be an effective carrier for the topical delivery of endoxifen as a potential alternative administration route.

14.5.5. Oridonin

Oridonin, a natural tetracycline diterpenoid isolated from *Rabdosia rubescens*, has been reported to be a potent cytotoxic agent [78]. Micellar carriers were prepared in order to increase aqueous solubility, and its permeation across excised mouse skin was studied [68]. It was shown that the micellar carrier of oridonin had higher transdermal delivery compared to its saturated solution. The authors indicated that the micelles of oridonin could be considered for intravascular administration as a transdermal drug delivery system in cancer chemotherapy.

14.5.6. Clotrimazole, econazole nitrate and fluconazole

Clotrimazole, econazole nitrate and fluconazole are azole group antifungal compounds which are widely used in their conventional formulations, such as ointment, topically [79] but the efficiency of topical antifungal treatment is affected due to the low aqueous solubility of drugs. Bachav *et al.* optimised the micellar carriers of these antifungal drugs to improve the deposition of these antifungals in the target layer after application on both porcine and human skin [21]. It was demonstrated that the deposition of econazole in both human and porcine skin was significantly higher than in its commercial liposome formulation. The authors reported, according to confocal laser scanning microscopy data, that the micellar carriers may increase targeted follicular delivery.

14.5.7. Benzoyl peroxide

Benzoyl peroxide is one of comedolytics frequently used in the treatment of mild and moderate acne [80]. Although commonly used by the patients, it has several side effects including skin irritation depending on doses of benzoyl peroxide, skin dryness, contact allergy, burning, scaling, itching and erythema, resulting in poor patient compliance. The deposition of anti-drugs is also required in the *pilosebaceous* units of the skin for the efficiency of topical acne treatment. benzoyl peroxide (BPO) loaded polymeric micelles were prepared using the thin film hydration method and various solvents to decrease the side effects of BPO and increase the deposition of BPO in the *pilosebaceous* units [69]. These were about 25 nm in hydrodynamic diameter with a narrow polydispersity index in the water and had encapsulation efficiency. Confocal laser scanning microscopy studies showed that Nile red loaded polymeric micelles were localized in the hair *follicles*.

14.5.8. Retinoic acid

The topical application of all-*trans* retinoic acid (ATRA) has been used in the treatment of several dermatological diseases such as photo-aging [81]. Polymeric micelles of retinol have been formulated to improve of photo-stability of retinol, because retinol is very sensitive to light, heat, and oxidizing agents. ATRA entrapped in polymeric micelles with various PEG and PE structures have thus been prepared [66]. The authors reported micelles of ATRA composed of PEG (molecular weight of 750 Da) and that conjugation of dipalmitoylphosphatidylethanolamine (PEG750-DPPE) showed the highest entrapment efficiency (82.7 %) among the tested micelles. It was demonstrated that up to 87 % of ATRA were measured following the storage of the PEG750-DPPE micelle solution in ambient media for 28 days. Based on that data PEG750-DPPE micelles of ATRA were proposed as an alternative carrier for the formulation of cosmeceutical formulations due to its improved photo-stability.

14.5.9. Quercetin and rutin

The flavonoids rutin and quercetin have been described as cell-protecting agents because of their antioxidant, antinociceptive, and anti-inflammatory actions [82]. Micellar carriers of quercetin and rutin which are antioxidant compounds were prepared and the permeation of these drugs across skin using Franz diffusion cells was examined *in vitro* [70]. The aqueous solutions of both quercetin and rutin were used as the control groups. The authors showed that quercetin and rutin loaded micelles had more efficient skin permeation than those of the control groups. In this study, a safety assessment of quercetin and rutin loaded micelles on skin was made to evaluate the application possibility of these polymeric micelles to cosmetics. No adverse symptoms were observed following the application.

14.6. CONCLUSIONS

Topical treatment of most skin disorders would be useful in terms of delivering drugs to the diseased layers of skin and preventing the systemic side effects of drugs, however, skin – particularly its *stratum corneum* layer – is a strong barrier to the effective drug concentrations in its deeper layers in cutaneous drug delivery. Conventional forms of dosage such as creams and gels are unsatisfactory for topical treatment due to the poor penetration of drugs into targeted layers of skin and inadequate deposition in the skin, resulting in low topical bioavailability. The development of novel drug carriers is an important challenge for delivering drugs topically to treat topical diseases. In recent years, nano-sized drug carriers have been used as a popular strategy for delivering drugs into the skin. Micelles are also one of the drug carriers that improve the delivery of drugs *via* skin. Improved cutaneous targeted delivery of several drugs has been observed for these systems when compared to conventional topical formulations in the studies. Based on recent findings, micelles seem to be promising carriers which provide the deposition of some drugs in particular superficial layers of the skin and hair *follicles*.

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**COLLOIDAL CARRIERS
IN THE TOPICAL TREATMENT OF
DERMATOLOGICAL DISEASES**

Sevgi Güngör*, M. Sedef Erdal, and Sinem Güngördük

Department of Pharmaceutical Technology, Faculty of Pharmacy,
University of Istanbul, 34116 Istanbul, Turkey

*Corresponding author: sgungor@istanbul.edu.tr

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15.1. INTRODUCTION

The common dermatological diseases, including acne, atopic dermatitis, eczema, psoriasis and microbial/fungal infections, affect the life quality of people worldwide. Skin disorders pose a continuous and serious threat to human health and life, and remain a major healthcare problem. Most of these skin disorders have been caused by inflammatory conditions and infectious pathogens. Topical treatment plays a crucial role in the treatment of skin diseases as it targets the drugs to the pathological sites within the skin and, minimises and/or prevents systemic side effects. The clinical efficiency of drugs applied topically depends on the concentration achieved in cutaneous tissues, which is mainly related to the ability of the compound to penetrate into tissue. The main limitation of topical treatment is mostly the poor penetration of drugs into deeper layers of skin, due to the unique barrier characteristics of *stratum corneum*, which is the outermost layer of the skin. The integrity of the skin barrier can be weakened in most dermatological diseases, and the enhancement of topical delivery efficacy is a great challenge for improving the localisation of drugs at the target sites for the treatment of dermatological diseases. As well as the barrier characteristics of skin, the physicochemical properties of drugs such as high lipophilicity and poor aqueous solubility also affect penetration across skin [1-3].

Different types of novel nano-sized drug carriers have been developed to overcome barriers to the dermal targeting of drugs in topical drug delivery [4]. Microemulsions are one of the colloidal carriers widely investigated for enhancing the localisation of the drugs at the targeted skin sites. Microemulsions are thermodynamically stable, optically isotropic, transparent dispersions of oil and water that are stabilised by an interfacial film of surfactant and co-surfactant. They offer the advantages of high drug solubilising capacity, long term stability, and ease of scale-up in manufacturing. Microemulsions have been intensively studied over recent decades by many scientists because of their great potential in pharmaceutical applications [4-6].

The present chapter focuses on background information about the structure of human skin, and microemulsions as colloidal drug carriers. The common dermatological diseases and the drugs used in their topical treatment are reviewed. Research studies in which focused on the development of microemulsions as a challenge for the effective treatment of skin disorders have also been summarised.

15.2. THE SKIN

The skin is the largest organ of the human body, accounting for more than 10 % of body weight and covering a surface area of approximately 2.0 m². It has a multi-lamellar structure that provides a physical barrier to protect organisms against environmental factors and to regulate heat and water loss from the body [7]. The skin also offers an ideal application site through which to deliver drugs for both local and systemic pharmacological effects, due to being easily accessible and having a large surface area [2]. It has excellent barrier functions against the penetration of drugs, however, due to the unique arrangement of its structure [3,8].

Morphologically, the skin consists of three main layers. From the outside to the inside these layers are the epidermis, the dermis, and the hypodermis (subcutaneous tissue). The epidermis is also composed of two distinct layers, the *stratum corneum* (non-viable epidermis) and viable epidermis [2,9]. The *stratum corneum* is located in the outermost layer of the epidermis. Although its thickness is only 10–20 µm, it is the main barrier of the skin [10]. It is formed primarily of keratin filled dead cells, which are called as corneocytes. The corneocytes are embedded in the bilayer lipid lamellar domain. The main lipids in the *stratum corneum* are ceramides, cholesterol and free fatty acids. The *stratum corneum* can be described as a brick and mortar organisation, in which the corneocytes are the bricks and the intercellular part of the lipid domain is the mortar [8,11]. The viable epidermis, ~ 100–150 µm thick, is responsible for formation of the *stratum corneum* and is itself also made up of different layers. From the outer to innermost, they are: *stratum granulosum*, *stratum spinosum*, *stratum germinativum (basale)* [12,13]. Cells proliferate in the basal layer, and viable cells differentiate and migrate through the skin surface. The morphological differentiation is formed between the *stratum granulosum* and *stratum corneum*, and the viable cells are changed to corneocytes. The viable epidermis layer is therefore a stratified epithelium composed of different layers. Each layer of the epidermis is defined by morphology, position and the differentiation state of keratinocytes [12,14]. As well as keratinocytes, there are melanocytes responsible for melanin production, merkel cells providing sensory perception and Langerhans cells with an immunological function [12]. In the epidermal barrier (epithelial layers and follicles), the tight junction proteins have been shown, and in diseased skin such as psoriasis, characterised by a compromised skin barrier, the localisation and expression of these proteins have been shown to be altered [15].

The dermis is about 1–2 mm thick, and located below the epidermis layer. It is made up of two main structures including fibrous proteins and glycosaminoglycans (GAG). Fibrous proteins are collagen, elastin, and fibronectin. In the GAG matrix, there are hyaluronic acid and chondroitin sulphates [2,9]. The dermis provides mechanical support for the skin. There

are also the fibroblasts, mast cells and dendritic cells in dermis layers. There are also several appendages including the pilosebaceous units (hair follicles and associated sebaceous glands), eccrine sweat and apocrine glands. The hypodermis (subcutaneous tissue) is located underneath the dermis layer. It acts as an anchor and provides support to connect the skin to the underlying muscle. Skin also has immunological functions due to having antigen-presenting cells, such as Langerhans cells, in the viable epidermis and dermal dendritic cells in dermis [16].

The diseased skin is mostly characterised by a diminished barrier function and altered lipid composition and organisation. Thus, the barrier features of the skin against penetration become less efficient in dermatological diseases such as atopic dermatitis, psoriasis, *etc.* The reduced barrier characteristics of the skin in atopic dermatitis are mainly due to a decrease in the amount of ceramides and the high proportion of hexagonal lateral packaging of the epidermal lipid [14,17]. There is also an irregular pattern of lipid organisation and irregular structure of protein particles of desmosomes [17]. In inflammatory skin disorders, namely dermatitis, psoriasis and fungal infections of the skin due to dermatophytes, leucocytes invade the skin. The thickness of the skin increases due to enhanced proliferation and the disturbed differentiation of keratinocytes. In the differentiation process, the nuclei and DNA of keratinocytes are degraded and, thus the amount of water in superficial skin is decreased to less than 20 % [18]. In psoriasis, the mitotic rate of the basal keratinocytes is ultimately high, and thus erythematous plaques with silvery scales at the skin surface are observed; the epidermis is thickened, and angiogenesis and the presence of inflammatory cells, such as dendritic cells, macrophages and T cells in the dermis, are increased [19].

15.3. BASIC CONCEPTS OF MICROEMULSIONS

Microemulsions are thermodynamically stable, fluid and isotropic colloidal nanocarriers with a dynamic microstructure that form spontaneously by combining appropriate amounts of oil, water, surfactant and co-surfactant [20,21]. The droplet size of microemulsions is usually in the range of 20–200 nm [22]. They are mostly prepared using the phase titration method and can be depicted with the help of pseudoternary phase diagrams. Pseudoternary phase diagrams provide the boundaries of the different phases (microemulsion, liquid crystalline, micelles) as a function of the component composition [5,22].

There has been increased interest in recent years in the use of topical vehicles that may modify drug penetration into the skin [23]. The most difficult aspect of the skin delivery of drugs is overcoming the barrier of the *stratum corneum*. Although the integrity of the skin barrier can be weakened in most of the

dermatological diseases, as mentioned above, there is still a need to deliver drugs into targeted skin layers in order to improve treatment efficacy and patient compliance. Various strategies have thus been employed with this aim, and among them microemulsion type colloidal carriers have been suggested as efficient promoters of drug localisation to skin [24,25].

15.3.1. Components of microemulsions

Microemulsions are mainly composed of oil and water phases which are successfully formulated using a combination of suitable surfactant and co-surfactant. A large number of oils, surfactants and co-surfactants are available which can be used as components of microemulsions, but a selection of the components suitable for pharmaceutical use involves a consideration of their toxicity and, if the systems are to be used topically, their irritation and sensitivity properties [22].

The optimal type of a microemulsion depends on the physicochemical properties of the drug; lipophilic drugs are preferably loaded to oil in water (o/w) type microemulsions whereas water in oil (w/o) type microemulsions are better carriers for hydrophilic drugs [21,26]. For lipophilic drugs, the ability of a microemulsion to maintain the drug in a dissolved state is strongly influenced by the solubility of the drug in the oil phase [23]. The ability of the selected oil to increase the region where the microemulsion is formed is equally important [20]. Fatty acids, alcohols, esters of fatty acids and alcohols, and medium chain triglycerides are among the most commonly used oil components in microemulsion systems [5].

The choice of the surfactant is another critical factor in the formulation of microemulsions, as it helps in the reduction of the interfacial tension by forming a film at the oil-water interface resulting in the spontaneous formation of microemulsions. The selected surfactant should microemulsify the oil and should also possess good solubilising potential for the selected drug candidate. Another crucial factor is the acceptability of the surfactant for the desired route of drug delivery. Non-ionic surfactants are mostly preferred for dermal delivery since they are well-known for their non-irritant nature [6,20]. Amongst the various surfactants, polysorbate 80 (Tween 80), sorbitan monooleate (Span 80), caprylocaproyl polyoxylglycerides (Labrasol) and phospholipids are widely used in the formulation of pharmaceutical microemulsions [20,26].

The incorporation of a co-surfactant, such as short- or long-chain alcohols or polyglycerol derivatives, in the microemulsion formulation reduces interfacial tension and increases the flexibility and fluidity of the interface by penetrating into the surfactant monolayer [5]. Diethylene glycol monoethyl ether (Transcutol), propylene glycol and poly(ethylene glycol) 400 are among the most preferred co-surfactants for dermal drug delivery [20].

15.3.2. Characterisation of microemulsions

In general, the effect of the surfactant to co-surfactant ratio, oil type and drug incorporation on the phase behaviour of the microemulsions have been characterised. Particle size and polydispersity index and the viscosity of the microemulsions are important parameters which should be identified. It has been shown that the size of the dispersed phase has an effect on drug transport into the skin [26,27]. The electrical conductivity of microemulsions has to be determined in order to identify whether the microemulsions are oil continuous or water continuous. Advanced techniques such as nuclear magnetic resonance (NMR) can be used to determine the location of the drug between the oil and water phase. From the skin delivery perspective, another important factor is the irritation potential of the oil, surfactant and co-surfactant. *In vitro* cytotoxicity tests have gained great interest for determining the biocompatibility and tolerability of microemulsions [24].

15.3.3. Enhancement mechanisms of microemulsions

The type of oil, surfactant and co-surfactant, the concentrations and ratios of these components and the type of microemulsion (o/w, w/o or bicontinuous systems) affect drug release and skin penetration from microemulsions [24]. Some of the potential mechanisms by which microemulsions would improve transport of drugs to the skin are described below and schematised in Figure 1 [21-23,27].

- Ingredients of microemulsions can modify the diffusional barrier of the *stratum corneum* either by perturbation/fluidisation of intercellular lipid bilayers or denaturation of intracellular keratin or modification of its confirmation.
- Due to the high solubilisation capacity of microemulsions an increased concentration gradient towards the skin can be reached.
- The ultralow interfacial tension and the continuously fluctuating interfaces of microemulsions can facilitate drug penetration into deeper skin layers compared to conventional formulations.
- The partitioning and solubility of drugs in *stratum corneum* could be increased depending on microemulsion composition.
- The internal phase can act as a drug reservoir resulting in controlled and sustained release from microemulsions.

Figure 1. The potential mechanisms by which microemulsions would improve the transport of drugs to the skin

Microemulsions have additional advantages in drug delivery over conventional dermatologic formulations (emulsions, gels, *etc.*) such as improved shelf life due to their thermodynamic stability, ease of manufacture and scale-up because of the spontaneity of formation, and ability to entrap hydrophilic and hydrophobic drugs either alone or in combination. The encapsulation of therapeutic agents in the microemulsion structure can offer improvements in their chemical, photochemical and enzymatic stability [24].

Due to the broadness of the subject and the great number of studies published, this chapter will mainly focus on the influence of microemulsions as topical carriers in several common dermatological diseases discussed below. Even though a precise separation between the topical and transdermal delivery from microemulsions is not possible, most of the studies demonstrate that a more pronounced cutaneous drug localisation in skin layers, rather than percutaneous permeation, can be obtained with microemulsions [25,26].

15.4. MICROEMULSIONS AS COLLOIDAL DRUG CARRIERS FOR SKIN DISORDERS

Common dermatological diseases, including acne, atopic dermatitis, psoriasis and microbial/fungal infections, affect the life quality of people worldwide. Most of these skin disorders have been caused by inflammatory conditions and infectious pathogens. Topical delivery of drugs is always preferred for treating mild and localised dermatological conditions. The success of topical dermatologic therapies is dependent upon many factors, such as correct diagnosis, type of lesion being treated and the vehicle in which the active agent is delivered. The clinical efficiency of drugs applied topically depends on the

concentration achieved in cutaneous tissues, which is mainly related to the ability of the compound to penetrate into tissue. The enhancement of topical delivery efficacy is therefore a great challenge for improving the localisation of drugs into target sites in dermatological diseases. Different types of novel drug carriers could be an option to overcome the problems associated with conventional vehicles and to overcome the skin barrier to dermal targeting of drugs in topical drug delivery. Extensive review articles have been published, dealing with the nano-sized drug carriers intended for topical delivery of dermatological drugs [4,15,18,28-30]. The common dermatological diseases and the drugs used in their topical treatment are reviewed and research studies focused on microemulsions as a challenge for the effective treatment of these skin disorders are summarised below and in Table 1.

Table 1. Overview of topical microemulsion studies in common dermatological diseases

TREATMENT: ACNE	Drug	Oil phase	Surfactant	Co-surfactant	Aqueous phase	<i>In vitro</i> (skin/ membrane) and <i>in vivo</i> studies	Ref.
	tretinoin	isopropyl myristate; transcutol P	tween 80 labrasol	propylene glycol	water	cellulose membrane	[31]
	retinoic acid	isopropyl myristate	soybean lecithin caprylyl/capryl glucoside	ethanol 1,2-hexanediol	phosphate buffer	polydimethylsiloxane membranes pig ear skin	[25]
	adapalene	oleic acid	tween 20	transcutol P	water	porcine ear skin	[32]
	salicylic acid	isopropyl myristate	tween 80	propylene glycol	water	–	[33]
	sodium salicylate	isopropyl myristate	tween 80	propylene glycol	water	cellulose membrane	[34]
	nadifloxacin	oleic acid	tween 80	ethanol	water	rat skin	[35]
	nadifloxacin	capryol 90	tween 80	transcutol P	water	rat abdominal skin	[36]
	clindamycin phosphate	isopropyl palmitate	aerosol OT	1-butanol	water	human epidermis	[37]
	nicotinamide	isopropyl palmitate	span 80	tween 80	water: isopropyl alcohol	newborn pig skin	[38]
	sodium ascorbyl phosphate	mygliol 812	labrasol	plurol oleique	water	cellulose membrane	[39]

Table 1. Continued

TREATMENT: ACNE	Drug	Oil phase	Surfactant	Co-surfactant	Aqueous phase	<i>In vitro</i> (skin/ membrane) and <i>in vivo</i> studies	Ref.
	isotretinoin	isopropyl myristate	labrasol	cremophor EL plurol oleique solutol HS5	water	pig skin	[40]
	metronidazole	isopropyl myristate	lecithin	butanol	water	dialyzing tubing adult male/female patients	[41]
TREATMENT: ATOPIC DERMATITIS							
	hydrocortisone acetate	plurol oleique	labrasol	transcutol P labrafil	water	pig ear skin	[42]
	hydrocortisone acetate	eucalyptus oil	tween 80	ethanol isopropanol propylene glycol	water	cellulose membrane rabbit ear skin	[43]
	tacrolimus	cetiol B	phospholipon 90 G plantacare 2000 span 80 tagat S2	1,2-pentylene glycol	water	human skin	[44]
TREATMENT PSORIASIS							
	betamethasone dipropionate	oleic acid sefsol	tween 20	isopropyl alcohol	water	rat skin	[45]
	methotrexate	ethyl oleate	labrasol	plurol isosteareique	sodium chloride solution	porcine skin	[46]
		myristic isopropyl ester	tween 80 span 80	1,2-octanediol	water		
	curcumin	eucalyptol	tween 80	ethanol	water	porcine skin	[47]

Table 1. Continued

TREATMENT: FUNGAL INFECTIONS							Ref.
	Drug	Oil phase	Surfactant	Co-surfactant	Aqueous phase	<i>In vitro</i> (skin/ membrane) and <i>in vivo</i> studies	
	econazole nitrate	labrafil M1944	solutol HS15 span 80	transcutol P	water	rat skin	[48]
	miconazole nitrate	1-decanol: 1-dodecanol	oramix® NS 10 lecithine	propylene glycol 1,2- -hexanediol	phosphate buffer	pig ear skin	[49]
	ketaconazole	lauryl alcohol	labrasol	ethanol	water	rat skin	[50]
	clotrimazole	lemon oil isopropyl myristate	tween 80	<i>n</i> -butanol	water	cellulose membrane mice abdominal skin	[51]
	sertaconazole	oleic acid	tween 80	propylene glycol	water	mice abdominal skin	[52]
	terbinafine	oleic acid	labrasol	transcutol P	water	human cadaver skin	[53]
	terbinafine	oleic acid	labrasol	transcutol P	water	rat skin	[54]
	naftifine	oleic acid	cremophor EL cremophor RH40	transcutol P	water	pig skin	[55]
TREATMENT: VIRAL INFECTIONS							
	oxyresvera trol	isopropyl myristate	tween 80	isopropyl alcohol	water	shed snake skin	[56]
	penciclovir	oleic acid	cremophor EL	ethanol	water	mouse skin	[57,58]
	acyclovir	isopropyl myristate captex 355 labrafac	tween 20	span 20	water dimethylsulfoxide	mice skin	[59]

15.4.1. *Acne vulgaris*

Acne vulgaris (acne) is a common skin disorder, affecting over 80 % of the population at some point in their lifetime. Acne is caused by follicular epidermal hyperproliferation and abnormal sebum production within pilosebaceous units in the skin. The anaerobic diphtheroid *Propionibacterium acnes* (*P. acnes*) has been demonstrated as the principal cause of inflammation in *acne vulgaris* [60,61]. *P. acnes* is naturally present deep within the follicle and contributes to the progression of acne realising pro-inflammatory mediators that lead to the formation of papules or pustules, which worsen the severity of the disease [62]. Other dermal bacterial flora such as *Staphylococcus epidermidis* and *Staphylococcus aureus* (*S. aureus*) may play a role in acne aetiology, particularly as secondary infections [60]. Acne can be classified as mild, moderate, or severe according to the morphology of lesions. The lesions of acne are clinically divided into inflammatory and non-inflammatory types [62].

When choosing medications to treat an individual with acne, several factors are considered, including the clinical type and severity of acne, skin type and the presence of scarring. Topical therapy is indicated for mild to moderate acne and mainly involves the application of retinoids, antibiotics, and antibacterial agents, whereas systemic therapy is required for severe acne (Table 2) [63].

Table 2. Topical agents in the treatment of *acne vulgaris*

Category	Antiacne compounds	
Retinoids	tretinoin	isotretinoin
	adapalene	tazarotene
	moltretinide	all- <i>trans</i> -retinoyl- β -glucuronide
Hydroxy acids	glycolic acid	mandelic acid
	lactic acid	salicylic acid
	lipohydroxy acid	
Antibacterial agents	benzoyl peroxide	erythromycin
	clindamycin	nadifloxacin
	azelaic acid	azithromycin
	chloroxylenol	sodium sulfacetamide
	metronidazole	5-amino levulinic acid
Antioxidants	nicotinamide	sodium ascorbyl phosphate

The active compounds in topical acne treatment are mainly delivered through conventional formulations such as solutions, gels, creams and lotions. The major disadvantages of topical treatment with conventional formulations are their potential to cause local side effects such as skin irritation. Drugs applied topically must also pass across the *stratum corneum* barrier and reach the site of action (the lipophilic environment of the pilosebaceous unit). Targeting the deeper layers of skin is not possible with conventional formulations, however. It could therefore be a good option to improve the skin uptake of antiacne drugs with a carrier facilitating skin targeting, while decreasing systemic exposure and toxicity. Microemulsion-type colloidal carriers have been used for the delivery of many drugs in topical dermatological therapy and they can be good alternatives to enhance the skin delivery of antiacne compounds.

15.4.1.1. Microemulsion formulations of retinoids

Retinoids normalise epidermal differentiation in skin, and topical retinoids perform many functions that directly affect the pathogenic steps associated with acne [64]. Tretinoin was the first retinoid used for the treatment of acne. The efficacy of conventional preparations is limited, however, by cutaneous irritation. Tretinoin microemulsions have been prepared and evaluated for their droplet size, stability, zeta potential, viscosity and conductivity. The optimised tretinoin microemulsion demonstrated an enhanced *in vitro* release profile compared to commercial gels and creams [31]. Trotta *et al.* evaluated the ability of microemulsion systems of both type, o/w or w/o, to deliver retinoic acid through *in vitro* pig skin. They observed a large increase in retinoic acid skin deposition from o/w microemulsion systems [25]. Adapalene was the first synthetic retinoid used in the treatment of acne. It is a highly lipophilic compound and it has been shown that adapalene penetration of the hair follicles is increased with microemulsions [32]. Microemulsion-type topical carriers for isotretinoin were investigated with the objective of improving skin uptake of the drug. After *in vitro* permeation studies, the dermal penetration of isotretinoin from microemulsions was investigated by tape stripping. Confocal laser scanning microscopy provided insights into the localisation of the drug in the skin. The interaction between the microemulsion components and *stratum corneum* lipids was studied by attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy. The results indicate that microemulsion-based novel colloidal carriers have the potential to enhance skin delivery and the localisation of isotretinoin [40].

15.4.1.2. Microemulsion formulations of hydroxy acids and antibacterial agents

Salicylic acid is a keratolytic agent used in topical products with antimicrobial actions. Microemulsions and gelled microemulsions have been reported as suitable carriers for the topical application of different concentrations of salicylic acid [33,34]. In order to develop alternative formulations for the

topical administration of the antibacterial compound nadifloxacin, microemulsions were evaluated by various researchers [35,36]. Nanocarrier-based microemulsion formulations of nadifloxacin have been found to be promising carriers, showing enhanced efficacy against *Propionibacterium acne*. Microemulsions were developed as alternative formulations to the topical delivery of clindamycin phosphate. Drug permeation through the human epidermis *via* microemulsions has been found considerably better than that *via* its solution, indicating the enhancement of skin permeation by clindamycin phosphate microemulsions [37]. A topical w/o type microemulsion of metronidazole was shown to be more effective in the reduction of inflammatory lesions and erythema compared to commercial gel products [41].

15.4.1.3. Microemulsion formulations of antioxidant agents

A microemulsion containing nicotinamide has been evaluated for its characteristics, stability and skin penetration and retention. The microemulsion system has been found stable and provided a greater amount of skin retention than skin penetration, resulting in its suitability as an antiacne product [38]. Sodium ascorbyl phosphate is the hydrophilic derivative of ascorbic acid and is used in many cosmetic and pharmaceutical formulations because of the antioxidant activity. Microemulsions were selected as carrier systems for the topical delivery of sodium ascorbyl phosphate and it was shown that the drug maintained its stability and demonstrated sustained release when incorporated in the inner phase of the microemulsions [39].

15.4.2. Atopic dermatitis

Atopic dermatitis (AD) is a chronic, pruritic, inflammatory skin disease with a wide range of severity [65]. It is one of the most common skin disorders and affects approximately 20 % of children and 1–3 % of adults in developed countries [66]. In AD, skin barrier abnormalities are associated with a deficiency in ceramides and antimicrobial peptides, and function mutations in the filaggrin gene, which encodes for the filament aggregating protein, are also reported. A filaggrin mutation contributes to a disrupted epidermal barrier, increased trans-epidermal water loss, and inflammation. There are also many exogenous factors that can exacerbate barrier dysfunction in AD, specifically soaps and surfactants in detergents that accelerate corneocyte and lipid degradation. In genetically predisposed people the activation and skin-selective homing of peripheral-blood T cells and effector functions in the skin represents sequential immunologic events in the pathogenesis of atopic dermatitis [67]. Patients with AD are susceptible to a variety of secondary cutaneous infections such as *S. aureus* infections. The cutaneous and nasal colonisation of *S. aureus* exacerbates AD symptoms and the density of *S. aureus* colonisation correlates with AD clinical severity [66].

AD treatment requires a multi-therapeutic approach including short-term treatment to control AD flares as well as longer-term strategies to control symptoms between flares and to prolong the time until the next flare. Topical corticosteroids and more recently topical calcineurin inhibitors are preferred in the topical treatment of AD [68,69]. The use of emollients in AD treatment is considered supportive because hydration of the skin helps to improve the dryness, the pruritus and restore the disturbed skin barrier, and may also lead to a reduction of steroid therapy [67]. In topical therapy the treatment and prevention of acute inflammatory processes is essential to avoid exacerbation of the disease. Novel colloidal carriers could be an option for overcoming the problems associated with conventional vehicles.

15.4.2.1. Microemulsion formulations of topical corticosteroids

Topical corticosteroids are recommended as first-line therapy in AD treatment. Although they provide rapid relief, the major problem in treatment is corticophobia, which is related to potential local side-effects (striae, petechiae, telangiectasia, atrophy, and acne or rosacea), associated with long term topical corticosteroid usage. They are therefore preferred for use over short periods (5–7 days) to settle eczema flare ups [67]. Topical corticosteroids are classified in Table 3 on the basis of their relative potency class. Clobetasol propionate is a super potent corticosteroid of the glucocorticoid class used to treat various skin disorders including atopic dermatitis, psoriasis and vitiligo. It suppresses the immune system by reducing immunoglobulin action and like other topical corticosteroids, clobetasol propionate has anti-inflammatory, antipruritic, and vasoconstrictive properties. Microemulsion and microemulsion-based gel formulations were evaluated as a vehicle for the dermal delivery of clobetasol propionate. Microemulsion based gel formulation showed significant changes in skin structure and the visualisation of cutaneous uptake *in vivo* using laser scanning microscopy-confirmed targeting of clobetasol propionate to the epidermis and dermis layers [70,71]. The permeation of hydrocortisone from microemulsions across *in vitro* animal membranes was examined and it has been proposed that gel and ointment formulations of hydrocortisone could be more suitable when it is desirable to restrict drug absorption only to the diseased skin area. Microemulsion carriers promote the transdermal permeation of hydrocortisone which in turn could lead to the occurrence of systemic side effects [42,43].

Table 3. Classification of topical corticosteroids

Relative potency class	Topical corticosteroid
1 – Super potent	clobetasol propionate halobetasol propionate
2 – Potent	betamethasone dipropionate desoximetasone fluocinonide
3 – Upper mid-strength	betamethasone dipropionate betamethasone valerate fluticasone propionate triamcinolone diacetate
4 – Mid-strength	hydrocortisone valerate mometasone furoate
5 – Lower mid-strength	betamethasone valerate fluticasone propionate hydrocortisone butyrate hydrocortisone valerate triamcinolone acetonide
6 – Mild	alclometasone dipropionate desonide fluocinolone acetonide
7 – Least potent	hydrocortisone

15.4.2.2. Microemulsion formulations of topical calcineurin inhibitors

Topical calcineurin inhibitors tacrolimus and pimecrolimus are approved for second-line therapy for the treatment of AD when the use of topical corticosteroids is ineffective or inadvisable [69,72]. They inhibit activation of T cells and mast cells by blocking calcineurin and suppressing inflammatory cytokines and other mediators of the allergic inflammatory reaction [64]. In contrast to corticosteroids, topical calcineurin inhibitors do not lead to skin atrophy [67]. Tacrolimus and pimecrolimus have demonstrated short-term (3 weeks) and long-term (24 months) safety and efficacy in the treatment of AD in adults and children [73]. Tacrolimus is a lipophilic and high-molecular weight (MW : 822.05) molecule and is commercially formulated as a lipophilic ointment. A sufficient bioavailability in the living epidermis is essential for the efficacy of tacrolimus, which cannot be achieved by conventional formulations. To overcome this, a microemulsion-type colloidal carrier has been developed, and it was shown that microemulsions provided significantly higher bioavailability of tacrolimus in the intended skin compartment than the commercially established reference preparation [44].

15.4.3. Psoriasis

Psoriasis is an immune-mediated disorder which is characterised by relapsing episodes of inflammatory lesions and hyperkeratotic plaques. It is known to be the most prevalent autoimmune disease in humans and ranges in severity from mild to severe [73,74]. The goals of psoriasis treatment are to gain initial and rapid control of the disease process, decrease the percentage of body surface area affected, decrease plaque lesions and improve a patient's quality of life. Topical therapies are preferred to treat mild and localised psoriasis, and phototherapy or systemic therapy is reserved for severe forms. Conventional topical formulations are inefficient in providing a targeted effect and patient noncompliance remains a critical limitation in the effective treatment of psoriasis [75,76].

For the past 60 years the mainstay of the topical treatment of psoriasis has been corticosteroids. They are available in a variety of formulations, with potencies ranging from superpotent to least potent (Table 3) and are frequently used in combination with other forms of topical treatment such as Vitamin D analogues. The Vitamin D3 analogues calcipotriene and calcitriol, topical retinoids (tazarotene), topical tars, anthralin and keratolytics (salicylic acid, urea and glycolic acid) are the other therapy options in mild psoriasis treatment [74,76]. Topical calcineurin inhibitors (tacrolimus and pimecrolimus) have been found less effective in treating psoriasis than AD and the most frequently reported adverse event is skin irritation at the application site [75,77].

Novel colloidal carriers such as microemulsions can be effective alternatives in alleviating the side effects associated with the available therapeutic agents. Microemulsions can provide enhanced administration of drugs to the epidermis and dermis and the excess growth of skin cells in psoriasis could be controlled more easily this way [78].

15.4.3.1. Microemulsion formulations in topical psoriasis treatment

Betamethasone dipropionate, an upper-mid strength topical corticosteroid, has been prepared as a microemulsion and a microemulsion-based gel formulation together with salicylic acid for the treatment of psoriasis. The microemulsion based gel formulation has been found safe and effective and permeation of both drugs was enhanced when compared to the conventional gel formulation [45]. Methotrexate is one of the most effective systemic agents for the treatment of severe psoriasis. The traversing efficiency of methotrexate from microemulsion and solution formulation has been studied and it was found that the microemulsion formulation may be of value in the topical administration of methotrexate [46].

15.4.4. Fungal infections

Infections caused by pathogenic fungi and limited to the human skin, nails, hair and mucosa, are referred to as superficial fungal infections. Dermatophytes are one of the most frequent causes of tinea and onychomycosis. Candidal infections are also among the most widespread superficial cutaneous fungal infections. Despite the fact that fungal infections are rarely life threatening, they are important because of their worldwide increased incidence, person-to-person transmission, and morbidity [79].

The topical treatment of fungal infections has several advantages, including targeting the site of infection, a reduction of the risk of systemic side effects, enhancement of the efficacy of treatment and high patient compliance. The main classes of topical antifungals are the polyenes, azoles, allylamine/benzylamines and hydroxypyridones (Table 4). Currently, these antifungal drugs are commercially available in conventional dosage forms such as creams, gels, lotions and sprays [80].

Table 4. Topical Antifungal Compounds

Category	Topical antifungal compound
Azoles	econazole, miconazole, ketoconazole clotrimazole, oxiconazole, sertaconazole sulconazole
Allylamines/benzylamines	terbinafine, naftifine, butenafine
Polyenes	nystatine
Hydroxypyridones	ciclopirox

The efficiency of topical antifungal treatment depends on the penetration of drugs through the target tissue. Antifungal drugs should reach effective therapeutic levels in viable epidermis after dermal administration. In this context, the formulation may play a major role in the penetration of drugs into skin [81]. New approaches to the topical treatment of fungal infections of the skin encompasses new delivery systems for approved and investigational compounds. Microemulsions are among those new carriers used to ensure effective drug concentration levels in the skin after the dermal administration of antifungals.

15.4.4.1. Microemulsion formulations of azole antifungals

Microemulsion formulations of econazole nitrate have been prepared, characterised and the percutaneous permeation of econazole nitrate *in vitro* through rat skin was investigated. It was concluded that microemulsions enhanced drug retention in the skin and may be promising vehicles for the effective percutaneous delivery of econazole nitrate [48]. It was reported that

skin accumulation of miconazole nitrate from positively charged microemulsions increased significantly when compared with accumulation from their negatively charged counterparts. The increased accumulation has been attributed to the interaction between the positive microemulsion system and negatively charged skin sites [49]. The percutaneous absorption of ketoconazole from microemulsions was enhanced, and histopathological investigations on rat skin demonstrated the safety of prepared microemulsions for topical delivery of ketoconazole [50]. Clotrimazole topical microemulsions and microemulsion based gels have been prepared and evaluated for their stability, droplet size, viscosity, *in vitro* release across cellulose membrane and drug retention in skin. Both prepared formulations achieved significantly higher skin retention for clotrimazole over clotrimazole commercial cream [51]. A microemulsion-based hydrogel has been studied as a topical delivery system of sertaconazole for effective treatment of cutaneous fungal infections. The inhibition zone of the microemulsion-based hydrogel formulation against *Candida albicans* (*C. albicans*) was found to be higher in comparison with sertaconazole commercial cream. The drug retention capacity of the microemulsion hydrogel was also higher than that of commercial cream and did not cause any irritation in skin sensitivity studies on rabbits [52].

15.4.4.2. Microemulsion formulations of allylamine/benzylamine antifungals

A microemulsion-based terbinafine gel has been developed for the treatment of onychomycosis. The optimised microemulsion-based gel formulation demonstrated better penetration and retention of terbinafine in the human cadaver skin as compared to the commercial cream. Terbinafine microemulsion in the gel form also showed better activity against *C. albicans* and *Trichophyton rubrum* than the commercial cream [53]. In another study, a microemulsion formulation of terbinafine was developed and optimised with a view to provide controlled drug release and to enhance the skin permeability of terbinafine. It was found that the optimised microemulsion formulation showed better anti-fungal activity against *C. albicans* and *Aspergillus flavus* than the marketed product [54]. The effect of microemulsion-type colloidal carriers of naftifine on pig skin has been investigated by attenuated total reflectance infrared spectroscopy *in vitro*. The results revealed that treatment with microemulsion formulations lead to intercellular lipid bilayer disruption in the *stratum corneum*. Tape stripping studies showed that naftifine was in the lower layers of the skin after 24 h of treatment with microemulsion formulations [55].

15.4.5. Viral infections of the skin

Herpes simplex viruses (more commonly known as herpes) are categorised into two types: *herpes simplex* type 1 (oral herpes) and *herpes simplex* type 2 (genital herpes). Most commonly, herpes type 1 causes herpetic lesions around

the mouth and lips. Following a primary infection, the virus may become latent within nerve ganglia and recurrent infections may occur. Topical antiseptics or antibiotics may prevent secondary infections and antiviral compounds may be effective in reducing the length and severity of attacks [82].

15.4.5.1. Microemulsion formulations in topical viral infection treatment

Microemulsion-based formulations for the topical delivery of acyclovir have been developed. *In vivo* antiviral studies showed that a single application of acyclovir microemulsion formulation resulted in complete suppression of the development of herpetic skin lesions [59]. The skin irritation potential and pharmacodynamics of penciclovir loaded microemulsion were investigated. Male guinea pigs were employed as animal models which were infected with *herpes simplex* virus type 1 in a pharmacodynamics study. The results indicated that compared with commercial penciclovir cream, penciclovir microemulsion could significantly inhibit the replication of *herpes simplex* virus type 1 in skin [57]. Microemulsion-based hydrogel as a topical delivery system for penciclovir has been investigated. The results of permeation test *in vivo* in mice showed that compared to the commercial cream, microemulsion-based hydrogel and microemulsion could significantly increase the permeation of penciclovir into both epidermis and dermis. Skin irritation tests in rabbits demonstrated that multiple applications of microemulsion-based hydrogel of penciclovir did not cause any erythema or oedema [58]. The permeating ability of oxyresveratrol in microemulsion was evaluated, and the efficacy of oxyresveratrol microemulsion in cutaneous *herpes simplex* virus type 1 infection in mice was examined. In cutaneous infection in mice, at 20 %, 25 %, and 30 % w/w, oxyresveratrol microemulsion topically applied five times daily for seven days after infection, was significantly effective in delaying the development of skin lesions and protection from death compared to the untreated control [56].

15.5. CONCLUSIONS

Dermatological skin disorders due to fungal, viral bacterial infections, and inflammatory reasons can seriously affect people's quality of life. Although there have been some great innovations in treatment, many of problems related to skin diseases remain difficult to treat efficiently. In particular, the adequate skin penetration of drugs in the target layers is a great challenge in topical therapy. The integrity of the skin barrier can be weakened in most of the dermatological diseases, such as atopic dermatitis and psoriasis. There is a need to target drugs into skin layers since the conventional dosage forms such as creams, ointments, and gels can be inefficient in achieving the required drug concentrations in the target cutaneous tissues. The development of novel carriers would have advantages in terms of the enhancement of both

therapeutic aspects and improvement of patient compliance. Today, only a few products with nano-sized carriers have been approved for topical treatment and are on the market, but there have been great efforts made, and a considerable amount of research focused on the optimisation of nano-sized novel carriers for skin delivery. Numerous strategies including optimisation of colloidal carriers such as microemulsions have emerged over recent years to optimise the targeted delivery of drugs into skin layers, and some promising data, to some extent, has been published.

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**BIOCOMPATIBLE VITAMIN D3
NANOPARTICLES IN DRUG DELIVERY**

Sandeep Palvai and Sudipta Basu*

Department of Chemistry, Indian Institute of Science Education and
Research (IISER)-Pune, Pune, 411008, Maharashtra, India

*Corresponding author: sudipta.basu@iiserpune.ac.in

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16.1. INTRODUCTION

Cancer remains one of the major causes of mortality in the world with 14.1 million new cases and 8.2 million deaths in 2012 [1]. Traditionally, surgical intervention, radiotherapy, chemotherapy, targeted therapy, and immunotherapy have been used extensively to cure cancer [2]. Chemotherapy using small molecule drugs is one of the best strategies in current cancer therapy. However, chemotherapy leads to unwanted bio-distribution into all tissues including the bone marrow, gut, lymphoid tissue, fetus, spermatogenic cells, as well as hair follicles in the body, causing systemic toxicity, and severe side effects in patients [3]. To overcome drug-related toxicities and to guide small molecule chemotherapeutic drugs specifically into tumor tissues, nanotechnology-based tool kits have emerged as an interesting strategy in next-generation cancer therapy.

16.2 DETAILED OVERVIEW OF THE FIELD

Nanotechnology is an advanced field of science which involves the design and synthesis of materials in the range of 1–100 nm, offering unprecedented applications in various fields including chemical engineering, biology, physics, food technology, environmental science and many other fields [4]. The advent of nanotechnology has had a great impact on the field of pharmaceutical science, especially in the development of nanomedicine [5]. The emergence of so-called nanomedicine (drug-loaded nanoparticulate systems) offers numerous advantages in treating cancer, including optimized drug loading, improved pharmacokinetic profiles, protection of the payload from premature degradation, controlled and sustained release of drugs, reduced toxicity and efficient in vivo behavior, whereas conventional chemotherapeutics fail to meet these challenges [6].

Figure 1. Nanoparticles used in drug delivery in cancer

Nanoparticles also have a high surface-area-to-volume ratio which offers surface modification with a variety of different moieties which impart to them certain properties and functionalities such as higher drug loading, prolonged circulation in the blood and further increases in their ability to accumulate in solid tumors via the enhanced permeability and retention (EPR) effect (passive targeting), targeted tissue delivery by attaching specific ligands (active targeting) or by attaching some diagnostic moieties [7]. With the aforementioned properties, nanotechnology is expected to have a dramatic impact on nanomedicine. To date, a large number of organic and inorganic nanoparticles have been developed to encapsulate therapeutic and imaging agents for delivery to the site of action such as liposomes [8,9], dendrimers

[10,11], polymeric micelles [12,13], polymerosomes [14], solid-lipid nanoparticles [15], gold nanoparticles [16], silicon nanoparticles [17], quantum dots [18] and carbon nanotubes [19] (Figure 1). Although these formulated nanomaterials provide therapeutic advantages over conventional chemotherapy, they also possess unique and complex physicochemical properties that can lead to potential toxicity, resulting in impaired translation of laboratory-based advances into commercial products [20]. Sometimes, the use of unreasonably high quantities of the carrier can lead to problems with carrier toxicity (often resulting from a lack of biodegradability/biocompatibility of vectors used in nanocarrier formulation), metabolism and elimination, or biodegradability [21]. Hence, the evaluation of the biodegradability/biocompatibility of carriers used in nanomedicine is essential for successful translation into the clinic. Very few nanomedicines are currently available on the market for the treatment of cancer. Hence, there is an unmet need to either engineering novel drug delivery systems or to improve existing systems to minimize carrier-related toxicities [22]. This can be achieved by carefully choosing the material composition used in the nanoparticle formulation. Naturally occurring materials are expected to have minimized toxicity and known metabolic clearance mechanism in the body. Hence, it could be anticipated that nanoparticle formulations with naturally occurring materials would be a better approach to overcoming carrier-related toxicities in the successful engineering of nanomedicines. One of the promising naturally occurring candidates for drug delivery purposes is vitamins, which are necessary for our body and are acquired from food sources every day. Recently, we have reported for the first time the synthesis and biological evaluation of biocompatible and biodegradable vitamin D3 nanoparticles for the delivery of various anticancer drugs as monotherapy as well as in combination therapy to target drug resistance in cancer [23].

16.3. VITAMIN D3 AS A DRUG CARRIER

Cholecalciferol (vitamin D3) is one of the major compounds of the vitamin D family, produced through ultraviolet irradiation of 7-dehydrocholesterol in the skin [24]. Since vitamin D3 is a naturally occurring, biocompatible, biodegradable and non-toxic molecule, we have chosen this as a vector in the formulation of a novel biocompatible nanoparticle to deliver various clinically approved and extensively used anticancer drugs such as doxorubicin (a DNA damaging agent), PI103 (a phosphatidylinositol-3-kinase inhibitor) and paclitaxel (a microtubule stabilizing agent).

Figure 2. Synthetic scheme of vitamin D3-drug conjugates

The hydroxyl group of vitamin D3 enables the chemical conjugation of drugs. Firstly, vitamin D3 was treated with succinic anhydride in the presence of pyridine and 4-Dimethylaminopyridine (DMAP) to obtain the -COOH functional group (compound 2 in Figure 2), which further enables the covalent conjugation of drugs through either ester or amide bond formation. PI103 and paclitaxel were conjugated to the -COOH group by their phenolic and hydroxyl groups, respectively, forming ester bonds by using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDCI) and DMAP in the presence of dry dichloromethane as solvent to get compounds 3 and 4 (Figure 2). In the same way, doxorubicin was conjugated to the acid group of compound 2 through its secondary amine group by using *N,N,N',N'*-Tetramethyl-*O*-(1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) and diisopropylethylamine (DIPEA) in the presence of dry dimethylformamide (DMF) as the solvent to get compound 5 (Figure 2). These drugs conjugated to vitamin D (compounds 3, 4, and 5 in Figure 2) were further used in the synthesis of nanoparticles.

16.3.1. Synthesis and characterization of monodrug loaded vitamin D3 nanoparticles

Figure 3. Synthetic scheme for vitamin D3 nanoparticles

Biocompatible vitamin D3 nanoparticles were synthesized from vitamin D3-drug conjugates (compounds 3, 4, and 5) using a solvent evaporation-lipid film hydration-extrusion method (Figure 3). Each drug conjugate 3, 4 or 5 was mixed separately with L- α -phosphatidylcholine (PC), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[amino(poly(ethylene glycol)) 2000] (DSPE-PEG) in a 1 : 2 : 0.2 weight ratio to form a lipid film. This lipid film was hydrated with 1 mL of H₂O to get vitamin D3-PI103, vitamin D3-doxorubicin, and vitamin D3-paclitaxel nanoparticles.

Figure 4. Size distribution of vitamin D3-drug loaded nanoparticles by dynamic light scattering (DLS)

The size and shape of nanoparticles are very important as they play major roles during blood circulation, bio-distribution in the body and cellular internalization. Particles less than 10 nm in size are cleared easily from the body by kidney filtration, while larger ones have the tendency to be cleared by cells of the reticuloendothelial system (RES). Hence, nanoparticles with the optimal size should give favorable results. It was demonstrated that nanoparticles 100–200 nm size have the best properties for cellular uptake [25]. In order to synthesize monodispersed nanoparticles, a self-assembled polydispersed lipid suspension in water was further extruded thorough a 200 nm polycarbonate membrane (Step 4, Figure 3) repeatedly to get uniform particles with a size ranging from 100–200 nm. The nanoparticles synthesized from vitamin D3 were spherical in shape with a size less than 200 nm. The size and morphology of nanoparticles were characterized by DLS (Figure 4), atomic force microscopy (AFM), field emission scanning electron microscopy (FESEM), and transmission electron microscopy (TEM) (Figure 5).

The drug loading was found to be very high when the vitamin drug conjugates were used in the nanoparticle formation compared to that of nanoparticles synthesized by the physical encapsulation of drugs.

Figure 5. AFM, FESEM and TEM images of vitamin D3-drug loaded nanoparticles

The loading of different drugs was quantified from a standard concentration versus absorbance graph in characteristic $\lambda_{\max}$ = 340 nm, 480 nm and 270 nm for PI103, doxorubicin and paclitaxel, respectively, from ultraviolet-visible spectroscopy (UV-VIS). The loading efficiency of the drugs in the nanoformulation was calculated using the following equation.

$$\text{Drug loading efficiency} = \frac{\text{Amount of drug present } (\mu\text{g}) \text{ in nanoparticle} \times 100 \%}{\text{Amount of vitamin D3-drug conjugate } (\mu\text{g}) \text{ taken for nanoparticle synthesis}}$$

The loading efficiency of drugs in vitD3-doxorubicin NPs, vitD3-paclitaxel NPs and vitD3-PI103 NPs was 21.7 %, 36.8 %, and 26.9 %, respectively.

16.3.2. Release of drugs from nanoparticles

The release of drugs from nanoparticles is another important concern in drug delivery. Slow and sustained release of drugs is preferred over uncontrolled burst release, as burst release might lead to higher active drug concentrations near or above the toxic level in the blood circulation before reaching the tumor site [26]. Moreover, there is the possibility of active drug being metabolized and excreted without utilization, leading to wastage of drugs, both therapeutically and economically. When the drug is released slowly from nanoparticles over a period of time, this greatly reduces both the number and frequency of administrations necessary to maintain a therapeutic level of the drug in the body.

Figure 6. Schematic representation of drug release profile determination experiment by the dialysis method

To observe the release profile of drugs from the nanoparticles, we incubated the nanoparticles at pH 5.5 (to mimic the acidic lysosomal compartments inside the cells) and at physiological pH 7.4 (to mimic the blood circulation environment) inside a dialysis membrane with molecular weight cut-off (MWCO) = 1000 Daltons (Figure 6). Aliquots of known amounts were taken at pre-determined time points from outside the dialysis bag and the release of the drugs was quantified by a concentration versus absorbance calibration graph from UV-VIS spectroscopy. From this release kinetics experiment, it was found that vitD3-paclitaxel NP released 75.56 % of paclitaxel in 48 h at pH 5.5, whereas only 34.88 % paclitaxel was released at pH 7.4 in 130 h. On the other hand, vitD3-PI103-NP released 77.74 % of PI103 in 69 h, whereas at pH 7.4 a similar amount, 74.03 % of PI103, was released in 50 h. Finally, 66.35 % of doxorubicin was released in 120 h at pH 5.5 from the vitD3-Dox-NP, whereas at pH 7.4 only 9.47 % of doxorubicin was released in 72 h, which is seven-fold less release than at pH 5.5. From the release profile, it is clear that drugs were released in a slow, sustained manner over a period of time, which is expected to show better therapeutic efficacy when nanoparticles are injected intravenously into the body. It also showed enhanced release at pH 5.5 compared to pH 7.4; it was rationalized that the phenolic ester linkages, ester linkages and amide linkages in the vitD3-PI103 conjugate (3), vitD3-paclitaxel conjugate (4) and vitD3-doxorubicin conjugate (5), respectively, were more labile at pH 5.5 compared to pH 7.4, which resulted in enhanced release of PI103, paclitaxel and doxorubicin at an acidic pH.

16.3.3. *In vitro* cytotoxicity assay

These drug-loaded vitamin D3 nanoparticles showed good cytotoxicity in an *in vitro* cell viability assay performed using the HeLa cervical cancer cell line. VitD3-paclitaxel-NPs showed cell death with $IC_{50} = 0.25 \mu M$, inducing 17.78 % cell viability, whereas free paclitaxel showed $IC_{50} = 0.092 \mu M$ inducing 5.03 % cell viability at $25 \mu M$. VitD3-Dox-NPs showed cell death with $IC_{50} = 0.26 \mu M$ compared to free doxorubicin with an $IC_{50} = 0.21 \mu M$. VitD3-Dox-NPs induced 7.87 % cell viability whereas free doxorubicin induced 1.37 % cell viability at $25 \mu M$. Finally, vitD3-PI103-NPs showed cell death with an $IC_{50} = 12.8 \mu M$ inducing 38.65 % cell viability, whereas free PI103 showed cell death with an $IC_{50} = 0.76 \mu M$ inducing 5.38 % cell viability at $25 \mu M$ in 48 h.

Figure 7. Internalization of vitD3-Dox-NP in HeLa cells in 1 h, 3 h and 6 h time points. Lysosomal compartments and nuclei were stained by LysoSensor (green) and Hoechst (blue) fluorescent dyes. The images were taken by confocal laser scanning microscopy (CLSM). Size bar = 30 μ m.

It is known that nanoparticles enter into cells by different pathways such as phagocytosis, macropinocytosis, clathrin and caveolin mediated endocytosis, depending on their size, surface charge, *etc.* [27]. After internalization by different pathways, nanoparticles are trafficked to endosomes and then to sorting endosomes; from here, a fraction of nanoparticles is sorted back to the extracellular milieu through recycling endosomes while the remaining fraction is transported to secondary endosomes, followed by fusing with lysosomes where nanoparticles are expected to release their payloads [28]. To understand the cellular uptake mechanism of vitamin D3-NPs, we treated HeLa cells with vitD3-Dox-NPs and observed the internalization of NPs by CLSM; it was found that vitD3-Dox-NPs were internalized into HeLa cells through a low pH lysosomal compartment, whereas free doxorubicin internalized through the diffusion pathway (Figure 7). It can be anticipated that vitD3-paclitaxel-NPs and vitD3-PI103-NPs will also be internalized by a similar endocytosis mechanism through the low pH lysosomal compartment.

16.3.4. Dual drug loaded vitamin D3 nanoparticles

The majority of successful cancer therapies are limited by the development of drug resistance [29]. Several cancers develop resistance to chemotherapy drugs by a mechanism called multidrug resistance (MDR), leading to the failure of many forms of chemotherapy [30]. One of several reasons for MDR is repeated treatment with single drug agents, resulting in resistance to chemotherapy. Moreover, since cancers are dependent on multiple altered molecular pathways, single agent therapies alone might not work to offer long-lasting benefits to patients [31]. Therefore, combination chemotherapy with two or more drugs is used to treat cancer cells to circumvent tumor drug resistance [32]. However, as discussed previously, current chemotherapy is limited by non-specific interactions, resulting in severe side effects which are even more severe when drug cocktails are administered. Also, it is very important to maintain controlled drug ratios to obtain optimal drug activity by combination therapy. However, this is difficult due to the different pharmacokinetic and pharmacodynamic profiles of drugs. Different types of polymeric nanoparticles [33-35], lipidic nanoparticles [36,37], iron oxide nanoparticles [38], nanocomplexes [39], and graphene oxide nanoparticles [40] have been used as delivery vehicles for the co-delivery of different anticancer drugs with unified pharmacokinetic profiles and controlled loading ratios.

To exploit these novel biocompatible vitamin D3 nanoparticles as a versatile platform, we also synthesized dual drug loaded nanoparticles by using vitamin D3-drug conjugates for the combinational drug delivery [41].

To obtain dual-drug loaded vitamin D3 nanoparticle, we mixed the vitD3-PI103 conjugate (3 in Figure 2) with PC and DSPE-PEG in a 1 : 2 : 0.2 weight ratio and the lipid film was formed in the same manner discussed in section 16.3.1. This drug loaded lipid film was further hydrated with a 1 mg mL⁻¹ water solution of the second drug (doxorubicin or cisplatin or proflavine) to obtain vitD3-PI103-Dox-NP, vitD3-PI103-CDDP-NP and vitD3-PI103-proflavine-NP, respectively.

These dual drug-loaded vitamin D3 nanoparticles also showed a considerable degree of improved loading. The mean drug loading in vitD3-PI103-CDDP-NP was found to be equal to 23.8 µg of PI103 and 306.4 ± 17.6 µg of cisplatin (cisplatin encapsulation efficiency = 67.2 %) *per* mg of the formulation. Moreover, the mean drug loading in vitD3-PI103-Dox-NP was 42.0 ± 2.5 µg for PI103 and 158.0 ± 7.4 µg for doxorubicin (doxorubicin encapsulation efficiency = 34.2 %) *per* mg of the final formulation. Finally, the mean drug loading for PI103 and proflavine in vitD3-PI103-proflavine-NP were found to be equal to 66.4 ± 9.4 µg and 149.5 ± 11.7 µg, respectively, (proflavine encapsulation efficiency = 32.2 %) *per* mg of the final formulation.

These nanoparticles also showed pH-triggered release, where nanoparticles were incubated at pH 5.5; vitD3-PI103-CDDP-NP showed a slow and sustained

release profile for 72 h, having released 79.22 % of cisplatin in 23 h and 78.73 % of PI103 in 47 h. However, vitD3-PI103-proflavine-NP released 86.14 % of proflavine in 48 h and 72.88 % of PI103 in 96 h. Finally, vitD3-PI103-Dox-NP demonstrated 47.05 % release of doxorubicin in 72 h and 91.5 % of PI103 release in 11 h. At pH 7.4 and 37 °C, it was noticed that vitD3-PI103-CDDPNP released 41.5 % of cisplatin in 47 h and 61.4 % of PI103 in 25 h. However, vitD3-PI103-Dox-NP showed 48.8 % PI103 and 35.2 % doxorubicin release in 72 h. Finally, vitD3-PI103-proflavine-NP released 45 % of proflavine in 96 h and 48 % of PI103 in 72 h.

Dual drug-loaded NPs also showed significant cytotoxicity in human hepatocellular carcinoma cells (Hep3B), and the cytotoxicity was considerably higher than single drug loaded nanoparticles, indicating synergistic effects. The vitD3-PI103-Dox-NP were cytotoxic to Hep3B cells with an $IC_{50} = 6.5 \mu M$ compared to free doxorubicin showing an $IC_{50} = 18.4 \mu M$. The vitD3-PI103-CDDP-NP showed a considerably lower $IC_{50} = 28.15 \mu M$, or more cytotoxicity, compared to $IC_{50} = 49.08 \mu M$ for free cisplatin. Finally, in contrast, vitD3-PI103-proflavine-NP showed a higher $IC_{50} = 30.5 \mu M$ compared to $IC_{50} = 10.1 \mu M$ for free proflavine. Whereas single drug encapsulated vitD3-Dox-NPs, vitD3-CDDP-NPs, and vitD3-Proflavine NPs showed an $IC_{50} = 87.7 \mu M$ with 42.1 % cell viability at 100 μM , $IC_{50} = 93.7 \mu M$ with 44.5 % cell viability at 100 μM and $IC_{50} = 47.5 \mu M$ with 12.6 % cell viability at 80 μM , these cytotoxicities are considerably lower than the dual drug-loaded NPs.

Moreover, dual drug-loaded vitamin D3 nanoparticles showed enhanced cytotoxicity in a cisplatin resistant human hepatocellular carcinoma (Hep3B-R) cell line when compared to either monodrug loaded nanoparticles or the free single drug in a synergistic manner. VitD3-PI103-CDDP-NP showed a considerably lower $IC_{50} = 36.8 \mu M$ with 24.0 % viable cells compared to $IC_{50} = 70 \mu M$ with 63.1 % viable cells for free cisplatin in resistant cells, while vitD3-PI103-Dox-NP showed a considerably lower $IC_{50} = 9.66 \mu M$ with 12.8 % viable cells compared to $IC_{50} = 29.49 \mu M$ with 13.9 % viable cells for free doxorubicin. Finally, vitD3-PI103-proflavine-NP showed an $IC_{50} = 19.14 \mu M$ with 8.7 % viable cells compared to an $IC_{50} = 11.27 \mu M$ for free proflavine with 2.5 % viable cells. VitD3-Dox-NP, vitD3-CDDP-NP and vitD3-proflavine-NP induced 42.6 % cell viability at 100 μM with an $IC_{50} = 86.4 \mu M$, 40.3 % cell viability at 100 μM with an $IC_{50} = 79.3 \mu M$, and 9.5 % cell viability at 80 μM with an $IC_{50} = 27.2 \mu M$ respectively. Interestingly, vitD3-PI103-NP showed almost negligible cytotoxicity in Hep3B-R cells with an $IC_{50} = 92.9 \mu M$. Finally, vitD3-PI103-CDDP-NP also showed considerable cytotoxicity in 5-fluorouracil (5-FU) resistant Hep3B cells (Hep3B-5FU-R) when the cell viability was evaluated at 24 h postincubation, showing an $IC_{50} = 37.9 \mu M$ compared to $IC_{50} = 70 \mu M$ for free 5-FU.

Dual drug-loaded nanoparticles showed anticancer activity in a synergistic way in both the Hep3B and Hep3B-R cell lines; this was calculated by the Chou-Talalay method [42]. In Hep3B cells, vitD3-PI103-CDDP-NP and vitD3-PI103-

-Dox-NP showed combination index (CI) values that varied from 0.17–0.31, which clearly indicated the strong synergistic effect. However, vitD3-PI103-proflavine-NP showed slight synergy to a nearly additive effect with CI values varying from 0.62–1.02. Similarly, vitD3-PI103-CDDP-NP and vitD3-PI103-Dox-NP showed strong synergy (CI = 0.14–5.0) in Hep3B-R cells in contrast to vitD3-PI103-proflavine-NP, which showed weak synergy to a nearly additive effect (CI = 0.60–0.91).

These dual drug loaded vitamin D3 nanoparticles induced cytotoxicity by DNA damage, which was confirmed by western blot analysis with the quantification of poly(ADP-ribose) polymerase (PARP) as a marker for DNA damage repair [43]. For vitD3-PI103-CDDP-NP and vitD3-PI103-Dox-NP, greater cleaved PARP expression was observed (the carboxy-terminal catalytic domain has a molecular weight of 89 kDa) compared to that without treatment and with 5-fluorouracil (5-FU, a positive control) treatment in Hep3B cells.

Hence, in conclusion, we have developed a biodegradable-biocompatible novel vitamin D3 nanoparticle with a size less than 200 nm, ideal for tumor homing through the EPR effect. This vitamin D3 nanoparticle can be used as platform to load single or multiple drugs with high loading. Drugs are released in a slow and sustained manner over a long period of time in a pH dependent manner. Furthermore, these nanoparticles were internalized into the tumor cells *via* endocytosis into the acidic lysosomal compartment and showed efficacy in cervical cancer cells as well as drug resistant hepatocellular carcinoma. We anticipate that these vitamin D3 nanoparticles can be translated to the clinic as a biocompatible and biodegradable vector for delivering drug combinations to reduce the toxic side effects of drug cocktails and to offer a better quality of life to cancer patients.

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***IN SITU* DRUG SYNTHESIS AT CANCER CELLS
FOR MOLECULAR TARGETED THERAPY BY
MOLECULAR LAYER DEPOSITION
-CONCEPTUAL PROPOSAL-**

Tetsuzo Yoshimura*

Tokyo University of Technology, School of Computer Science
1404-1 Katakura, Hachioji, Tokyo 192-0982, Japan

*E-mail: tetsu@cs.teu.ac.jp, kekyoandyoritan@gmail.com

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17.1. INTRODUCTION

Molecular targeted drugs have been developed as ideal substances to overcome cancer. Antibody drugs are promising for efficient and safe drug delivery. Antibodies with attached strong cancer killing drugs enable exact targeting to attack cancer cells with small side effects. Antibodies with attached quantum dots enable the imaging of cancer cell distributions [1], those with attached paramagnetic agents enable labeling for a magnetic resonance imaging system (MRI) [2], and those with attached radioactive compounds enable enhanced radiotherapy [3]. One drawback of antibody drugs is their large molecular weight. This prevents them from attacking the inside of cancer cells, limiting the efficacy of these drugs to areas outside cancer cells.

On the contrary, low molecular weight drugs can pass through cell membranes, attacking the inside of cancer cells. One drawback of these drugs is the side effects caused by imperfect targeting selectivity.

Recently, it was revealed that the destruction of cancer stem cells is essential to achieve perfect healing, and many approaches have attempted to attack cancer stem cells. The difficulty in destroying cancer stem cells is based on several factors [4] such as the excretion of drugs by adenosine triphosphate (ATP)-binding cassette (ABC) transporters, cell protection *via* cell cycle arrest, and reducing systems to protect from oxidative stress.

In the present chapter, the concepts of molecular targeted drug delivery utilizing *in situ* drug synthesis at cancer cell sites by the molecular layer deposition (MLD) [5-7] are described. MLD is a monomolecular-step synthesis process for tailored organic materials, in which molecules are connected one by one in designated sequences. This technique is expected to provide improved ways to carry drugs to cancer cells and cancer stem cells without attacking normal cells [8].

In addition, the concept of laser surgery utilizing a self-organized lightwave network (SOLNET) [9-11], which enables self-aligned optical waveguide construction toward luminescent targets, is proposed.

MLD and SOLNET are technologies that have been developed in the photonics, optoelectronics, and electronics fields. All the experimental results presented in this chapter were obtained in these fields, including photovoltaics, electro-optic devices, and optical interconnects within computers. It would be author's great pleasure if the concepts proposed here would be evaluated by researchers in the biomedical field.

17.2. MOLECULAR LAYER DEPOSITION (MLD)

MLD is a monomolecular-step synthesis process for tailored organic materials. Although MLD was developed for applications in the fields of photonics, optoelectronics, and electronics, MLD may also be applicable to molecular targeted drug delivery to cancer cells. In this section, the concept and featured capabilities of MLD are reviewed.

17.2.1. Concept

MLD is a precisely-controlled synthesis process for tailored organic materials with designated molecular arrangements [5-7]. The concept of MLD is shown in Figure 1, where four kinds of source molecules, Molecules A, B, C, and D, are used. MLD is achieved by selective chemical reactions or electrostatic forces between source molecules. They are prepared under the following guidelines, that is, the same kind of molecules cannot be combined, and different kinds of molecules can be combined. Therefore, in Figure 1, Molecules A-B, B-C, C-D, and D-A can be formed, while Molecules A-A, B-B, C-C, and D-D cannot be formed.

Figure 1. Concept of MLD

First, Molecule A is provided to a substrate in order to connect it to the connecting sites of the substrate. Once the connecting sites are covered with Molecule A, the deposition of Molecule A is automatically terminated because the same source molecules cannot be combined. This is called the self-limiting effect, which is utilized in atomic layer deposition (ALD) [12]. Next, molecules are switched from A to B to connect Molecule B to Molecule A. When Molecule A is covered with Molecule B, the deposition of Molecule B is automatically terminated. By repeating this process from B to C, from C to D, and so on, a tailored organic material with a monomolecular-step sequence of A/B/C/D/--- is synthesized.

MLD utilizing selective chemical reactions can be performed with source molecules having two or more reactive groups such as $-\text{NH}_2$, $-\text{CHO}$, $-\text{NCO}$, $-\text{OH}$, $-\text{COOH}$, acid anhydride groups, and epoxy groups. MLD utilizing the electrostatic forces can be performed with molecules possessing an electric charge. MLD can be carried out either in the vapor phase or in the liquid phase.

Figure 2 shows an experimental proof-of-concept of MLD using pyromellitic dianhydride (PMDA) and 4,4'-diaminodiphenyl ether (DDE) [5]. When *p*-phenylenediamine (PPDA) is provided onto a DDE surface, the film thickness, which was monitored by a quartz crystal microbalance, rapidly increases and is then saturated. When the molecules are switched from PPDA to DDE, again, the film thickness rapidly increases and is saturated. By repeating molecule switching, step-like film growth is observed. The thickness change for one growth step is close to the sizes of PMDA and DDE. These results indicate that monomolecular-step synthesis is performed by MLD.

Figure 2. Experimental demonstration of MLD utilizing two kinds of source molecule, PMDA and DDE

17.2.2. Capabilities

Three featured capabilities of MLD are schematically depicted in Figure 3. Due to the self-limiting effect, MLD enables “ultra-thin/conformal organic material synthesis” on arbitrary structures, including porous, deforming, and floating objects. MLD enables “tailored organic material synthesis” with artificially-controlled molecular sequences as explained above in Figure 1. MLD also

enables “selective organic material synthesis.” Due to these capabilities, MLD has provided a variety of applications in the fields of photonics, optoelectronics, and electronics. Bent *et al.* succeeded in forming copper diffusion barriers in large-scale integrated circuits [13] and depositing photoresists [14] by MLD. Weimer and Liang achieved a uniform polymer film coating on surfaces of nano-particles [15]. The George group developed a growth process of hybrid organic-inorganic polymer films by combining MLD with ALD [16]; the films were used as gate insulators for organic thin-film transistors [17] and thin-film encapsulations for organic light-emitting diodes [18]. MLD has also been applied to photovoltaic devices [6,7,19-21] and electro-optic devices [6,7,22,23].

The ability of MLD to synthesize tailored organic materials at selected sites is especially important in the application to molecular targeted drug delivery to cancer cells. In Sections 17.3 and 17.4, details of the tailored organic material synthesis and the selective organic material synthesis, namely, *in situ* organic material synthesis at selected sites, are reviewed.

Figure 3. Three featured capabilities of MLD

17.3. TAILORED ORGANIC MATERIAL SYNTHESIS

One of the examples of tailored organic material synthesis by MLD is the fabrication of polymer multiple quantum dots (MQDs) [20,21,24] using three source molecules, *i.e.* terephthalaldehyde (TPA), *p*-phenylenediamine (PPDA) and oxalic dihydrazide (ODH). TPA has two $-CHO$ groups, while PPDA and ODH have two $-NH_2$ groups. TPA and PPDA are connected with a double bond generated by a reaction between $-CHO$ and $-NH_2$, allowing π -electron delocalization over the entire produced molecule. TPA and ODH produce a molecule containing a series of single bonds, which severs the π -electron wavefunction. Quantum dots (QDs) can be formed in polymer wires using these bond characteristics.

Figure 4 shows an example of the MLD process in which source molecules are connected in the sequence -ODH-TPA-PPDA-TPA-ODH---, to construct a polymer QD with an OTPT structure.

Figure 4. MLD process to construct a polymer QD with an OTPT structure

Figure 5. 3QD polymer MQD containing QDs with OT, OTPT and OTPTPT structures

Figure 5 shows a polymer MQD named “3QD” containing three kinds of QDs: OT, OTPT, and OTPTPT. The 3QD polymer MQD is synthesized with a molecule switching sequence of -ODH-TPA-ODH-TPA-PPDA-TPA-ODH-TPA-PPDA-TPA-PPDA-TPA-ODH---. The regions involving ODH are barriers. The region between the two ODHs is regarded as a QD, where the π -electron wavefunction is delocalized. In the region of OTPTPT, molecules are connected in the sequence -ODH-TPA-PPDA-TPA-PPDA-TPA-ODH-, and the QD length is ~ 3 nm. For OTPT, the QD length is ~ 2 nm, and for OT ~ 0.8 nm.

Figure 6 shows the absorption peak shifts to shorter wavelengths (higher energies) in the trend OTPTPT, OTPT, then OT. This trend follows that of decreasing QD length, and is attributed to the changing degree of quantum confinement of π -electrons in the QDs.

Figure 6. Absorption spectra of OT, OTPT, OTPTPT, and 3QD polymer MQD

Figure 7. Absorption peak energy of OT, OTPT, and OTPTPT plotted as a function of QD length

In Figure 7, experimental results of the absorption peak energy of OT, OTPT and OTPTPT structures are shown as a function of QD length. Results derived from the quantum confined model for QDs are also presented. The experimental and calculated values are in good agreement, suggesting that the absorption peak shift is attributed to the electron confinement by the QDs.

As can be seen in Figure 6, the 3QD polymer MQD exhibits a broad absorption band extending from ~ 300 to ~ 480 nm, which is attributed to the superposition of component absorption bands of OT, OTPT and OTPTPT structures.

Thus, we have successfully controlled the molecular arrangement in polymer wires with designated sequences of three molecules using MLD, and fabricated polymer MQDs, suggesting the possibility of synthesizing drugs in monomolecular steps by MLD.

17.4. IN SITU ORGANIC MATERIAL SYNTHESIS AT SELECTED SITES

We have developed several techniques to synthesize organic materials at selected sites. In this section, selective growth processes utilizing hydrophilic/hydrophobic surface characteristics and anchoring molecules are described.

17.4.1. Hydrophilic/hydrophobic surfaces

Figure 8 shows examples of selective growth utilizing hydrophilic/hydrophobic surface characteristics [7]. When TPA and PPDA molecules are provided onto a glass substrate with a patterned triphenyldiamine (TPD) coating, poly-azomethine (poly-AM) is selectively grown on the hydrophilic glass surface as shown in Figure 8(a). No poly-AM is grown on the hydrophobic TPD surface.

Figure 8. Selective growth utilizing hydrophilic/hydrophobic surface characteristics

The same selective growth of poly-AM can be observed by using hexamethyl-disilazane (HMDS) instead of TPD [25]. Such selectivity occurs due to the fact that the PPDA and TPA molecules weakly adsorb on hydrophobic surfaces while they strongly adsorb on hydrophilic surfaces.

Figure 8(b) shows selective growth of polymer MQDs of the OT structure grown by MLD. It was found that the polymer MQD film grows only on TiO_2 . On ZnO, no film grows. This might be because ZnO exhibits weak hydrophilic characteristics.

17.4.2. Anchoring molecules with chemical reactions

Figure 9 shows a process for selective growth utilizing anchoring molecules with chemical reactions [26]. First, molecules such as amino-alkanethiol are distributed. The molecules are selectively adsorbed on sites with Au atoms. Next, TPA molecules are provided, then they connect to amino-alkanethiol molecules by chemical reactions between $-\text{CHO}$ and $-\text{NH}_2$. Next, PPDA molecules are provided to connect them to TPA molecules. By repeating these steps, poly-AM is selectively grown at Au sites. In this process, the amino-alkanethiol molecules act as anchoring molecules to initiate material synthesis at the sites.

Figure 9. Process of selective growth by MLD utilizing anchoring molecules with chemical reactions to synthesize organic materials on Au

Figure 10. FTIR-RAS spectra for poly-AM grown on Au by MLD

In order to examine the anchoring effect of amino-alkanethiol molecules on selective growth, poly-AM was grown on glass substrates with Au films by MLD in the following two procedures. The first is denoted by “with amino-alkanethiol,” in which poly-AM grew after distributing amino-alkanethiol over the Au film surface in a solvent. The second is denoted by “without amino-alkanethiol,” in which poly-AM grew on the Au film surface without amino-alkanethiol. Figure 10 shows the Fourier transform infrared reflection absorption spectroscopy (FTIR-RAS) spectra of the Au film surface after providing TPA to it to perform Step 1 in Figure 9. The absorption peaks attributed to TPA in wavenumber regions around 820 cm^{-1} and 780 cm^{-1} are larger with amino-alkanethiol than without amino-alkanethiol. When the substrate temperature was raised to $45\text{ }^{\circ}\text{C}$, the peak height without amino-alkanethiol decreased while that with amino-alkanethiol did not decrease. These results indicate that, in the case with amino-alkanethiol, more TPA molecules existed on the Au film surface with stronger adsorption strengths *via* the amino-alkanethiol anchoring molecules compared to the case without amino-alkanethiol.

From the TPA connected to the amino-alkanethiol molecule, poly-AM was grown in Step 6, confirming the anchoring effect of the amino-alkanethiol molecules toward Au sites [26]. Such an anchoring effect that initiates material synthesis in MLD might be applied to the *in situ* drug synthesis at cancer cell sites, as discussed in Section 17.5.

Figure 11. Concept of seed-core-assisted MLD for three-dimensional synthesis

In addition, the concept of seed core-assisted MLD is briefly described. As Figure 11 shows, by depositing anchoring molecules of amino-alkanethiol on patterned Au objects, self-assembled monolayers (SAMs) are formed on the top and side walls of them. We call the objects with SAMs “seed cores.” Vertical growth of polymer wires are initiated by the SAM on the top of the seed core while horizontal growth occurs *via* the SAM on the sidewall. The regions, where polymer wires should not grow are covered with, for example, an SiO₂ film. Thus, the seed cores can be used to control polymer wire growth locations and orientations. By distributing the seed cores with designated patterns, polymer wires are expected to grow with designated configurations, constructing three-dimensional polymer wire networks.

17.4.3. Anchoring molecules with electrostatic force

Selective growth utilizing anchoring molecules with electrostatic forces can be performed by liquid-phase MLD (LP-MLD) [7,19,27], in which source molecules in the solvent are provided to objects in the liquid phase.

The proof-of-concept was demonstrated by using source molecules illustrated in Figure 12. The terms “p-type” and “n-type” were defined by Meier [28]. Rose Bengal (RB), eosin (EO) and fluorescein (FL) are p-type molecules, which tend to have a negative charge by accepting electrons. Crystal violet (CV) and brilliant green (BG) are n-type molecules, which tend to have a positive charge by donating electrons.

An LP-MLD process to synthesize organic materials on ZnO, which is an n-type semiconductor, is shown in Figure 13. The synthesis is performed with a molecule switching sequence of p-type molecule (p1) → n-type molecule (n1) → p-type molecule (p2) to construct a p1/n1/p2 structure. Here, due to the attractive force induced by the positive charge of ionized donors in the n-type ZnO and the negative charge in the p-type molecules, the p-type molecules are strongly connected on ZnO. Similarly, due to the attractive force induced by the positive charge in the n-type molecules and the negative charge in the p-type

molecules, the n-type molecules and the p-type molecules are strongly connected to each other. The same type of molecule does not connect due to the repulsive force between them. This interaction scheme between p-type molecules and n-type molecules satisfies the condition for MLD depicted in Figure 1.

Figure 12. Source molecules for selective growth utilizing anchoring molecules with electrostatic forces by LP-MLD

Figure 13. Process of selective growth by LP-MLD utilizing anchoring molecules with electrostatic forces to synthesize organic materials on ZnO

In the LP-MLD process shown in Figure 13, molecule p1 can be regarded as an anchoring molecule toward ZnO to initiate the synthesis of the p1/n1/p2 structure at the ZnO site.

Figure 14 shows photographs of the LP-MLD cell during the synthesis of a two-molecule stacked structure of ZnO/RB/CV. A ZnO substrate was placed in the cell, and solutions of source molecules of RB and CV were sequentially injected into the cell. The substrate was exposed to each solution of source molecules for ~ 5 min. The molecule concentration of the solution was 1.6 mol L^{-1} . Rinse processes were used prior to source molecule switching. Namely, LP-MLD was carried out with sequential steps of RB injection, rinse, CV injection, and rinse.

Figure 14. LP-MLD to synthesize a two-molecule stacked structure of ZnO/RB/CV

To analyze the stacked structures of molecules synthesized on ZnO by LP-MLD, the surface potential was measured. Source molecules were introduced to ZnO powder layers formed on glass substrates with indium tin oxide (ITO) electrodes.

The surface potential of the plain ZnO layer before LP-MLD was found to be about -200 mV , which is attributed to negative electric dipole moments generated on the ZnO surface by electrons donated from zinc atoms in interstitial sites to oxygen adsorbed on the surface. When p-type molecules are adsorbed onto the ZnO layer, as shown in Figure 15, the surface potential becomes more negative. This is attributed to the additional negative electric dipole moments generated by the negative charge in the p-type molecules on the ZnO surface [29]. Conversely, when n-type molecules are adsorbed onto

the ZnO layer, the surface potential becomes less negative. This is attributed to the positive charge in the n-type molecules on the ZnO surface [29].

For the two-molecule stacked structure of ZnO/RB/CV, the surface potential becomes less negative compared to the surface potential of the plain ZnO. This is caused by the positive charge in the n-type molecules on the top of the stacked structure [29]. For the three-molecule stacked structure of ZnO/RB/CV/EO, the surface potential becomes more negative compared to the surface potential of the plain ZnO. This is caused by the negative charge in the p-type molecules on the top.

From these results, it is suggested that multi-molecule stacked structures are definitely synthesized on ZnO by LP-MLD using p-type and n-type molecules.

Figure 15. Surface potential of ZnO with single- and multi-molecule stacked structures synthesized by LP-MLD

Using RB for p-type molecules and CV for n-type molecules, we demonstrated the anchoring effect. As shown in Figure 16, when CV is provided on a ZnO layer in the solvent, the ZnO surface remains white, indicating that little CV is adsorbed on ZnO. This means that immobilization of CV on ZnO sites is not possible.

When RB is provided on a ZnO layer in the solvent, the ZnO surface becomes pink, indicating that RB is adsorbed on ZnO. When CV is provided on the

RB-adsorbed ZnO layer in solvent, the ZnO surface becomes blue, which is the color of CV, indicating that much CV is adsorbed on ZnO *via* RB. These results indicate that immobilization of CV on ZnO sites is achieved by RB. This implies that RB acts as the anchoring molecule to immobilize CV on ZnO sites.

The anchoring mechanism demonstrated in Figure 16 is expected to be applied to the molecular targeted drug delivery utilizing *in situ* drug synthesis at cancer cell sites by MLD, as discussed in Section 17.5.

Figure 16. Immobilization of CV on ZnO sites by anchoring molecules of RB

17.5. MOLECULAR TARGETED DRUG DELIVERY BY *IN SITU* SYNTHESIS AT CANCER CELLS

As mentioned in Section 17.2.4., MLD has the potential to be applied to molecular targeted drug delivery. The anchoring mechanisms shown in Figures 9 and 13 can be used to initiate *in situ* drug synthesis at particular sites. LP-MLD is analogous with *in situ* drug synthesis within a human body [7,8,19,30] because the human body is a liquid system. The human body is regarded as the MLD cell and cancer cells are the substrate. In the present section, three examples of selective drug delivery with *in situ* drug synthesis at

cancer cells by LP-MLD are proposed for low molecular weight drugs and antibody drugs.

17.5.1. Low molecular weight drugs into cancer cells

Low molecular weight drugs have the advantage that they can pass through cell membranes and attack the inside of cancer cells. However, as illustrated in Figure 17, they have a drawback in that they attack normal cells while attacking cancer cells due to their imperfect targeting selectivity, resulting in side effects.

For example, gefitinib shuts down intracellular signal transduction in cancer cells by binding to the epidermal growth factor receptor (EGFR). This is an effective molecular targeted drug for lung cancer; however, it causes interstitial pneumonia as a side effect.

Figure 17. Side effects caused by low molecular weight drugs

If *in situ* synthesis of a toxic drug can be done selectively within cancer cells by connecting small non-toxic component molecules using LP-MLD, selective delivery of the toxic drug into targeted cancer cells might be achieved without attacking normal cells, namely, without side effects.

Figure 18 shows a conceptual illustration of an LP-MLD process for molecular targeted drug delivery. The toxic drug is divided into several non-toxic component molecules. In this example, the toxic drug is decomposed into five

component molecules; Molecules A, B, C, D, and E, which have reactive groups for performing the LP-MLD process.

Figure 18. Conceptual illustration of an LP-MLD process for *in situ* synthesis inside cancer cells for low molecular weight drug delivery

First, Molecule A is injected into a human body. Molecule A is selectively connected to the ATP-binding sites of the tyrosine kinase domain of EGFR. Here, Molecule A acts as the anchor at the ATP-binding site. After excess

Molecule A is excreted from the human body, Molecule B is injected to be selectively connected to Molecule A. By successive injections of Molecules C, D, and E, the synthesis of the toxic drug is completed. The synthesized drug acts as a tyrosine kinase inhibitor, which disconnects intracellular signal transduction in cancer cells by protecting ATP from binding to ATP-binding sites, thus suppressing cancer growth.

17.5.2. Antibody drugs to cancer cells

Figure 19 shows an LP-MLD process to stack different kinds of functional molecules on cancer cells one by one with designated arrangements. First, Molecule A, which is an antibody, is injected into the human body to be selectively attached to cancer cells as an anchor for the initiation of synthesis, then excess molecule A is excreted from the body. Next, Molecule B, which is a luminescent agent for imaging, is injected to be connected to Molecule A. Similarly, by successively injecting Molecule C, which is a sensitizer for photo-dynamic therapy (PDT), and D, which is a radio-enhancement agent, multi-functional materials having the structure A/B/C/D can be constructed on cancer cells.

Figure 19. Conceptual illustration of an LP-MLD process for *in situ* synthesis on cancer cells in antibody drug delivery

17.5.3. Drugs into cancer stem cells

It is known that the destruction of cancer stem cells is important to achieve perfect healing of cancer. The difficulty in the destruction of the cancer stem cells is due to several factors such as the excretion of drugs by ABC transporters, cell protection by cell cycle arrest, and reducing systems to protect from oxidative stress. In the present subsection, a proposal for the suppression of drug excretion by ABC transporters is proposed.

Figure 20. Model of drug excretion by ABC transporters

Figure 20 shows a model for drug excretion by ABC transporters. Rapamycin, which is an inhibitor of the mammalian target of rapamycin (mTOR), is known as a molecular targeted drug against leukemic stem cells. The drug is carried away from cancer stem cell sites by ABC transporters distributed in the surrounding region. This reduces the ability of the drug to attack cancer stem cells.

One possible way to solve this problem may be drug delivery by LP-MLD, as conceptually illustrated in Figure 21. The drug is divided into several component molecules, say, five component molecules of Molecules A, B, C, D, and E, that can easily pass by ABC transporters and reach cancer stem cells. First, Molecule A is injected into the human body to be selectively connected to mTOR within cancer stem cells. Molecule A acts as an anchor for mTOR. After excess Molecule A is excreted from the body, Molecule B is injected to be connected to Molecule A. By successive injections of Molecules C, D, and E, the drug is synthesized. It acts as an mTOR inhibitor, which disconnects intracellular signal transduction in cancer stem cells, enabling cancer growth suppression.

Figure 21. Conceptual illustration of the LP-MLD process for *in situ* synthesis inside cancer stem cells in molecular targeted drug delivery

17.6. LASER SURGERY BY A SELF-ORGANIZED LIGHTWAVE NETWORK (SOLNET)

A reflective self-organized lightwave network (R-SOLNET), which enables optical waveguides to be automatically formed toward luminescent targets [31-33], has been developed for self-aligned optical coupling in optical interconnects within computers. In this section, the possibility of R-SOLNET application to laser surgery is proposed.

17.6.1. Concept and demonstrations of SOLNET

SOLNET utilizes attractive force induced between light beams in photo-induced refractive-index increase (PRI) materials such as photopolymers, whose refractive index increases upon light beam exposure. Figure 22 shows a concept of R-SOLNET utilizing luminescent targets [31-33]. An optical device such as an optical fiber and luminescent targets are put in a PRI material. A write beam is introduced from the optical device into the PRI material. A part of the write beam is absorbed by the luminescent targets followed by luminescence from them. The luminescence induces the “pulling water” effect to grow R-SOLNET between the optical device and the luminescent targets. Namely, because the refractive index increases more rapidly in the region where the write beam and the luminescence overlap than in the surrounding region, the write beam and luminescence attract each other to merge by self-focusing. This enables us to construct self-aligned coupling waveguides between the optical device and the luminescent targets automatically.

Figure 22. Concept of R-SOLNET utilizing luminescent targets

We have performed experimental demonstrations of R-SOLNET formation between a multimode (MM) optical fiber with core diameter of $50\text{ }\mu\text{m}$ and a luminescent target of tris(8-hydroxyquinolino)aluminum (Alq3) powder dispersed in polyvinyl alcohol (PVA). As shown in Figure 23, a luminescent target put on an optical fiber edge is placed in a PRI material, which is a mixture of Norland Optical Adhesive NOA65 ($n = 1.52$), NOA81 ($n = 1.56$), and a sensitizer of crystal violet (CV), together with an MM optical fiber.

Figure 23. Experiment of R-SOLNET formation between an MM optical fiber and a luminescent target of Alq3-dispersed PVA

When a write beam with a wavelength of 405 nm is emitted from the MM optical fiber, green/blue luminescence is generated from the luminescent target. At the same time, red luminescence from CV doped in the PRI material is observed, which enables us to trace R-SOLNET formation. With writing time, SOLNET is formed toward the luminescent target, and finally the optical fiber and the target are connected by R-SOLNET, providing a proof-of-concept of R-SOLNET utilizing luminescent targets. This indicates that the R-SOLNET can be constructed toward a micrometer-order scale target. Figure 24 shows an experimental demonstration of R-SOLNET toward two luminescent targets. It is found that a branching R-SOLNET connects an MM optical fiber on the left to two luminescent targets on the right.

These results suggest the possibility of SOLNET-assisted laser surgery. The method is expected to be applied for the selective removal of scattered small cancer cells, to which luminescent molecules are adsorbed before laser exposure.

Figure 24. Experimental demonstration of R-SOLNET formation toward two targets

17.6.2. SOLNET-assisted laser surgery

Figure 25 shows the concept of the SOLNET-assisted laser surgery [7,8]. First, luminescent molecules are adsorbed onto cancer cells by LP-MLD. After inserting an optical fiber and a PRI material into the region surrounding the cancer cells, a write beam is introduced from the optical fiber to form R-SOLNET that connects the optical fiber to the cancer cells. By introducing surgery laser beams into the R-SOLNET *via* the optical fiber, cancer cells are

destroyed selectively. By detecting the backward luminescence emitted from the luminescent molecules, *in situ* monitoring of the degree of cancer cell destruction might be possible.

For practical applications of SOLNET to laser surgery, it is necessary to select appropriate non-toxic PRI materials.

Figure 25. Concept of SOLNET-assisted laser surgery

Figure 26 shows the concept of SOLNET-assisted photodynamic therapy (PDT), in which two-photon photochemistry is used [7,30]. In molecules with two-photon photochemistry, an electron is excited by a photon with a wavelength of λ_1 from the S_0 state to the S_n state, when then transfers to the T_1 state, and is finally excited to the T_n state by a photon with another wavelength of λ_2 to induce chemical reactions for attacking cancer cells. This mechanism enables us to widen the region where PDT is effective, as mentioned below.

In conventional PDT, the excitation light cannot reach the deepest regions containing cancer cells. By using two-photon photochemistry, three-dimensional attack on cancer cells might be possible because the chemical reactions occur only in regions where photons with λ_1 and photons with λ_2 coexist. Therefore, the chemical reactions can be selectively induced in any

region we want by controlling the position of the light beams of the two different wavelengths.

As illustrated in Figure 26, first, luminescent molecules with the two-photon photochemistry are adsorbed into cancer cells. After forming the R-SOLNET that stretches toward the cancer cells, surgery beams of λ_1 are emitted from the R-SOLNET toward the cancer cells. At the same time, surgery beams of λ_2 are introduced so that the λ_1 beams and the λ_2 beams overlap in the area containing cancer cells. Thus, cancer cells located in deeper parts are destroyed.

Although many molecules with two-photon photochemistry are known, such as porphyrin, biacetyl, comphorquinone, benzyl, *etc.* [34], in order to apply them to the human body, more advanced molecules that can be safely dissolved in blood and exhibit light absorption with λ_1 and λ_2 in a range of 600–1000 nm, where light absorption due to hemoglobin is weak, should be researched.

Figure 26. Concept of SOLNET-assisted PDT utilizing two-photon photochemistry

17.7. SUMMARY

MLD enables the *in situ* synthesis of tailored organic materials at selected sites utilizing the self-limiting effect and anchoring molecules to initiate synthesis at selected sites. This function of MLD is expected to be applied to molecular targeted drug delivery.

Because the human body is a liquid system, it is regarded as the MLD cell and cancer cells as the selected sites. By dividing a toxic drug into several non-toxic component molecules and injecting them into the body sequentially, the toxic drug is synthesized at sites where the drug should be delivered. This synthesis process may reduce side effects. The synthesized drug may be a tyrosine kinase inhibitor, an antibody with functional molecules, or an mTOR inhibitor with the goal of disrupting intracellular signal transduction in cancer stem cells to suppress cancer growth.

In addition, SOLNET-assisted laser surgery, which might be applicable to the selective removal of scattered small cancer cells, was proposed.

MLD and SOLNET have been developed in the photonics, optoelectronics, and electronics fields. The author expects that the proposed concepts will be evaluated by researchers in the biomedical field.

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Chapter

18

DRUG-DELIVERY SYSTEMS USING MACROCYCLIC ASSEMBLIES

Yu Liu^{1,2*}, Kun-Peng Wang¹, and Yong Chen^{1,2}

¹ Department of Chemistry, State Key Laboratory of Elemento-Organic Chemistry, Nankai University, Tianjin 300071, P. R. China

² Collaborative Innovation Center of Chemical Science and Engineering (Tianjin), Nankai University, Tianjin 300071, P. R. China

*Corresponding author: yuliu@nankai.edu.cn

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18.1. INTRODUCTION

Recently, the design of advanced drug-delivery systems with high therapeutic efficacy toward malignant tumours and insignificant toxicity to normal tissues has become one of the most challenging tasks in medicinal chemistry [1]. For an effective drug-delivery system, a high level of water solubility, controlled release, targeted delivery, biocompatibility, biodegradability and simplified delivery are all necessary. In recent decades, a variety of nano-supramolecular systems, including liposomes [2], inorganic nanoparticles [3], polymeric micelles [4] and carbon nanomaterials [5], have been constructed from multiple functional components through molecular assembly induced by host-guest complexation.

Macrocycle-based host-guest inclusion complexes consist of a host molecule with a cavity and a guest molecule inside the cavity. Generally, such a host has external features that interact with the solvent and internal features that foster binding of the guest through its specific shape and favourable environment [6]. As typical macrocyclic hosts, cyclodextrins (CDs) and sulfonatocalixarenes are non-toxic, biocompatible and have strong binding ability in water, which enables them to act as excellent platforms for drug delivery.

CDs represent a class of cyclic oligosaccharides composed of D-glucose units linked by α -1,4-glucose bonds, which are water soluble, non-toxic, commercially available compounds with a low price, and their structures are rigid and well defined. Commonly used CDs are α -, β - and γ -CDs, which are composed of 6, 7 and 8 D-glucose repeating units, respectively (Scheme 1) [7].

Scheme 1. Schematic representation of α -, β - and γ -CDs ($n = 6, 7$ or 8 , respectively)

Importantly, the three-dimensional structure of CDs can be represented as a truncated cone, with the secondary hydroxyl groups on the wider end of the cone and the primary hydroxyl groups on the smaller cone rim. This particular arrangement makes the interior of the CD cavity less hydrophilic relative to the

aqueous media and favours the hosting of hydrophobic molecules. Therefore, CDs can bind various inorganic/organic/biological molecules and ions in both aqueous solution and the solid state through a series of non-covalent interactions including hydrophobic or van der Waals interactions, the release of CD ring strain, changes in solvent-surface tensions or through hydrogen bonding with the CD hydroxyl groups [8]. The inclusion complex can alter the physical and chemical properties of the guest molecule, but typically results in the enhanced water solubility of the guest. Owing to their low price, good availability and capability of forming inclusion complexes with high water solubility, CDs are extensively studied as convenient building blocks to construct nano-structured functional materials, especially bioactive materials [9]. Since the 1970s, numerous potential applications of CDs in medicinal chemistry have been studied in terms of increasing the availability of insoluble substrates, reducing substrate inhibition, limiting product inhibition and as delivery agents. For example, CDs are very useful for solubilising drugs and their carriers. In our previous work, we found that the water solubility of paclitaxel could be increased to 2 mg mL^{-1} in a supramolecular assembly formed of tetraethylenepentaamino-bridged bis(β -CD) and two paclitaxel complexes [10].

Scheme 2. Schematic representation of the SCnAs ($n = 4-8$) family

Another typical macrocyclic host is the *p*-sulfonatocalix[*n*]arene (SCnA, $n = 4-8$) family of water-soluble calixarene derivatives that bind guest molecules to their cavities in aqueous media (Scheme 2) [11]. SCnAs possess three-dimensional and π -electron-rich cavities with multiple sulfonate groups, which endow them with fascinating affinities and selectivities, especially toward organic cations [12]. They can also serve as scaffolds for functional and responsive

host-guest systems [13]. Moreover, SCnAs are biocompatible, which makes them potentially useful for diverse life-science and pharmaceutical applications. In this part, we highlight some typical nano-structured assemblies based on CDs and SCnAs as well as their important applications in drug delivery.

18.2. CYCLODEXTRIN-BASED SUPRAMOLECULAR SYSTEMS FOR GENE DELIVERY

Gene therapy is the use of genes (DNA and RNA) instead of conventional drugs to treat diseases. Gene therapy has drawn more and more attention in recent years as a potential means of treatment. Considering that naked genes are not effectively endocytosed by cells and are easily degraded by serum nucleases [14], it is important for scientists to find efficient and safe gene delivery systems [15]. Different from viral carriers, non-viral carriers have low immunogenicity, good biocompatibility and satisfactory DNA-loading capability. Therefore, non-viral gene carriers such as liposomes, polymers and dendrimers have been widely used as vectors for gene therapy. Recently, CD-based supramolecular systems have attracted great attention for gene delivery, because CDs can bind with nucleic acids and increase their stability against nuclease as well as improve cellular uptake. For example, the controlled condensation of DNA is one of the key steps in gene delivery and gene therapy [16]. Recently, to increase transfection efficiency and decrease toxicity, cationic CDs and CD-modified polycations have been used as novel vectors for gene delivery [17].

CD-based polypseudorotaxanes are a type of supramolecular assembly with CDs threading onto the polymer chains, and they are stabilised by hydrogen bonding between adjacent CD cavities as well as through non-covalent interactions between the long-chain molecule and the threaded CD cavities [18]. Interestingly, CD-based polypseudorotaxanes can be converted to CD-based polyrotaxanes by introducing bulky terminals (bulky organic or organometallic groups) at the chain ends in order to prevent the de-threading of CDs. These bioactive CD-based polypseudorotaxanes or polyrotaxanes constructed by threading CDs with polycations and/or fused-ring aromatic substituents onto the polymer chain have widely been used to interact with DNA.

As a good bioactive precursor, anthryl-modified CDs are good chemically switched DNA intercalation materials [19]. By threading anthryl-modified β -CDs onto the poly(propylene glycol) bis(2-aminopropyl ether) (PPG-NH₂, molecular weight (*MW*) = 2000 Da) chains, polypseudorotaxanes bearing several anthryl groups can be obtained easily with an average of ten CD units per PPG chain [20]. This polypseudorotaxane can condense the originally loose, free DNA into solid particles with an average diameter of approximately 100 nm (Figure 1), as demonstrated by fluorescence titration and atomic force microscopy (AFM) (Figure 2). From molecular modelling studies, one can find that

anthryl-modified β -CDs in polypseudorotaxane can intercalate both the minor and major DNA grooves. Therefore, the driving force of DNA condensation should not only be the electrostatic interactions between the protonated amino groups in polypseudorotaxane and the negatively charged phosphates

in DNA, but also the intercalation of multiple anthryl groups in the DNA grooves.

Figure 1. Structure of anthryl-modified CD-based polypseudorotaxanes

Figure 2. AFM images of (a) free calf-thymus DNA and (b) condensed DNA induced by CD-based polypseudorotaxane with anthryl grafts

Another typical example is a two-dimensional cationic polypseudorotaxane constructed by threading 6-[(6-amino-6-deoxy- β -CD dichloride molecules onto the polymer backbone, followed by complexing cucurbit[6]uril

(CB[6]) units on the branches of the modified CDs (Figure 3) [21]. Owing to the strong binding between hexane-1,6-diamine and CB[6] ($K = 4.49 \times 10^8 \text{ M}^{-1}$) in aqueous solution [22], the degree of CB[6] substitution can be controlled. This two-dimensional cationic polypseudorotaxane displays controllable DNA condensation ability by adjusting the amount of CB[6] in the polypseudorotaxane, as CB[6] on spermidine and spermine affects their ability to adjust the activity of a DNA enzyme. The DNA condensation ability of this polypseudorotaxane reaches its highest efficiency with 70 % CB[6]. Further investigations using agarose-gel electrophoresis, ethidium-bromide displacement and AFM experiments demonstrate that the effective charges of polypseudorotaxane interacting with DNA and the changing rigidity of polypseudorotaxane with the addition of CB[6] jointly lead to the unusual DNA condensation ability of the two-dimensional polypseudorotaxane.

Figure 3. Structure of the two-dimensional polypseudorotaxane

CD-based polyrotaxanes are also used in gene delivery. Cationic CD-containing polyrotaxanes constructed by threading cationic CD derivatives onto polymer backbones show good DNA-binding ability, low cytotoxicity and high gene-transfection efficacy [23]. For example, a type of polyrotaxane constructed from oligoethylenimine-grafted β -CDs threading onto the polymer chain, which possesses a high cation density, shows high gene-transfection efficiency with and without serum. Moreover, the transfection efficiency of these cationic polyrotaxanes, in most cases, increases with elongation of oligoethylenimine grafts on the β -CD units.

Through the strong Au-S binding, thio- or polythio-modified CDs can be absorbed on the surface of gold to form three-dimensional supramolecular

assemblies. In a typical example, a supramolecular assembly is constructed by adsorbing oligo(ethylenediamino)-CDs on gold nanoparticles (Figure 4) [24]. Possessing many CD cavities at the outer space, this assembly is demonstrated to be a good vector for DNA binding as well as having moderate plasmid transfection efficiency as a carrier in cultivated cells *in vitro*, which are sufficiently investigated by means of circular-dichroism spectroscopy, transmission electron microscopy and visual GFP expression.

Figure 4. Oligo(ethylenediamino)-CD-modified gold nanoparticles (AuNPs)

By further introducing anthryl adamantanes into the supramolecular assembly containing AuNPs and β -CDs, the obtained secondary supramolecular assembly exhibits good condensation ability toward calf thymus DNA (ct-DNA) owing to the good DNA reactivity with anthracene moieties (Figure 5) [25]. AFM images show that, with an increasing guest-to-host ratio, more and larger aggregates are formed (Figure 6), demonstrating that the CD–AuNP/anthryl adamantane system can act as a promising DNA concentrator and give good binding abilities toward ct-DNA. In addition, the condensation efficiency can be conveniently controlled by adjusting the ratio between the AuNPs and anthryl adamantane grafts. The larger size of the DNA supramolecular aggregates is beneficial to their intracellular uptake, and the smaller size of free complexes of CD-modified AuNPs/anthryl adamantane means that the complexes can be eliminated from the cell more quickly after completion of the delivery mission. Therefore, this supramolecular nanostructure may have exciting applications in gene therapy with the promising potential to control gene expression and delivery.

Figure 5. Construction of the CD-AuNP/anthryl adamantine host-guest system

Figure 6. AFM images of ct-DNA (0.37 g L^{-1}) in the absence (a) and presence of CD-modified AuNPs/anthryl adamantine with different guest/host ratios: 1/20 (b), 1/5 (c) and 1/1 (d)

In addition, the amino-terminated polypseudorotaxane can also attach to the surface of the AuNPs to form three-dimensional nanocages through electrostatic interactions between the amino terminals of polypseudorotaxane and the gold nuclei (Figure 7). Interestingly, this type of nanocage constructed by the attachment of numerous L-Try-CD-based polypseudorotaxanes onto the surface of the AuNP only gives weak DNA cleavage ability [26]. However, after being saturated with buckminsterfullerene (C_{60}), the nanocage exhibits a much

higher DNA cleavage activity under visible-light irradiation, and most of the closed supercoiled DNA strands are cleaved to form nicked circular DNA [27].

Figure 7. Structure of L-Try-CD-based polypseudorotaxane

In addition to gold particles, carbon nanotubes are also used as templates to construct three-dimensional CD-based supramolecular assemblies. Many linear macromolecules, including organic polymers and biomacromolecules, are able to couple with carbon nanotubes through non-covalent wrapping or adsorption. Therefore, nanotube/CD supramolecular assemblies can be constructed conveniently by wrapping or adsorbing CD-polymers on the carbon nanotubes.

As the surface of the nanotube is hydrophobic, it hardly interacts with the double-stranded DNA, where the hydrophilic sites (phosphates) are exposed on the surface. However, after wrapping an anthryl CD-based polypseudorotaxane on the surface of a carbon nanotube, the resultant nanotube/polypseudorotaxane supramolecular assembly shows good ability in terms of wrapping and cleaving double-stranded DNA (Figure 8) [28]. The adsorption of CDs onto the carbon nanotube and the intercalation of anthryl groups into the DNA grooves may play important roles in DNA wrapping.

Figure 8. Schematic representation of DNA wrapping for a nanotube/polypseudorotaxane supramolecular assembly

In another typical example, a β -CD-modified chitosan moiety shows moderate DNA condensation ability and is able to condense free DNA to form uniform hollow loops [29]. After associating adamantanyl pyrene molecules to β -CD-modified chitosan, the dyad with exposed pyrene grafts is more effective in condensing DNA than β -CD-modified chitosan, and the free DNA strands are condensed to solid particles with an average diameter of approximately 200 nm by the dyad, rather than forming hollow loops by the β -CD-modified chitosan (Figure 9). The enhancement in the DNA condensing efficiency is ascribed to the cooperative contribution of aromatic pyrenes and inherent ammonium cations on the chitosan surface. Interestingly, by wrapping β -CD-modified chitosan on the carbon nanotube, the resultant dyad can condense free DNA to compact particles with an average diameter of approximately 80 nm. The wrapping of β -CD-modified chitosan rearranges the β -CD-modified chitosan on the surface of the carbon nanotube into highly dispersed polymers, which enables more active ammonium cation interactions with DNA grooves. Inspired by the improved DNA condensation shown by chitosan/pyrene and nanotube/chitosan dyads, a nanotube/chitosan/pyrene triad is tested as a combinatorial vector, which shows a promoted DNA condensation ability compared with that of the nanotube/chitosan dyad.

Figure 9. AFM images (a-d) of DNA condensation induced by CD-modified chitosan, chitosan/pyrene, nanotube/chitosan or nanotube/chitosan/pyrene dyads

Figure 10. Structure of PEI-Ada-LCD@DNA assemblies

Poly(ethyleneimine) (PEI, 25 kDa), one of the most effective gene-delivery vectors studied to date, has a high buffer capacity that can protect DNA from the degradation of nuclease, but it also induces higher toxicity in the biological process on account of their non-biodegradability. To construct safe and

effective gene-delivery carriers, a CD-based cross-linking system of low-molecular-weight PEI is designed as an effective way to reduce the cytotoxicity at a high gene expression level (Figure 10) [30]. Herein, the supramolecular cross-linking system, composed of adamantyl-modified PEI and L-cystine-bridged bis(β -CD), is used as a bioavailable recycling DNA carrier through host-guest interactions, and shows better DNA condensation, transfection ability and lower cytotoxicity than 25 kDa PEI. Significantly, the disulfide bond in the cross-linking sites can be cleaved easily by reductive enzymes to promote DNA release. This assembly not only avoids the complicated synthesis/separation steps that are always involved in covalently modifying PEI, but also provides a non-viral gene carrier with stronger gene condensation affinity, higher gene transfection efficiency and lower cellular toxicity, which will energise the potential use of CD-based bioactive supramolecular nanostructures in the construction of safe and highly efficient gene carriers.

18.3. CYCLODEXTRIN-BASED SUPRAMOLECULAR SYSTEMS FOR DRUG DELIVERY

In recent years, the construction of carrier-mediated artificial systems through a supramolecular methodology has offered a powerful strategy to design and construct drug formulations and delivery agents [31]. CDs possess well-recognised biocompatibility and ability to form stable complexes, making them attractive as building blocks for the construction of nano-scale functional and bioactive materials [32]. CD-based materials, including amphiphilic CDs, CD-polymers, CD-pendant polymers and CD-based polyrotaxanes, can form nano-structured assemblies such as micelles, nano-gels and vesicles, which exhibit multiple hydrophilic/hydrophobic domains and recognition sites, and, therefore, are potential nanocarriers for both hydrophilic and hydrophobic bioactive molecules [33].

In order to construct versatile nano-assembled drug carriers, a supramolecular assembly of folic acid (FA)-modified β -CD and graphene oxide (GO) non-covalently linked by an adamantane-grafted porphyrin is constructed [34]. Herein, GO, with a thickness of one atom and a large two-dimensional structure, can strongly bind to various organic or biological molecules through chemical modifications, thus promoting practical innovations in biological systems [35], such as nanometre-sized carriers of drugs and genes. Benefiting from strong π - π stacking between the porphyrin and GO and the high hydrophobic affinity of CD for adamantane, the resulting quaternary supramolecular nanoarchitecture can be employed as a delivery platform to efficiently carry doxorubicin hydrochloride (DOX) (Figure 11). Owing to the targeting effect of FA, the concentration of the assembly in normal tissues may remain at lower level, thereby reducing toxicity to normal cells. Therefore, this

system may represent a general protocol for a large library of multifunctional supramolecular biomaterials and can provide a new potential pathway to comprehensively understand the applicability of bioactive, nanoscale materials.

Figure 11. Complex of GO, DOX, adamantane-modified porphyrin and FA-modified CD

Furthermore, hydrophobic camptothecin (CPT) can also be delivered using a CD-based system [36]. As the hyaluronic acid (HA) skeleton can specifically recognise various cancer cells that over-express HA receptors on the cell surface [37], hyaluronated adamantine (HA-ADA) is chosen as the target molecule, whereas CD-modified GO (GO-CD) is used as a scaffold (Figure 12). Benefiting from the supramolecular complexation of the β -CD cavity with the adamantyl group and the π - π stacking interaction between the planar GO surface and the drug molecule, a ternary assembly of CPT@GO-CD/HA-ADA is successfully constructed, and CPT is successfully endowed with water solubility. The inclusion complex of CD/ADA prevents the GO skeletons from intermolecular aggregation in water, which then facilitates the disruption of GO sheets into small-sized components. In the cytotoxicity experiments, CPT@GO-CD/HA-ADA exhibits a higher curative effect and a lower

cytotoxicity than a free drug. The conventional chemotherapeutic drugs can be specifically delivered to their intended sites of action, and their general toxicity can be reduced, to a significant extent, for wider clinical applications.

Figure 12. Structure of the CPT@GO-CD/HA-ADA supramolecular assembly

Based on the target ability of HA, new conjugated polysaccharides: CD-grafted hyaluronic acid (HACD) composed of an HA main chain and CD side chains are constructed. Then, HACD and the adamplatins prodrug can form hydroxyapatite (HAP) nanoparticles that possess a hydrophilic HA backbone for recognising cancer cells as a delivery system for an adamplatins prodrug both *in vitro* and *in vivo* (Figure 13) [38]. The anti-tumour activity of HAP in mice is comparable to the commercial anticancer drug cisplatin, but the toxicity to normal cells is much lower. The specific binding of HA on the backbone of HAP to the HA receptors that are over-expressed on tumour cells not only allows receptor-mediated endocytosis of HAP into tumour cells and tissues, but also prevents normal cells and tissues from being damaged. The present methodology provides a versatile HA platform for targeted drug delivery and transport into cancer cells, while exhibiting minimal uptake into normal tissues.

Figure 13. Structure of conjugated HAP

Figure 14. Schematic illustration of the chemical structures of the HACD-AuNPs

Through the high affinity of the β -CD cavity for adamantane moieties, polysaccharide-gold nanocluster supramolecular conjugates (HACD-AuNPs), which consist of AuNPs bearing adamantane moieties and HACD, can also be constructed as the delivery platform (Figure 14) [39]. Owing to their porous

structure, this supramolecular conjugate can serve as a versatile and biocompatible platform for the loading and delivery of various anticancer drugs, such as DOX, paclitaxel (PTX), camptothecin (CPT), irinotecan hydrochloride (CPT-11) and topotecan hydrochloride (TPT). DOX encapsulation and its loading efficiency are calculated to be 78.68 and 11.03 %, respectively. Significantly, the DOX@HACD-AuNPs displays slow and controlled release of the drug, with the release rate measured to be 3–4 times lower than that of free DOX in acidic or neutral environments. Owing to the high efficiency of their cellular uptake by HA-reporter-mediated endocytosis, the resulting drug@HACD-AuNP system effectively inhibits the growth of MCF-7 cells, enables pH-responsive drug release in cells and decreases drug toxicity toward normal cells. Furthermore, this carrier can provide new possibilities in the development of targeted drug delivery and biomedical applications.

18.4. SULFONATOCALIXARENE-BASED NANOPARTICLES FOR DRUG DELIVERY

Different to CDs, SCnAs can promote the self-aggregation of aromatic and amphiphilic molecules by lowering the critical aggregation concentration (CAC), enhancing aggregate stability and compactness as well as regulating the degree of order in the aggregates. This unique self-assembly strategy has been defined as calixarene-induced aggregation (CIA) [40]. The number of guest species has been divided into four categories: aromatic fluorescent dyes [41], amphiphilic surfactants [42], drugs [43] and proteins [44].

Owing to the biocompatibility of SCnAs, a lot of research on the use of CIA is devoted toward fabricating supra-amphiphiles, which are of fundamental interest for drug-delivery applications. For example, *p*-sulfonatocalix[5]arene (SC5A) as the host and 1-pyrenemethylaminium (PMA) as the guest were first used to fabricate self-assembled binary supramolecular vesicles (Figure 15), which can successfully load DOX [45]. This amphiphilic self-assembly has an average diameter of 99 nm and a narrow size distribution according to a dynamic laser-scattering experiment. Transmission electron microscopy (TEM) shows a hollow spherical morphology, convincingly indicating a vesicular structure. The thickness of the bilayer membrane is about 3 nm, which is on the same order of magnitude as the sum of one PMA length (7 Å) and two SC5A heights (14 Å), indicating that the vesicle is unilamellar. From the obtained details, the model of supramolecular vesicles can be deduced to have hydrophobic pyrene segments packed together, with inner- and outer-layer surfaces consisting of hydrophilic phenolic hydroxyl groups of SC5A, which are exposed to water. SC5A and PMA are connected together by host-guest and charge interactions. After purification by ultracentrifugation and dialysis, DOX is successfully loaded into the vesicle. The loaded DOX molecules

can be released upon warming, together with the disassembly of the vesicles, as proven by detecting the amplification of the fluorescence signal of DOX that is accompanied with a temperature increase. Excess SC5A leads to the formation of a 1 : 1 inclusion complex, accompanied by the disassembly of the amphiphilic aggregation.

Figure 15. Construction of the SC5A+PMA supramolecular binary vesicles and temperature-responsive drug release from the vesicles

Many biomacromolecules, such as proteins and nucleic acids, change their behaviour in response to a combination of environmental stimuli, rather than to a single stimulus. The construction of materials that can mimic this feature is of great interest. Therefore, multi-stimuli-responsive supramolecular vesicles are constructed through the CIA theory of *p*-sulfonatocalix[4]arene (SC4A) (Figure 16) [46]. The resulting vesicles respond to multiple stimuli, including temperature, the addition of CD and redox reactions, benefiting from the intrinsic advantages of supramolecular species. The architecture of these vesicles that contain entrapped DOX can be disrupted through the reduction of viologen to its neutral form, by increasing the temperature or upon the addition of CDs; the disruption triggers the efficient release of the entrapped DOX from the vesicle interior. During *in vitro* experiments, the loading of DOX into the vesicles does not affect its toxicity to cancer cells, whereas encapsulation reduces damage to normal cells.

Figure 16. Formation of a multi-stimulus-responsive supramolecular binary vesicle composed of SC4A and an asymmetric viologen

Amphiphilic self-assemblies that respond to enzymatic reactions represent an increasingly important topic in biomaterial research, and applications of such assemblies for the controlled release of therapeutic agents at specific sites where a target enzyme is located are feasible. For example, cholinesterase-responsive supramolecular vesicles based on SC4A and myristoylcholine are used as a targeted drug-delivery system (Figure 17) [47]. Amphiphilic myristoylcholine cannot be used alone to fabricate an enzyme-responsive assembly, because the CACs of the substrate (myristoylcholine) and the product (choline) are similar. Complexation of SC4A with myristoylcholine directs a supramolecular binary vesicle and decreases the CAC of myristoylcholine by a factor of approximately 100. As the components are held together by non-covalent interactions, the assembled and unassembled states are in dynamic equilibrium, and the enzymatic cleavage of free myristoyl chloride results in the disintegration of the self-assembled vesicles. The binary vesicles consisting of SC4A and myristoylcholine respond specifically and efficiently to cholinesterase, and the cholinesterase-induced cleavage of myristoylcholine disrupts the hydrophilic–hydrophobic balance of the binary super-amphiphiles, resulting in vesicle disassembly. In addition, the release of

a drug, such as the Alzheimer's drug tacrine, encapsulated in the vesicles can be triggered by this enzymatic cleavage. Cholinesterase is over-expressed in Alzheimer's disease and, therefore, this system has potential utility in the delivery of Alzheimer's drugs [48].

Figure 17. Enzymatic responsive of amphiphilic assemblies of myristoylcholine fabricated in the presence of SC4A

More recently, a trypsin-responsive supramolecular vesicle was further fabricated by employing SC4A as the macrocyclic host and protamine as the enzyme-cleavable guest. Differing from the small-molecule species employed in CIA previously, the protamine guest is a non-amphiphilic natural biological cationic protein, which greatly expanded the range of engaging substrates in fabricating CIA assemblies. The obtained vesicle is conceptually applicable as a controllable-release model at over-expressed trypsin sites. Prospectively, this proof-of-concept is adaptive to build various enzyme-triggered self-assembled materials as smart controlled-release systems that are capable of a site-specific response.

18.5. CONCLUSION

Nano-scaled supramolecular systems have been considered the most promising carriers for drug and gene delivery. With delicate design and well-controlled manipulation, nano-supramolecular systems possess a variety of functions, such as prolonged circulation, broad loading spectrum, suitable size and shape for tissue penetration and passive targeting, which are easy to tailor for active targeting at different levels and controllable release.

In recent decades, supramolecular assemblies constructed from CDs and SCnAs for drug and gene delivery have increasingly attracted the attention of chemical and biological scientists. The past two decades have witnessed a significant harvest in CD-based bioactive supramolecular assemblies. The future of CD-based supramolecular systems in drug and gene delivery is promising, in view of the notable clinical success of new pharmaceuticals based on parent CDs, their small-molecule derivatives and CD-containing polymers as well as other controlled delivery systems. In the next section, we highlight various stimulus-responsive vesicles based on CIA theory for drug delivery. These results demonstrate the feasibility of using SCnAs in disease therapy.

Finding methods to utilise host-guest interactions is a challenge, and such methods can be expected to permit the establishment of novel strategies for molecular recognition, sensing and assembly. In the future, more exciting findings and the potential of macrocyclic supramolecular assemblies are going to be discovered.

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Chapter

19

ULTRASOUND-MEDIATED DRUG DELIVERY

Yufeng Zhou*

School of Mechanical and Aerospace Engineering, Nanyang
Technological University, Singapore

*E-mail: yfzhou@ntu.edu.sg

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19.1. INTRODUCTION

Drug or therapeutic agent delivery to the target is a common problem in medicine. The demand for drug delivery systems in the United States is predicted to have an annual growth rate of more than 10 % and to reach \$132 billion in 2012 [1]. Nanocarriers accumulate passively within tumors that have a leaky vasculature *via* the enhanced permeation and retention (EPR) effect [2], and can bind to selected tumor cells through interactions between the vehicle and the target by specific ligands and receptors. Once the carriers have been endocytosed into tumor cells, the release of the drug occurs. Conventional delivery modalities, such as injections and oral administration, have significant advantages in terms of convenience and cost, but have significant limitations. Drugs can be targeted in an either passive or active manner. The major limitation of systemic chemotherapy administration is the exposure of all tissues [3]. Only a small amount of the dose ($< 5\%$) reached the target (*i.e.*, cancerous, infected or inflamed tissues). Both the structural heterogeneity of biological tissues and the limited accessibility of target cells, which is usually due to an exaggerated desmoplastic reaction, excessive interstitial pressure, and the poor status of the blood vessel endothelium, are detrimental to drug targeting. Therefore, temporally and spatially controlled drug delivery remains an important avenue of research and application.

A “magic bullet” was first proposed by Paul Ehrlich in the early 20th century [4]. To achieve this, great efforts have been made by scientists or physicians to selectively target a disease-causing organism and then deliver therapeutic molecules without damage to healthy tissue in response to a stimulus from an external force or internal microenvironment. The stimulus can be the overexpression of receptors on tumor cells or a physical stimulus such as temperature, pH, light, pressure, ultrasound, electric or magnetic fields. The therapeutic agent should be protected to prevent unintended degradation during its transportation within an organism, concentrate exclusively at the desired site, and then be taken up mostly in the target tissue [5]. Although some nanoparticles have shown promising results *in vitro*, only a few of them have demonstrated enhanced tumor accumulation and pharmacological efficacy *in vivo*.

Among all the diagnostic imaging modalities, ultrasound (US) imaging has the unique advantages of real-time data acquisition, low cost, portability, and non-ionization. Since blood has a similar acoustic impedance as that of surrounding soft tissue, it has very low echogenicity. However, the acoustic impedances of most gases are usually six orders of magnitude lower. So, complete reflection occurs at the interface of gas and soft tissue. Microbubble-based ultrasound contrast agents (UCAs) have been developed to improve echogenicity by increasing acoustic scattering and reflection in arteries or

perfused tissues, especially in cardiosonography. Contrast-enhanced ultrasound (CEUS) has made a significant contribution in clinical diagnosis. Microbubbles must be sufficiently small in order to exit the heart through the pulmonary capillaries and serve as surrogate red blood cells acting as true, non-diffusible, intravascular indicators. A variety of UCAs have been developed and undergone preclinical and clinical trials. Only a few have received Food and Drug Administration (FDA) approval for clinical use. The first approved UCA was Albunex in 1994 for left ventricular opacification [6-8]. Optison (GE Medical Diagnostics) and Definity (Lantheus Medical Imaging) were approved by the FDA in 1997 and 2001, respectively [9]. SonoVue (Bracco Imaging) and Optison have been approved in Europe for clinical diagnosis. Sonazoid (GE Medical Diagnostic) is approved in Japan and Korea [10]. Figure 1 shows an example of nodular peripheral enhancement in a 2.5 cm hemangioma in the right lobe of the liver using transverse CEUS scan after Sonovue injection. Although unparalleled images of the heterogeneity of tissue perfusion can be provided when intravenously infused UCAs circulate freely throughout the circulatory system [11], CEUS imaging has not yet been able to quantify organ perfusion (*i.e.*, cardiac system, liver, kidney, and brain) [12].

Figure 1. Transverse CEUS scan (a) 6 seconds and (b) 12 seconds after Sonovue injection during the early arterial phase shows nodular peripheral enhancement and very quick centripetal fill-in of the lesion (arrow), respectively, in a 39-year-old woman with a 2.5 cm hemangioma in the right lobe of the liver, courtesy of [13]

Recently, theranostic technology with concurrent and complementary diagnostic and therapeutic capabilities has become an emerging and promising modality in clinical treatment. Agents are involved to generate signals in response to specific pathological stimuli (*i.e.*, disease diagnosis) and simultaneously release a therapeutic particle (*i.e.*, drug, protein, gene, nucleic acids) to the pinpointed targeted areas. Theranostics may be a revolution in medicine and in the pharmaceutical industry. Ultrasound has been used widely

in clinics for therapy, such as physiotherapy, hyperthermia, and high-intensity focused ultrasound (HIFU) for tumor ablation. Due to greatly increased interest and the sophistication of imaging and molecular biology techniques, the growth of therapeutic ultrasound is rapid. The first successful application of therapeutic ultrasound in human skin metastases was reported in 1944 [14] despite its first failure on Ehrlich's carcinoma in 1933 [15]. There is now considerable interest in combining ultrasound exposure with microbubbles that act as the vehicle for localized drug delivery [16]. Compared with other approaches, this approach may change the structure of cell membranes and then release encapsulated drugs and molecular mediators (*i.e.*, dextran, pDNA, siRNA, and peptides) into the cytosol upon exposure to ultrasound waves, thus bypassing the degradative endocytotic pathway both *in vitro* and *in vivo* [17–21]. As a result, the therapeutic index of agents could be increased, and the use of agents with high toxicity or therapeutic inefficiency may be reconsidered and reintroduced. The transformation of microspheres into powerful therapeutic systems by simple application of external acoustic energy has great potential and has attracted a great deal of research interest [22]. Its technical advantages include the use of non-ionizing acoustic waves, with high spatial and temporal resolution, real-time monitoring, affordability, easy operation, portability, wide availability in clinics, and favorable economics.

19.2. MECHANISM OF ULTRASOUND-MEDIATED DRUG DELIVERY

The absorption and dissipation of acoustic energy in a medium will cause an elevation in temperature. In soft tissue, ultrasound-induced hyperthermia, at a temperature of 40–45 °C, has been found to decrease DNA synthesis, alter protein synthesis (*i.e.*, heat shock proteins), disrupt the microtubule organizing center, vary expression of receptors and binding of growth factors, and change cell morphology and attachments at the both the subcellular and cellular levels [23,24]. Thermo-sensitive drugs can be activated by hyperthermia. Even non-thermosensitive polymeric carriers and drugs exhibit increased localization in heated tumors because of increased tumor blood flow and vascular permeability. Subsequently, the cytotoxicity of the chemotherapeutic agent is enhanced.

Propagation of acoustic waves in the medium results in cyclic bubble compression and expansion and significant energy deposition around the bubbles, as shown in Figure 2. The driving frequency and acoustic pressure amplitude determine the relative contributions of thermal and mechanical mechanisms in the sonication region. The mechanical index (MI) is usually used to describe the possibility of acoustic cavitation [25].

$$MI = \sqrt{\frac{p}{f}} \quad (1)$$

where p is the peak negative pressure and f is the driving frequency. At a low acoustic amplitude, microbubbles oscillate in a linear manner. Mechanical resonance effects amplify microbubble scattering by an order of magnitude. With an increase of amplitude, significant non-linear responses are evoked. Extraction of the non-linear acoustic response could highlight the signal from microbubbles, which is now available in some sonographic systems. A higher MI (0.3–0.6) causes forced expansion and compression of microbubbles and results in violent bubble collapse [26]. There is less collateral damage to surrounding tissue induced by stable cavitation, while inertial cavitation, using either native or introduced bubbles, may produce significant effects on the extracellular membrane (*i.e.*, permeability) that facilitate drug and gene delivery, generate nanocarrier destabilization (*i.e.*, drug release), directly affect intracellular vesicle morphology, and induce several biological effects to enhance endosomal escape, all leading to the cellular uptake of therapeutic molecules [27]. Acoustic cavitation plays a potentially key role both in achieving targeted and localized drug release and enhanced extravasation at modest output levels, whilst simultaneously enabling real-time monitoring of the drug delivery process. However, 0.5–2.5 MHz ultrasound with up to 2.0 MPa pressure alone showed no significant difference in cell viability [28].

Figure 2. Schematic diagram of oscillation and collapse of bubble in the acoustic field, which is termed as acoustic cavitation phenomenon

Meanwhile, ultrasound may also increase the convection of liquid by acoustic streaming in the direction of sound propagation [29,30] or microstreaming

and shear flow due to stable or inertial cavitation of oscillating bubbles (*i.e.*, repeated expansion and shrinkage) [30,31]. Both of them may increase microvascular leakage and enhance drug delivery by extravasation.

If inertial cavitation occurs in proximity to the target cell, transient pores may be formed in the cell plasma membrane, as shown in Figure 3, which offers an efficient way for intracellular uptake of a drug/gene *via* enhanced membrane permeability or endocytosis, although its transfection efficacy is not so high as with viral vectors, and the time window is limited [32,33]. Those relatively small pores (from tens to hundreds of nanometers) will seal by energy- and calcium-dependent repair within a few minutes [33-35]. Otherwise, cell viability may not be maintained. Microbubbles serving as cavitation nuclei greatly enhance sonoporation. The concentration of microbubbles at a target site needs to be optimized; too high a concentration poses the potential risk of embolism and may also produce an excessive acoustic shielding effect, preventing exposure of the target tissue. In order to avoid irreversible membrane disruption and a disrupted cell cycle and consequently significantly reduced detrimental cellular bio-effects [36], a series of relatively short ultrasound pulses are generally delivered. The thresholds of inertial cavitation depend on the shell elasticity of microbubble. Thus, sonoporation may ultimately be most effective in promoting the extravasation of large macromolecules to improve delivery to tissue beyond the vasculature [37-40]. Sonoporating the tissue first and then releasing the nanoparticle before the pores on the cell membrane reseal may be advantageous. Sonoporation is suited for site-specific drug delivery by controlling ultrasound exposure under the guidance of a certain imaging modalities.

Figure 3. Representative pores at (a) MAT B III and (b) red blood cells after sonication in the presence of microbubbles illustrated by scanning electron microscope, courtesy of [41]

One of the major obstacles to non-viral gene delivery is nuclear entry. Passive diffusion of macromolecules in the cytoplasm is restricted by the complex

network of microtubules, proteins, and various subcellular organelles. For non-dividing cells, molecules larger than 40 kDa are actively transported into the nucleus through a nuclear pore complex. In contrast to plasmid DNA delivery, inefficient RNA interference (RNAi) is preferred in the transnuclear localization of siRNA since siRNA acts in the cytosol.

In addition to membrane channel transport, endocytosis, an energy-requiring process by which cells absorb molecules by engulfing them, also plays an important role [36,42]. The induction of surface pores or depressions may enhance the effectiveness of endocytosis; the process is illustrated in Figure 4. Increased local hyperpolarization, endocytosis, and pinocytosis of the cell membrane favor the absorption of macromolecular substances in the size range of 70–500 kDa [43].

Figure 4. Schematic diagram of different types of endocytosis

Apoptosis (programmed cell death) is the initiative programmed death of gene-controlled cells under physiological or pathological conditions, as shown in Figure 5. This may occur in the developmental process of tissues and organs, or in stressed cells to get rid of irreversible damage or harmful cells (*i.e.*, malignant tumors). Molecular pathways of cell apoptosis are influenced by an array of stimuli, such as the lack of cell growth factors, ionizing radiation, DNA damage, immunoreactions, ischemic injury, anti-hormonal therapy, and the expression of genes and the intracellular distribution of a cytotoxic agent. Most apoptotic pathways involve aspartate-specific cysteine protease family members (the caspases), cell senescence, pyroptosis, and poly(ADP-ribose) polymerase-1 (PARP-1)-mediated cell death. Sonoporation may lead to apoptosis and cell cycle arrest to suppress cancer cell growth. With plasmid transfection and ultrasound irradiation, the apoptosis rate is about 13 %; the apoptosis rate with ultrasound targeted microbubble destruction (UTMD) is 43.86 % $\pm$ 4.44 % [44].

Figure 5. The pathways of apoptosis, courtesy of [45]

The use of light for therapy began in 1900, combining acridine orange and light to destroy a paramecium [46]. The cytotoxic product of the photochemical reaction of non-porphyrin photosensitizers was identified to be singlet oxygen [47]. The terminology of sonodynamic therapy, which combines ultrasound with a sonosensitive agent derived from chlorophyll, appears contextually aligned with photodynamic therapy. The use of ultrasound is more complicated than using light because it can potentially produce free radicals and light (sonoluminescence) during acoustic cavitation. The agents themselves have no antitumor ability, but exhibit it only in the context of sonochemistry. Therefore, much less risk of adverse effects is expected for normal tissues.

19.3. DRUG VEHICLE CARRIER

Contrast agents (Echovist®, agitated saline containing air bubbles) were first used in in 1968 for echocardiography; improved aortic delineation was reported. However, large air bubbles disappeared within a few seconds following intravenous injection due to the high solubility of air in the blood, and the inability of the bubbles to pass through pulmonary capillaries. With continued interest and technological advances in CEUS, efforts have been made in the design and manufacturing of microbubble contrast agents, especially in terms of their clinical safety, stability, and size. The second generation of microbubbles have been developed, using high molecular weight hydrophobic and poorly diffusive gases (*i.e.*, perfluorocarbons, perfluorobutane, and sulfur

hexafluoride) surrounded by a thin and stabilizing shell composed of phospholipids and biocompatible and biodegradable polymers (*i.e.*, pLGA), proteins, or surfactant molecules [48,49]. Longer-chain lipids with a higher phase transition temperature can improve the stability of microbubbles. The circulation half-life of Optison® and SonoVue® is more than 15 minutes [50,51]. Such microbubbles can circulate a few times after injection. The microbubbles (0.5–8 μm in size) have resonance frequencies within the range of a sonographic system (0.2–15 MHz). If microbubbles are less than 0.5 μm in diameter, there is no significant contrast effect at clinical concentrations. Enhancing cross-linking and/or chain entanglement in the shell, such as by using synthetic polymers, further enhances the stability of microbubbles, but reduces the elasticity of the shell and attenuates their oscillation patterns.

The simplest way of enhancing drug delivery by ultrasound is to introduce microbubbles and the therapeutic agent of interest simultaneously. For example, blood clots could be dissolved more quickly for decanalization in stroke patients under ultrasonic exposure in the presence of microbubbles and tissue plasminogen activator (tPa) or urokinase. However, transfection is poor if the target is not in the circulatory system.

The various physicochemical properties of microbubbles allow for a variety of bioactive substances (*i.e.*, genes, drugs, proteins, antisense constructs, gene silencing constructs, and stem cells) to be attached to or incorporated in order to increase the ability to be effectively and specifically introduced into different targets. There are various ways of entrapping drugs within a microbubble (see Figure 6) [52,53]. Drugs may be incorporated into the membrane or in a shell of microbubbles. A monolayer lipid shell (2–3 nm for phospholipid microbubbles) limits loading the hydrophobic pharmaceuticals, and may lead to a premature release [54]. Although a thicker triglyceride lipid shell can increase the loading capacity, it is only available for hydrophobic drugs (*i.e.*, paclitaxel). Polymeric microbubbles have a much higher loading capacity of both hydrophobic and hydrophilic drugs; the release rate depends on the drug properties (*i.e.*, lipophilicity and water solubility). Negatively charged drugs can have stable and strong deposition in or onto a cationic microbubble shell by electrostatic interactions. However, Küppfer cells, leukocytes, and macrophages may capture these charged microbubbles. Because of the short half-life, UTMD has mainly focused on to the cardiovascular system, the central nervous system, and tumor endothelium. Multiple drug reservoirs (*i.e.*, nanoparticles encapsulated with different types of therapeutics) can attach to the microbubble surface or be enclosed within the microbubble.

Figure 6. Different approaches to loading drugs/DNA into microbubbles by (A) attaching to the membrane, (B) embedding within the membrane, (C) bounding non-covalently to the surface, (D) enclosing inside, and (E) incorporating into an oily film surrounded by a stabilizing layer with a ligand for targeting, courtesy of [55]

Nucleic acids are rapidly degraded in a biological environment. Deposition methods for nucleic acids include direct incorporation of the DNA into the microbubble shell [56], the use of cationic lipids in the microbubble shell [32], deposition of single [57] or multiple layers [58] of cationic polymers on the microbubble shell, covalent linking of DNA-nanoparticle carriers [59], and the use of complementary DNA strands to load nanoparticles. The drawbacks of incorporated naked plasmid DNA (pDNA) and pDNA-polymer complexes released from microbubbles are the large microbubble size (3–7 μm with a consequently short circulation time and ineffective extravasation into the tumor); the necessity to complex pDNA with cationic polymers to prevent degradation; the low loading efficiency of pDNA (~ 6700 molecules/bubble) due to the limited number of cationic lipids; and premature release of more than 20 % of the encapsulated pDNA [60]. pDNA and siRNA have been covalently bound *via* biotin-avidin-biotin linkages to the microbubble shell. The capacity of a 3 μm bubble is more than 12,000 DNA molecules.

pDNA is bound to cationic lipid shelled microbubbles *via* electrostatic charge coupling [61,62]. Mixing a cationic lipid in the aqueous phase with other lipid components uniformly is a simple method of preparation. Such electrostatic interactions are controlled by the ionic strength of the incubation media, the concentrations of the reactants, and their order of mixing. The much smaller size and lower cationic charge of the resulting polyplexes facilitate cellular

uptake. However, aggregation between cationic polyplexes and anionic blood proteins (*i.e.*, albumin) leads to rapid clearance by the reticuloendothelial system (RES). Immature decomplexation of cationic polymers and anionic nucleic acids occurs because of the high ionic strength of the blood.

The cationic polymer polyethylenimine (PEI) has high cationic charge, which enables the polymer to bind and condense DNA, as well as inhibit enzymatic degradation, prolong the *in vivo* lifetime, promote endocytosis for cellular uptake, facilitate endosomal escape of DNA into the cytoplasm, and enhance the degradation of nucleic acids by acid activated enzymes in the cytoplasm. After crossing the membrane, PEI can induce osmotic swelling and trigger the release of DNA [63]. PEI-based vectors are rapidly cleared by the RES and are cytotoxic in high doses. Ameliorating the surface charge by adding non-ionic polyethylene glycol (PEG) can significantly improve their performance. Covalently coupling PEI polymers to the lipid microbubble shells *via* PEG-tethered maleimide groups (PEI-PEG-SH) creates polyplex-microbubble hybrids [64]. PEI and DNA loading into microbubbles can be controlled by modulating the maleimide concentration in the microbubble shell. *Ex vivo* studies on excised tumors have shown 40-fold higher expression, while a 10-fold increase was found *in vivo* [65-67].

Anionic bubble lipopolyplexes, 450–600 nm in diameter, deliver pDNA into cells without endocytosis and lead to high gene expression in liver non-parenchymal cells following US exposure. In addition, anionic bubble lipopolyplexes do not show any severe hepatic toxicity and do not enhance the production of proinflammatory cytokines. Because of their neutral electric charge, anionic bubble lipopolyplexes can be prepared without aggregation even under high concentration conditions.

In order to selectively adhere microbubbles to cellular epitopes and receptors of target cells and subsequently increase drug delivery specificity and transfection, one or several specific ligands, such as antibodies, carbohydrates, and peptides, are coupled to the shell (see Figure 7) [68]. Monoclonal antibodies have a very high specificity and selectivity for a large range of epitopes. In contrast, peptides are low-cost and less immunogenic. Simultaneous targeting to multiple ligands could synergistically increase adhesion strength [69]. There are two ways of coupling ligands to the microbubble shell: covalent binding by being attached to the head of phospholipids directly or *via* an extended polymer spacer arm and non-covalent binding by avidin-biotin bridging and streptavidin-biotin bonding. However, since avidin carries a strong positive charge in the glycosylate layer, the bio-distribution of microbubbles may be altered, resulting in non-specific adhesion and initiation of an undesired immune response. Furthermore, several washing steps required in the loading process influence microbubble stability and reproducibility. In comparison, streptavidin may be a better alternative. Using a PEG molecular tether as an intermediary spacer arm between the ligand and the lipid shell indirectly is

feasible and results in high specificity and targeting. Folate receptors are expressed in large numbers on many cancer cells. Folate that is attached to a PEG tether may undergo enhanced interaction with receptors on the cell membrane, leading to intracellular uptake.

Figure 7. Targeting microbubbles to cancer cells by connecting the receptors on the surface with (A) an antibody, (B) an avidin bridge, or (C) a flexible spacer arm, courtesy of [70]

Magnetic microbubbles have been developed which are capable of carrying a drug payload and can be moved to the target site by means of an external magnetic field gradient under the guidance of magnetic resonance imaging (MRI); these are then disrupted by a focused ultrasound beam [3,71,72]. Superparamagnetic materials are used to build to the shell because of a compromise between strong magnetization and avoidance of particle aggregation [73]. Superparamagnetic iron oxide nanoparticles SPION loaded bubbles have been shown to improve the contrast of both ultrasound and MR images [74]. A mixture of non-magnetic microbubbles and magnetic micelles (containing magnetic nanoparticles but no gas) have in fact shown slightly higher transfection efficiency upon exposure to both ultrasound and a magnetic field. In an alternating magnetic field (AMF), heat will be generated in magnetic nanoparticles because of hysteresis loss and/or Neel relaxation. These effects alter the nanocarrier structure, *i.e.* by increasing the shell or bilayer porosity, disintegrating the Fe_3O_4 core, or deforming the single-crystal nanoshell lattice, leading to pulsatile drug release on demand. However, magnetic guidance is hampered by the complexity of the set-up and the high strength and gradient of the magnetic field that needs to be applied against the hydrodynamic forces of blood flow [75].

However, the accessibility of microbubbles is restricted because of their size through vasculature barriers. Advances in nanotechnology could benefit drug and gene delivery, since nano-sized carriers (<500 nm) are optimal for

extravasation into the tumor interstitial space, passing through cell membranes, and delivering both small molecules and macromolecules [76].

Liposomes, made by hydrating a dry lyophilized precursor matrix, have pockets of entrapped air within the lipid bilayer of the liposome membrane and can deliver both hydrophilic and hydrophobic drugs and larger molecules (*i.e.*, pDNA, siRNA, and mRNA) by an ultrasound trigger [77,78]. A significant merit of nanoparticles over microbubbles is their small size (usually <250 nm in diameter) and PEG coating, which allow them to circulate in the bloodstream for hours and then extravasate in tumors, ischemic cardiac tissue, and skeletal muscle [50].

Thermosensitive liposomes (TSLs) were first synthesized in 1980 to treat solid L1210 xenograft tumors in mice [79], but it was found that therapeutic ultrasound can serve as a source of hyperthermia and trigger doxorubicin (DOX) release [80]. Such a thermal response arises from a phase transition of the constituent lipids and conformational variations in the lipid bilayer at around 40 °C (see Figure 8). These are the most advanced thermoresponsive nanosystems and have been used in several clinical trials [81,82]. TSLs can be mixed with microbubbles to synergize both the thermal and cavitational mechanisms (cavitation and radiation force) induced by ultrasound [83,84].

Figure 8. Release of doxorubicin from temperature-sensitive liposomes at their phase transition temperature, which is typically chosen to be 40–42 °C

Conjugating liposomes loaded with both hydrophilic and hydrophobic drugs to a microbubble shell *via* avidin–biotin interactions, as shown in Figure 9, combines the advantages of liposomes and microbubbles and overcomes their respective individual limitations [85,86]; $\sim 10^5$ liposomes can be bound to each microbubble. This 1.5 μm liposome-microbubble hybrid oscillates in a similar manner to unconjugated microbubbles. After disruption of the microbubble, the liposomes are detached. Positively charged lipoplexes or polyplexes can be placed on the surface of the microbubbles using a similar approach [59,87].

The FDA-approved liposomal drugs are listed in Table 1, while there are many more drugs still at the stage of clinical trials [88,89].

Figure 9. Schematic diagram of PEGylated nanoparticles attached to a microbubble *via* avidin-biotin binding, courtesy of [90]

Table 1. Liposomal drugs approved for clinical use, courtesy of [88,89]

Product	Drug	Approved indication
Ambisome	Amphotericin B	Severe fungal infections leishmaniasis
Abelcet	Amphotericin B	Severe fungal infections
Amphotec	Amphotericin B	Severe fungal infections
DaunoXome	Daunorubicin	Kaposi's sarcoma
Doxil/Caelyx	Doxorubicin	Kaposi's sarcoma, ovarian/breast cancer, multiple myeloma+Velcade
Lipo-Dox	Doxorubicin	Kaposi's sarcoma, ovarian/breast cancer
Myocet	Doxorubicin	Combination therapy with cyclophosphamide in metastatic breast cancer
Visudyne	Verteporfin	Age-related molecular degeneration, pathologic myopia, ocular histoplasmosis
Depocyt	Cytarabine	Neoplastic meningitis and lymphomatous meningitis
DepoDur	Morphine sulfate	Pain following surgery
Diprivan	Propofol	Anesthesia
Marqibo	Vincristine	Acute lymphoblastic leukemia
Estrasorb	Estrogen	Menopausal therapy

(continued) Table 1. Liposomal drugs approved for clinical use, courtesy of [88,89]

Product	Drug	Approved indication
Expaxal	Inactivated hepatitis A virus (stain RG-SB)	Hepatitis A
Inflexal V	Inactivated hemagglutinin of influenza virus strains A and B	Influenza

Alternatively, microbubbles (1–5 μm) can be formed *in situ* from a small liquid emulsion droplet (200–300 nm) by acoustic droplet vaporization (ADV) using ultrasound frequencies above 1 MHz applied in microsecond-length pulses [91–93]. Perfluorocarbon droplets have a low boiling point and high stability. The Laplace pressure imposed upon the emulsion droplets due to their high curvature is given by:

$$\Delta p = p_{\text{in}} - p_{\text{out}} = \frac{2\gamma}{R_e} \quad (2)$$

where p_{in} and p_{out} are the pressures inside and outside the droplet, respectively, γ is the surface tension, and R_e is the radius of the droplet. When the temperature is above the boiling point of PFC5 (29 °C), the vapor pressure is lower than the internal pressure of the nanoemulsion droplet, so the droplet remains in the liquid state.

PEG-modified liposomes entrap nanodroplets in the size range of 600–700 nm and are termed bubble liposomes. The preparation of bubble-liposomes is quite simple: existing aqueous liposomes are placed in a vial with pressurized perfluoropropane gas, and the vial is sonicated in a bath for several minutes. Perfluoropropane inside bubble liposomes may be in the liquid form in the size range of several tens of nanometers and be subjected to excessive Laplace pressure. Liquefied superheated perfluoropropane droplets can be triggered by ultrasound to become much larger gas bubbles; this releases the liposome contents, as shown in Figure 10 [94,92]. Although cationic liposomes often cause the agglutination of erythrocytes and high levels of hemolysis due to the interaction between the lipid and the erythrocyte membrane, bubble liposomes containing cationic lipids have little effect on erythrocytes [95]. The loading of pDNA onto the surface of bubble liposomes (30–40 %) containing cationic lipids, by either electrostatic interactions or the fixed aqueous layer formed with PEG, provide effective protection of pDNA against serum, resulting in high transfection efficiency due to the retention of the pDNA-supercoiled structure [96]. Furthermore, adding pDNA to bubble liposomes does not result in a significant change in the physical properties.

Figure 10. A schematic illustration showing the composition/structure of liposomes that generate bubbles upon heating, and how they can be used to rupture cancer cells by transient cavitation, thus resulting in cell death, courtesy of [97]

eLiposomes are similar to bubble liposomes, as they encapsulate emulsions and therapeutic agent in liposomes for targeted delivery. Phase transition of emulsion droplets ruptures the lipid bilayer of the eLiposome to release the therapeutic agent after sonication. To prepare eLiposomes, a plasmid and lipid-stabilized perfluoropentane nanoemulsion is co-entrapped in carrier liposomes. There are no cationic eLiposome components and or toxic molecules in this formulation.

19.4. APPLICATIONS

19.4.1. Sonothrombolysis

Clot formation within a vessel in the brain causes ischemic stroke, within the coronary arteries causes myocardial infarction, and within the peripheral veins causes deep vein thrombosis. Acute ischemic stroke occurs frequently worldwide and is the third leading cause of death. The dissolution and breaking up of a clot using thrombolytic drugs, such as tissue plasminogen activator (tPA), could limit the damage caused by the blockage of blood vessels,

and is termed thrombolysis. Currently, the only FDA-approved therapy for acute ischemic stroke is intravenous (IV) administration of rt-PA within 3 h of stroke onset. In order to improve the clinical efficacy of thrombolysis, ultrasound has been investigated in combination with thrombolytic drugs to enhance recanalization by creating high-speed microjets during the asymmetric collapse of microbubbles [98]. The process is illustrated in Figure 11.

Figure 11. An illustration of sonothrombolysis process: (a) an intracranial occlusion, (b) microspheres reach the site of the intracranial occlusion and permeate around or through the thrombus, (c) an ultrasound pressure wave oscillates and destroys the microspheres, leading to (d) complete arterial recanalization, courtesy of [99]

Two clinical trials have investigated sonothrombolysis. The Combined Lysis of Thrombus in Brain Ischemia Using Transcranial Ultrasound and Systemic t-PA (CLOTBUST) trial used 2 MHz ultrasound at an intensity of 750 mW cm^{-2} with a sample volume of 3–6 mm for power Doppler and 10–15 mm for transcranial Doppler (TCD), respectively [100]. Early and complete recanalization of the arterial occlusion was more significant in the group exposed to continuous TCD than the control (46 % vs. 18 % within 2 h, $p < 0.001$), and both clinical recovery and complete recanalization were 25 % vs. 8 % ($p = 0.02$). In addition, both groups had similar rates of intracranial hemorrhage. The other trial was the Transcranial Ultrasound in Clinical Sonothrombolysis (TUCSON) trial using perflutren lipid microspheres [99]. Microbubbles decreased the time to arterial recanalization and increased the rate of recanalization. Low doses of microbubbles used in sonothrombolysis may be safe, but higher doses

increased the rate of symptomatic intracranial hemorrhage (SICH) [101]. Furthermore, reverberations of the low frequency ultrasound (*i.e.*, 120 kHz) inside the skull lead to hot spots of energy and more SICH [102].

Targeted Arg-Gly-Asp-Ser tetrapeptide (RGDS) microbubbles prepared by lyophilization have also been prepared to encapsulate t-PA [103]. The envelopment rate of tPA was $81.12 \pm 2.44\%$, detected by enzyme-linked immunosorbent assay (ELISA). The conjugation rate of RGDS was $94.49 \pm 6.19\%$, detected by flow cytometry. In addition, sonothrombolysis using intermittent high MI pulses guided by a 3-D sonography is effective at improving myocardial blood flow and decreasing infarct size [104]. Upstream pressure decreased progressively during US delivery, indicating the thrombolysis process. More rapid and complete lysis occurred with increasing peak negative acoustic pressure (1.5 MPa vs. 0.6 MPa) and increasing pulse length (5000 cycles vs. 100 cycles).

19.4.2. Tumor/cancer treatment

Tumor tissues have atypical microvascular permeability, leading to the enhanced permeability and retention (EPR) effect, which enhances passive drug targeting. However, the larger amount of collagen in the tumor extracellular matrix might require a higher infusion pressure to initiate the flow of medicine or a gene into the tumor mesenchyme. UTMD is a promising new method and has the advantages of non or minimal invasiveness, high gene and drug transfer efficiency, and high organ and tissue specificity [105]. It can change the tumor microenvironment and provide felicitous conditions to achieve even and sufficient drug delivery effectively [106]. Whilst there is a concern that ultrasound-induced inertial cavitation may create shear forces that induce the breakup of primary cancer cells, thereby enhancing metastasis, this has been recently assuaged. It was found that there was no difference in the observed metastases between control and ultrasound-treated xenograft mice [107]. This conclusion is also valid in human patients, as no increased chance of metastasis has been reported [108]. Tissue with thick veins could be transfected with US exposure, given enough time when the mixture of pDNA and bubble liposomes is injected. In contrast, nanosized pDNA loaded bubble liposomes (p-BLs) are a potentially superior carrier during transfection *via* microvessels or transfection into tissues with low blood flow to deliver various negatively charged molecules [109].

In human cancers, malignant cells possess defects in apoptosis that increase their resistance to anti-tumor treatment; this plays a critical role in tumorigenesis. Ultrasound irradiation has been shown to induce apoptosis in tumor cells. A significant reduction in tumor size was observed using cisplatin and cetuximab-coupled microbubbles in immune deficient mice with xenografts; the subsequent histological study illustrated increased numbers of apoptotic cells following UTMD compared with chemotherapeutic agents

alone. The induction of apoptosis mediated by UTMD is safer and more effective than other methods, by inactivating tumor cells, restraining cell proliferation, and improving the therapeutic effects of genes or chemotherapeutic drugs.

Multidrug resistance (MDR) is a major barrier to chemotherapy and is responsible for the overall poor efficacy of drug therapies. It is mediated by the overactivity of several MDR-associated proteins and P-glycoproteins. UTMD may significantly decrease P-glycoprotein and MDR-associated protein expression at the mRNA level rather than increase the sensitivity of MDR cells to sonoporation. Thus, ultrasound exposure could be used as an alternative, physical method to reverse MDR in cancer cells [110].

Doxorubicin loaded TSLs (ThermoDox, Celsion Corporation) have been assessed in phase II trials for the treatment of breast cancer and colorectal liver metastasis, and in phase III trials for the treatment of hepatocellular carcinoma in association with hyperthermia or radiofrequency ablation. An example of spheroid viability *in vitro* after treatment with TSLs and ultrasound is shown in Figure 12.

Figure 12. Microscopic observations of U-87 MG spheroids after treatment with TSLs and focused ultrasound (FUS) mediated hyperthermia, courtesy of [111]

Sonoporation and microbubbles were first utilized in clinics for local therapeutic delivery in 2013 [112]. Five patients with inoperable pancreatic cancer were administered gemcitabine followed by sequential doses of microbubbles and ultrasound within a 30 min interval. This study established a sonoporation protocol in a clinical setting toward further development of this promising technique.

The drugs used together with ultrasound to treat solid tumors and cancer are listed in Table 2. Most of them are still in animal experiments using xenograft models in mice or rats. However, a genetically modified mouse model appears

to more closely recapitulate the human disease compared to transplantation mouse models (xenograft), suggesting that insufficient drug delivery into the tumor is an important mechanism for apparent chemoresistance [113]. One of the barriers may be the presence of a prominent stromal matrix that separates blood vessels from tumor cells. In addition, there are a few reports on clinical applications.

Table 2. Summary of drugs used with ultrasound in the treatment of tumors

Protoporphyrin IX (PPIX) and hematoporphyrin (Hp)	S180 solid tumors in mice
Photofrin II (PF)	AH130 solid tumors in male Donryu rats, colon 26 carcinoma
ATX-70	Colon 26 carcinoma tumor and Walker 256 tumor in BALB/c mice
F39/ATX-70	Nude mouse xenograft model
ATX-S10	Colon 26 tumors in mice
Pheophorbide-a	Squamous cell carcinoma
C1A1-phthalocyanine tetrasulfonate	Colon 26 carcinoma
Chlorin PAD-S31	Neointimal hyperplasia
Cyclophosphamide, thiotepa	TCT-4909 bladder or hepatoma tumor in mice
Adriamycin	Yoshida rat sarcoma, fibrosarcoma RIF-1, melanoma B-16
Daunomycin	Yoshida rat sarcoma
Chlorin-e6 (Ce6)	Hepatoma-22 solid tumors in mice
Cisplatin	Murine renal function
Dihydroxy(oxybiguanido)boron	Carcinoma
5-fluorouracil	Carcinoma, Ehrlich carcinoma
Hematoporphyrin	Sarcoma 180
Cisplatin or cetuximab with microbubble	Immunodeficient mice bearing 4 HNSCC
Micellar paclitaxel formulation (SYP-PM)	MCF7/ADmt breast cancer in mice
Cisplatin liposomal	Colon adenocarcinoma tumor in BALB/c mice
Liposome-encapsulated doxorubicin (S-DOX)	RIF-1 tumor in C3H mice

(continued) Table 2. Summary of drugs used with ultrasound in the treatment of tumors

Bleomycin	B-16 melanoma in SCID mice
Apo-2LTRAIL with Smac peptides	Malignant glioma in athymic mice
Paxlitaxel-liposome-microbubble complexes (PLMC)	4T1 breast carcinoma in BALB/c mice
Plasmid DNA with SonoVue	Hepa1-6 tumor in C57BL/6J female mice

19.4.3. Angiogenesis

Angiogenesis is the physiological process by which new blood vessels form from pre-existing vessels; this process is mostly responsible for blood vessel growth during development and in disease. Delivery of hepatocyte growth factor, either in the form of recombinant protein [114] or a plasmid [115], to rodents by UTMD has been found to result in proliferation and improvement in cardiac function [116]. The mRNA levels of various angiogenic factors as well as the blood flow rate were shown to increase significantly in the group treated with bFGF gene-loaded p-BLs and US exposure compared to the control group, which could be applicable to hind limb ischemia therapy. Skeletal muscle is a versatile tissue for UTMD, due to its ease of access (*i.e.*, hind limb skeletal muscle) without surgery. The ability of intravascularly administered microbubbles to home specifically to the skeletal muscle vasculature is a key advantage over competing systems.

19.4.4. Virotherapy

Virotherapy is an important emerging therapeutic modality, especially when direct intratumoral injection of these self-amplifying tumor cell killing agents can be achieved [117]. The FDA has approved several adenoviruses for Phase III clinical trials. However, the use of virotherapy for the systemic treatment of metastatic disease may ultimately be limited by poor extravasation into tumors and inefficient intratumoral spread. It now seems that therapeutic ultrasound may offer a solution to this limitation and approach clinical applicability rapidly. However, the amount of virus delivered and the intratumoral distribution of these viruses should be measured quantitatively to evaluate the clinical outcome.

19.4.5. Gene transfection

Gene therapy has great potential to overcome the limitations of conventional drug and protein therapeutic delivery modalities for certain diseases, such as inherited immune deficiencies, cardiovascular disease, genetic disorders, and cancers. Several steps, such as cellular internalization, endosomal escape, nuclear transfer and intracellular transcription, should be rationally regulated

to achieve efficient gene transfection. Among them, endosomal escape is considered one of the most important steps, because most carriers internalize into cells *via* the endocytic pathway, but cannot escape from endosomes, resulting in gene degradation in lysosomes. Therefore, the key factor in the success of gene therapy is an efficient and safe vector. In gene transfection, the efficient delivery of a few strands should be enough to provide an effect (one DNA molecule is sufficient to synthesize thousands of protein molecules) and subsequently obtain a sufficient therapeutic response. In the past, viral vectors predominated in gene therapy due to their ease of use, optimized internalization, efficient cytosolic release, direct and fast intracellular transport toward target, ready disassembly, and high transfection efficiency. However, several safety and immunogenicity concerns have restricted their clinical use [118]. Therefore, non-viral vectors are preferred. The ability of non-viral vectors to release nucleic acids intracellularly, while surviving low pH and digestive processes and avoiding premature decomplexation, is essential. Pharmacokinetic properties (*i.e.*, distribution, circulation, and clearance) and gene transfer conditions (*i.e.*, nucleic acid concentration, presence of serum, transfection time, and post-transfection time for gene expression) concomitantly affect the gene transfection efficiency.

Initial studies in the 1980s showed that US can enhance gene transfection by directly delivering DNA into the cytoplasm rather than simply co-injecting microbubbles with the unprotected plasmid intravenously, intraarterially or locally [119-123]. Without ultrasound exposure, very low transfection could be achieved *in vivo*. UTMB technology further increases transfection efficiency with more targeting specificity and gene protection. The integrity of the target is maintained, without infection. Acoustic energy can induce obvious apoptosis and restrain tumor cell proliferation *via* molecular mechanisms involving Bak, Bcl-2, and caspases, but not reactive oxygen species (ROS) [124], and the transitory expression level of NF- κ B [125]. Figure 13 shows significant *in vitro* gene expression in cervical cancer cells using plasmids and microbubbles after ultrasound irradiation [126].

Figure 13. (A–C) TdT-mediated dUTP nick end labeling (TUNEL) and (D–F) diaminobenzidine (DAB) expression in cervical cancer cells in C: control group; P + US: plasmid + ultrasound irradiation group; P + UTMD: plasmid + microbubble + ultrasound irradiation group, courtesy of [126]

Nanoparticle-loaded microbubbles (mRNA-lipoplex loaded microbubbles) can result in significant expression of reporter genes, but essentially no gene expression can be found a week after ultrasound exposure. However, PEI/DNA complexes could prolong transgene expression for at least 15 days *in vitro* and 30 days *in vivo* [127]. Ultrasound-mediated gene therapy has been used efficiently to deliver both stem cell factor and vascular endothelial growth factor (VEGF) to infarcted heart muscle, resulting in increased expression of specific genes associated with increased vascular density derived from VEGF, along with improved myocardial perfusion and ventricular function [128]. Repeated ultrasound treatment for endostatin or calreticulin gene delivery to muscles can also produce a significant anti-tumor effect on distant orthotopic tumors in other organs, including the liver, brain, and lungs. Vaccination in the lymph nodes (intranodal vaccination) leads to a stronger immune response. Microbubbles are able to migrate to the lymph nodes after subcutaneous injection. Therefore, sonoporation has the potential of direct intranodal transfection *in vivo* with much less cost and time consumption in comparison to the *ex vivo* procedure [129].

19.4.6. Blood-brain barrier (BBB) disruption

The blood-brain barrier (BBB) is an endothelial barrier present in the brain microvasculature. It consists of tight junctions around all capillaries in the central nervous system (CNS), which separates circulating blood from the brain extracellular fluid and therefore hampers the movement of various substances (*i.e.*, polar molecular) into the brain in a chemically stable environment. Microbubble cavitation alters the integrity of BBB components with no evidence of discrete lesions [130-132]. The disrupted BBB of the sonicated rabbit brain can repair itself within approximately 6 hours, and long-term follow-up showed nearly no damage [133]. So ultrasound-induced BBB disruption is a transient and reversible event [134]. The advance in this technology provides the opportunity to treat many formerly terminal conditions of the brain, not just limited to cancer.

An intravenously administered antibody, which targets the dopamine D4 receptor in the brain, has been shown to cross the BBB in a mouse model through the intact skull and recognize its antigens using MR-guided focused ultrasound (690 kHz, burst length of 10 ms, repetition frequency of 1 Hz, sonication duration of 40 s, and varied acoustic pressure of 0.6 to 1.1 MPa) and urocanic acid (UCA) injection [135]. BBB disruption was confirmed by contrast-enhanced T1-weighted MR and post-mortem by trypan blue staining. Little or no hemorrhage in the ultrasound focal region was observed at the pressure amplitude of lower than 0.8 MPa. Using the same protocol, the feasibility of delivering the chemotherapeutic agent Herceptin (150 kDa), which is effective in the treatment of breast cancer metastases, to the normal murine brain was also investigated [135]. In addition, the delivery of DOX, encapsulated in long-circulating pegylated liposomes, was also examined in the normal rat brain, as shown in Figure 14 [136].

Figure 14. (a) Contrast-enhanced transversal (axial) T1-weighted MR fast spin-echo image after sonication at acoustic power of 0.6 W. (b) The leakage of Trypan blue (arrows) from the vasculature into the brain parenchyma fixed in 10 % buffered formalin phosphate 4 h after sonication, courtesy of [136]

19.5. FUTURE WORK

There is plenty of evidence that ultrasound-mediated drug delivery and gene transfection in the presence of microbubbles is safe and effective. Ultrasound can be applied easily with a physical therapy apparatus or diagnostic equipment or using more complicated MRI-guided focused ultrasound systems. However, a lot of work is required to demonstrate the underlying mechanisms of action. For example, a clear understanding of the interaction between microbubbles and pDNA and the nuclear translocation mechanism of pDNA in the cytoplasm remains elusive and is ripe for investigation. New insights into sonoporation and the associated cellular mechanisms need to be further explored to allow optimal use of ultrasonic drug delivery [42]. The exact mechanism of ultrasound-mediated BBB penetration also needs to be elucidated. The molecular mechanism is subtle and elusive; it likely involves underlying DNA damage response pathways induced by ultrasound, which need further study. *In vitro* systems allow for the assessment of different mechanisms of action and good ultrasound dosimetry, but have little clinical relevance. It is difficult to quantify ultrasound exposure *in vivo* and to identify the individual elements responsible for the illustrated effects. New types of apoptosis-inducing methods specific to the molecular mechanism (*i.e.*, caspases) have become a new research direction and represent an important strategy for anticancer therapy. To further develop the applications of ultrasound and introduce them into the clinic, interdisciplinary collaboration is in great need. Researchers in cellular physiology, genetics, physical chemistry, and acoustic physics should collaborate with new strategic partnerships to be established among industry, pharmaceutical companies, and academia in order to accelerate the development of this novel technology [137].

A new generation of delivery vehicles which are tailor-made for cavitation-enhanced drug delivery for cancer or other targets is under continuous development. On-demand drug delivery can be improved using multiple stimuli-responsive systems, which recognize the microenvironment and mimic the responsiveness of living organisms. The diversity of responsive materials can be assembled in different formats with great flexibility. However, despite of *in vitro* proof of concept, only a few stimuli-responsive systems have been tested in *in vivo*, and very few have reached the clinical stage. The complexity of architectures and difficulties in scaling up the synthesis are likely to hamper their translation from bench to bedside. Moreover, toxicity is multi-factorial, depending on the composition, physicochemical properties, route of administration, and dose. Therefore, the benefit-to-risk ratio has to be balanced [138].

In the near future, the regulatory status of ultrasound-mediated therapies will need to be defined for clinical use. To prepare a way for future clinical therapy, a large number of animal studies are required to evaluate the feasibility and efficacy of UTMD technology according to the regulations of medical

authorities. Adverse effects, such as tissue damage and hemorrhage, should be avoided or minimized by decreasing microbubble concentrations and ultrasound energy. Moreover, careful monitoring is demanded in clinical settings to assure patient safety [139]. Passive acoustic mapping provides spatio-temporal information of cavitation activity using conventional ultrasound imagers, and could thus inform clinicians when and where a therapeutic agent has been successfully delivered [140,141]. To translate UTMD into clinical trials, more robust ultrasound parameters must be defined, such as the pressure threshold for cavitation initiation [142]. Both the enhanced permeability and retention effect and ligand recognition, which may be a consequence of the stochastic nature of ligand–receptor interactions, which is difficult in the control of drug release from targeting nanocarriers, still remains questionable in the clinic. The use of viral structures (*i.e.*, adenovirus), regarded as therapeutic biological products by the FDA, in combination with microbubbles and ultrasound, might also reach clinical trials within a few years, even if negligible viral transfection occurs in remote organs due to safety concerns [116]. More complicated structures, which combine nucleic acids and ultrasound-triggered carriers into micro or nanoparticles, will take many years to bring to clinic because they will be regarded as drugs by the FDA.

19.6. CONCLUSION

Ultrasound-mediated drug delivery has been investigated for decades. With the development and application of micro or nanobubbles, its efficiency and efficacy have been enhanced significantly due to cavitation effects. The design of specifically-targeted bubbles with loaded with drugs and/or genetic material makes theranostics possible, which will not only increase local transport but also reduce immature release and unexpected side effects. Because of the popularity of sonographic systems in hospitals and their easy operation, this technology may have great potential for clinical translation. Although most successful reports are at the stage of animal experiments, several drugs are in different phases of clinical trials and may be approved within a few years. In order to further develop this technology, the mechanisms of bubble interactions with drugs and tumor cells should be better understood. Extensive collaboration between academia, hospitals, the pharmaceutical industry, the ultrasound industry, and medicine regulatory authorities is necessary to push this technology forward.

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COPPER SULFIDE NANOPARTICLES: FROM SYNTHESIS TO BIOMEDICAL APPLICATIONS

Yuanyuan Qiu^{1,2}, Lei Lu^{1,2}, Dehui Hu^{1,2}, and Zeyu Xiao^{1,2,3*}

¹ Department of Pharmacology, Institute of Medical Sciences, Shanghai Jiao Tong University School of Medicine, 280 South Chongqing Road, Shanghai, 200025, PR China

² Translational Medicine Collaborative Innovation Center, Shanghai Jiao Tong University School of Medicine, 280 South Chongqing Road, Shanghai, 200025, PR China

³ Collaborative Innovation Center of Systems Biomedicine, Shanghai Jiao Tong University School of Medicine, 280 South Chongqing Road, Shanghai, 200025, PR China

*Corresponding author: zxiao@sjtu.edu.cn

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20.1. INTRODUCTION

The dream of developing nanoscience and nanotechnology started in 1959, when Richard Feynman presented his vision of manipulating and controlling things on a small scale at the annual meeting of American Physical Society. In 1968, molecular beam epitaxy was discovered in the Bell Laboratories, followed by the invention of scanning tunneling microscope, eventually contributing to the sophistication in the fields of nanoscience and nanotechnology [1].

As an important branch of the nanoscience and nanotechnology field, the development of nanomaterials for biomedical applications has received great attention in the past thirty years. Many nanomaterial-related products, such as textiles and cosmetics, have entered the market at an increased pace. Design and synthesis of multifunctional nanomaterials that combine two or more functions have provided potential applications in the diagnostic and treatment of cancer and a myriad of other diseases. Tunable physicochemical properties and their abilities to be efficiently applied in biological systems upon appropriate modification, make nanomaterials among the most promising systems for a range of biomedical applications, such as biosensing, molecular imaging, drug delivery, and therapy [2].

Copper sulfide nanoparticles (CuS NPs) are a class of semiconductor nanomaterials that demonstrate interesting characteristics for biomedical applications. Besides possessing the common features of semiconductor nanomaterials, including the variable energy structure and enhanced surface properties, CuS NPs are capable of forming various stoichiometries with unique optical and electrical properties, and thus they are explored as a potential platform for biosensing [3,4], molecular imaging [5], photothermal therapy [6], drug delivery [7], and theranostic agents [8].

In this review article, we summarize recent progress of CuS NPs in biomedical research. Firstly, we illustrate the detailed synthesis procedures of CuS NPs with distinct morphology and spatial orientation. Subsequently, we discuss the emerging role of CuS NPs for biomedical applications. Finally, we envision the major challenges and future directions of CuS NPs.

20.2 SYNTHESIS OF CuS NPs

Many studies reported the synthesis strategies of CuS NPs with all three dimensions, including (i) one dimensional nanorods, nanotubes, and nanowires, (ii) two dimensional nanoplates, and (iii) three dimensional hollow/solid nanospheres, core-shell NPs, and nanocages (Figure 1). The synthesis methods may vary with the morphology, which in turn contributes to

diversified properties and biomedical applications. One dimensional CuS nanostructures are widely used as biosensors, mainly due to their excellent electrochemical and catalytic properties; two dimensional CuS nanostructures are investigated for improving photothermal conversion efficiency; and three dimensional CuS nanostructures are suitable for biomedical applications, resulting from their easy-to-decoration properties and favorable physicochemical characteristics.

Characterization of CuS NPs can be performed using a wide variety of techniques, such as scanning electron microscopy (SEM), X-Ray diffraction (XRD), energy dispersive X-Ray spectroscopy (EDS), transmission electron microscopy (TEM) and high resolution TEM (HRTEM), atomic force microscopy (AFM), dynamic light scattering (DLS), Fourier transform infrared spectroscopy (FTIR), UV-visible and photoluminescence (PL) spectroscopy, *etc.* These techniques provide important information on the elemental, structural (*e.g.*, size and shape), and optical properties of CuS NPs [2].

Figure 1. (A) SEM image of solution-based synthesized CuS nanotubes. This image is adapted with permission from [9] © (2014) American Chemical Society. (B) TEM image of CuS nanoplates. This image is adapted with permission from [10] © (2011) American Chemical Society. (C) TEM image of hollow CuS NPs. This image is adapted with permission from [11] © (2014) American Chemical Society. (D) TEM image of CuS nanospheres. This image is adapted with permission from [12] © (2012) American Chemical Society

20.2.1. Hydrothermal method

Hydrothermal method offers a cheap, green and highly scalable way to prepare inorganic nanomaterials, and it is mainly applied to synthesize metal oxide based materials. This first report of using hydrothermal method was to synthesize metal sulphide nanomaterials [13]. Subsequently, this method was applied to manufacture a wide range of binary metal sulphides (*e.g.*, ZnS, CdS, PbS, CuS, $\text{Fe}_{(1-x)}\text{S}$, and Bi_2S_3). By varying the reaction conditions, two mechanisms may be involved in the synthesis process: a growth dominated route which permits the formation of nanostructured sulphide materials, and a nucleation driven process which produces NPs with temperature dependent size control. Consequently, this method offers a viable route to construct a wide range of metal sulphide NPs with flexible size and shape control [14].

Hydrothermal method can be used to fabricate uniform CuS NPs, and the reaction is performed at a relatively low temperature without using complex and toxic organometallic reactants. For example, CuS nanospheres (Figure 1d) could be synthesized simply by mixing aqueous solutions of CuCl_2 , sodium citrate, and Na_2S together at room temperature and the following reaction at 90 °C for 15 min [8]. The size and shape of the nanospheres could be tuned by changing parameters such as the category of the precursors, temperature of the reaction, reaction time, and *etc.* In another example, hollow CuS NPs was based on the self-assembly of nanoflakes derived from Cu(II)-thiourea complex into hollow nanospheres, with the size of several microns in diameter. Another study demonstrates the formation of hollow nanospheres and nanotubes from 5–10 nm CuS NPs at room temperature [15]. It was suggested that such hollow structures may be formed as a result of decomposition of thiourea into H_2S , which further reacts with the Cu precursor to produce CO_2 . The CO_2 produced forms gaseous cavities which act as heterogeneous nucleation centers under hydrothermal conditions for the aggregation of CuS nanoflakes, finally giving rise to hollow nanostructures. Two dimensional CuS nanostructures which are relatively rare and restricted mainly to nanoplates (Figure 1b) can also be prepared with hydrothermal methods. CuS nanoplates, the thickness and edge length of which are about 15 nm and 60 nm, have been successfully prepared in the presence of cation surfactant cetyltrimethylammonium bromide (CTAB) by hydrothermally treating the solution of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ and $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ at 180 °C for 24 h. The as-prepared CuS nanoplates are of hexagonal phase and are single crystal [16].

20.2.2. Microwave irradiation method

Microwaves are electromagnetic waves containing electric and magnetic field components. The electric field applies a force on charged particles, which was driven to migrate or rotate. The movement of charged particles further polarizes the particles. The concerted forces applied by the electric and magnetic components of microwaves rapidly change in direction, which

creates friction and collisions of the molecules. Microwave irradiation has been developed and widely used in the fields such as molecular sieve preparation [17], radio pharmaceuticals [18], preparing inorganic complexes and oxide, organic reactions, plasma chemistry, analytical chemistry, catalysis, and recently in the preparation of nanocrystalline particles. Microwave irradiation has shown very rapid growth in its application to materials science due to its unique reaction effects such as rapid volumetric heating and the dramatic increase in reaction rates, *etc.* Compared with conventional methods, microwave synthesis has the advantages of short reaction time, small particle size, narrow particle size distribution and high purity.

For the synthesis of CuS NPs, this method mostly uses microwave irradiation (~ 180 W) in aqueous medium or ethylene glycol for ~ 20 min to carry forth the decomposition process [3]. An early report stated a route to prepare CuS nanorods via the microwave-induced heating in aqueous solution under ambient air. Powder X-ray diffraction pattern indicates that the product was CuS composed of uniform and regular rods with diameters ranging from 5–10 nm and lengths 30–50 nm. The product has good crystallinity, uniform morphology, and high purity [19]. In another report, pure CuS with flower-like, hollow spherical, and tubular structures (Figure 1a) were synthesis by controlling pH and irradiation rate. In this method, $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ and CH_3CSNH_2 were dissolved in ethylene glycol, and followed by the addition of NaOH to form solutions with different pH values. Reactions proceeded in surfactant-free solutions contained in an acid digestion bomb using a microwave irradiation at different conditions [20].

20.2.3. Sonochemical method

Sonochemical method is a facile route operated under ambient conditions. During sonication, ultrasonic sound waves radiate through the solution causing alternating high and low pressure in the liquid media. This leads to the formation, growth, and implosive collapse of bubbles in a liquid. The collapse of bubbles with short lifetimes produces intense local heating and high pressure, thus facilitating the preparation of NPs with uniform shape and narrow size distribution. This method has become an important tool in the synthesis of various nanostructures, including particles, hollow spheres, porous spheres, rods, tubes, and wires [21].

Sonochemical methods are extensively explored to synthesize CuS NPs. For example, Kuar *et al.* firstly reported to synthesize CuS/PVA NPs with the size of 165–225 nm by sonochemical irradiation of an ethylenediamine-water solution of copper acetate and sulfur under an argon flow [22]. In another report, single crystalline CuS nanoplates with average sizes of about 20–40 nm were successfully synthesized without any surfactant by a sonochemical approach under ambient conditions. $\text{Cu}(\text{OH})_2$ nanoribbons were used as the copper source and *in situ* template to prepare CuS nanostructure [21]. In

another example, CuS NP-decorated reduced graphene oxide (CuS/rGO) composites have been successfully prepared *via* a sonochemical method in the room temperature and mild reaction conditions. X-ray diffraction and electron microscopy observations confirm that CuS NPs of 10–25 nm are well distributed on the rGO nanosheets. Ultraviolet–visible spectroscopy reveals the CuS/rGO nanocomposites show a strong and broad light absorption.

20.2.4. Other preparation methods

Several other methods, though less frequently reported, can be used to synthesize CuS NPs. For example, the use of a paired cell at room temperature [23], thermolytic degradation of copper thiolate precursor without solvent [24], and amylose-directed synthesis [25] can be applied to synthesize one dimensional CuS NPs. Chemical vapor reaction, single source method [26], and high-temperature precursor injection method were investigated to prepare two dimensional CuS nanostructures. To construct three dimensional CuS nanostructure, some other strategies include enzymatic treatment of dextran stabilized CuS nanosuspensions in a green synthetic method [27], the use of carboxylic acids as solvents for high nucleation rate and stabilization of nanoparticle dispersion [28], and synthesis of CuS NPs based on surfactant [29].

20.3. BIOMEDICAL APPLICATIONS OF CuS NPs

The usage of nanomaterials in biotechnology merges the fields of material science and biology. NPs provide a particularly useful platform, demonstrating unique properties with potentially wide-ranging applications in biomedical areas, as described below [30].

20.3.1. Biosensors

20.3.1.1. DNA sensors

DNA detection is highly important for the diagnosis of infectious diseases, genetic mutations, and clinical medicines [31]. DNA biosensors provide a powerful means of recognizing specific DNA sequences, and different biosensors have been reported for the detection of specific DNA sequences. Among them, electrochemical DNA biosensors are attracting great interest with regards to simplification of the analysis, miniaturization, and microelectronic integration [32]. CuS is a p-type semiconductor and has been extensively applied in lithium rechargeable batteries, solar cells, biology markers, and biosensors because it is inexpensive, nontoxic, easily produced, and readily stored and has high specific capacitances [33]. However, it is not favorable for applications in electrode materials because of its low conductivity. To solve this problem, CuS nanocomposite was prepared on an

electronically conductive support to facilitate charge transfer for electrochemical biosensing applications. In one example, CuS-graphene nanocomposite was constructed as a DNA biosensor platform [34]. The nanocomposite consists of CuS, graphene nanosheets, gold NP-conjugated ssDNA probe, and mean cell hemoglobin (MCH) (Figure 2). The embedding of CuS on graphene nanosheets helps to improve the solubility and dispensability of graphene nanosheets, which in turn enhances the conductivity of CuS. Upon the hybridization of target DNA strand with DNA probe to form a double-stranded structure on the biosensor surface, the electrochemical response of $[\text{Fe}(\text{CN})_6]^{3-/4-}$ decreased, thus exhibiting a low detection limit for the target DNA.

Figure 2. The electrochemical DNA biosensor consists of AuNPs, target complementary DNA, MCH and 6-mercapto-1-hexane [34]

Single-nucleotide polymorphisms (SNPs) refer to point mutations that constitute the most common genetic variation, and the single base alternations are strongly related to various medical and physiological features of human beings [35]. Therefore, accurate and sensitive analysis of SNPs will play an important role for disease diagnosis, predisposition assessment, pharmacogenetic analysis, and evolutionary studies [36]. In one study, CuS-based nanostructures were assembled to analyze SNPs. In this study, CuS NPs are modified on the surface of gold NPs, and through which are conjugated with the complementary base strand. Once the CuS nanostructures are

hybridized with the mismatched bases, cupric ions will be dissolved from the CuS NPs, yielding an ultrasensitive chemiluminescence signal with the SNPs detection limit of 8.0×10^{-17} to 1.0×10^{-14} M.

20.3.1.2. Glucose sensors

Glucose detection plays an important role in monitoring diabetes. The rising demands for glucose sensor with high sensitivity, high reliability, fast response and excellent selectivity have driven enormous research efforts for decade [37]. Early efforts were focused on the development of enzymatic glucose biosensors, principled by the detection of glucose oxidase (GOD). However, GOD is paucity of longterm stability and its activity is affected by many factors (*e.g.*, temperature, pH value and toxic chemicals), thus limiting the applications of this enzymatic method. Alternatively, nonenzymatic glucose sensors are recently developed. In a report, a sensitive nonenzymatic glucose sensor based on CuS nanotube (CuS NTs) was fabricated. CuS NTs made up of CuS NPs were successfully *in situ* synthesized on Cu electrode by a simple self-sacrificial template method [38]. It was a useful method to avoid the defects of the sonication and widen linear range around micromolar concentration to meet the requirement of predilution of human serum. The electrochemical property of CuS NTs modified Cu electrode (CuS NTs/Cu) for glucose electrooxidation was thoroughly investigated by cyclic voltammetry and amperometric methods. The CuS NTs based glucose sensor exhibited an extraordinary detection limit as low as 45 nM and two wide linear ranges. A lower glucose concentration ranged from 0.2 to 2.5 mM with high sensitivity of $3135 \mu\text{A mM}^{-1} \text{cm}^{-2}$ and a higher glucose concentration ranged from 2.5 to 6 mM with relative low sensitivity of $2205 \mu\text{A mM}^{-1} \text{cm}^{-2}$. Furthermore, the proposed sensor also showed high anti-interference ability and was successfully used for glucose detecting in human serum with good accuracy and satisfactory recovery.

20.3.2. Molecule imaging

Early diagnosis of cancer is crucial to increase the chances of survival, and NP-based molecular imaging technology is emerging as a new paradigm for cancer diagnosis [39]. Compared with conventional agents, NP-based imaging probes provide several advantages [40], including enhanced circulation half-life, the integration of “smart sensing” properties to the probes [41], and the ability to load both imaging and drug components in one vehicle for multifunctional applications [42]. Generally speaking, molecular imaging includes the use of optical techniques such as bioluminescence and fluorescence imaging [43], molecular magnetic resonance imaging (MRI) [44], magnetic resonance spectroscopy (MRS), positron emission tomography (PET) [45], single-photon emission computed tomography (SPECT) [46], and increasingly popular photoacoustic tomography (PAT) [40].

20.3.2.1. PAT imaging

PAT, also termed as optoacoustic tomography, is based on the measurement of ultrasonic waves induced by biological tissues absorption of short laser pulses. PAT employs nonionizing laser light to acoustically visualize biological tissues with high optical contrast and high ultrasonic resolution. Photoacoustic effects were demonstrated in turbid medium by Kruge in 1994, in biological tissues by Oraevsky *et al.* in 1997, and in rats by Wang *et al.* in 2003 [12]. Since then, this imaging modality has been widely researched and advanced toward clinical applications. The most important chromophores in the human body are oxyhemoglobin and deoxyhemoglobin in red blood cells. The hemoglobins have absorption coefficients of more than 100 cm^{-1} for visible light; hence, they are capable of generating strong photoacoustic signals ($\sim 10 \text{ bar}$). Therefore, PAT has been successfully applied to image vascular structures and tumor angiogenesis a few millimeters under the skin [47]. Unlike other high-resolution optical imaging modalities, such as confocal microscopy, two-photon microscopy, and optical coherence tomography, PAT relies on both diffused and ballistic light and thus can be used to image deeper biological tissues. However, because of the overwhelming scattering effect of biological tissues on light, light intensity decreases exponentially with depth with a decay constant that is related to effective penetration depth. The light intensity attenuation can be minimized by choosing an excitation laser wavelength within the near-infrared region (NIR), in which biological tissues have a relatively low absorption coefficient and a low scattering coefficient. Successful translation of PAT to the clinic requires a practical laser source that efficiently penetrates biological tissues and a contrast agent whose optical absorption peaks at or near the wavelength of the laser source. Nowadays, various organic or inorganic NPs, including porphyrin shell, gold NPs, carbon nanotubes, nanographene, and CuS NPs, all with high NIR absorbance, have widely explored for *in vivo* PAT imaging [48].

Recent studies have demonstrated the usage of CuS NPs as excellent contrast agents for *in vivo* PAT imaging. In one example, CuS-based PAT imaging was applied to visualize deep tissue, including mouse brain, rat lymph nodes and chicken breast (Figure 3). CuS NPs in this study had the average diameter of $11 \pm 3 \text{ nm}$, yielding an absorption peak at around 990 nm . A 1064 nm -wavelength laser was chosen for irradiation, as the wavelength elicits a strong absorbance of CuS NPs while a low absorption of normal tissues, thus increasing the signal-to-noise ratio. The result shows that this CuS-based PAT imaging allows for the detection of deep tissue at a depth of $\sim 5 \text{ cm}$ with an in-plane imaging resolution of $\sim 800 \mu\text{m}$ and a sensitivity of $\sim 0.7 \text{ nmol}$ per imaging pixel [12]. In another example, CuS-based PAT imaging was used to detect the activity of matrix metalloproteinases (MMPs) *in vivo* [48]. MMPs are zinc-dependent endopeptidases that degrade proteins in the extracellular matrix and play an important role in the development of various diseases including cancer, inflammatory, neurological and cardiovascular diseases. CuS NPs are

conjugated with a red-light-absorbing organic dye, BHQ3, *via* a MMPs cleavable peptide. The obtained CuS-peptide-BHQ3 (CPQ) nano-probe exhibits strong PAT signal at the wavelengths of 680 nm and 930 nm before enzyme cleavage, resulting from the absorption of BHQ3 and CuS NPs, respectively. In the presence of MMPs expressed inside the tumor, CPQ nano-probe is recognized by the protease, releasing free BHQ3 which as a small molecule could be rapidly washed out from the tumor area, leaving bare CuS NPs which would retain inside the tumor for a much longer period of time. The PAT signal ratios recorded at two different wavelengths, 680 nm/930 nm, is then significantly decreased. This novel nano-probe demonstrates the first study of using PAT imaging to investigate MMP activity in the animal tumor model.

Figure 3. Photoacoustic tomography (PAT) imaging of CuS NPs in mice. (A) A representative PAT image of mouse brain, acquired using a 532 nm laser without injecting contrast agent. (B) A PAT image of mouse brain acquired using a 1064 nm laser, at 24 h after intracranial injection of CuS NPs. (C) A PAT image of mouse brain acquired using a 1064 nm laser, at 7 days after intracranial injection of CuS NPs. (D) A photograph of the head of the mouse. Images of (A) to (D) are adapted with permission from [12] © (2012) American Chemical Society

20.3.2.2. PET/CT imaging

PET/CT is a highly sensitive and quantitative imaging modality in clinical oncology for cancer diagnosis, staging, and evaluation of therapeutic responses. This imaging modality is principled by the detection of radioactive isotope decay, during which unstable atoms spontaneously convert to a more stable form with a lower overall energy and release radioactive energy [49]. Recently, the radiolabeling of NPs with PET isotopes has gained increasing interest for evaluating the pharmacokinetics, tissue distribution, and clearance. Various radiolabeled NPs have been reported, including quantum dots, gold NPs, carbon nanomaterials, and polymeric NPs.

Conventionally, radioisotopes are linked to NPs through chelators to form stable complexes and this radiometal-chelator methods have two limitations. One limitation is the potential detachment of radioisotopes from NPs *in vivo*, which can result in misleading findings since PET imaging detects the radioisotopes (whether they are on the nanoparticles or not) but not the NPs themselves. The other limitation is that the data obtained may not reflect the pharmacological properties of unlabeled NPs due to the influence caused by radiotracers on physicochemical properties of NPs. Alternatively, ^{64}Cu -labeled CuS NPs provide a chelator-free method for PET/CT imaging. ^{64}Cu ($T_{1/2} = 12.7$ h; β^+ , 0.653 MeV [17.8 %]; β^- , 0.579 MeV [38.4 %]) has decay characteristics that allow for both PET imaging and targeted radiotherapy for cancer [8]. In an interesting report, chelator-free, PEG modified, ^{64}Cu -labeled CuS NPs (~11 nm in diameter) were constructed to serve as both a PET tracer and a therapy agent in tumor bearing mice (Figure 4). $^{64}\text{CuCl}_2$ was used as the precursor for the synthesis of ^{64}Cu -labeled CuS NPs, in which ^{64}Cu is an integral building block of CuS rather than labeling through a chelator. This design with desirable properties such as ease of synthesis, small size, presents an enhanced stability, higher tumor accumulation and hence better imaging results. The PEG- ^{64}Cu CuS NPs showed high uptake and retention in U87 human glioblastoma xenografts in mice due to the enhanced permeability and retention effect, which was successfully visualized by non-invasive PET imaging.

Figure 4. Micro-PET/CT images of nude mice-bearing s.c. U87 glioma xenografts acquired at 1, 6, and 24 h after *i.v.* injection of PEG- ^{64}Cu -CuS NPs. Yellow arrow: tumor; orange arrow: bladder; red arrow: standard. These images are adapted with permission from [8] © (2010) American Chemical Society

20.3.3. Photothermal ablation

Photothermal ablation represents a powerful technique for a variety of applications. This technique usually relies on a NIR laser to irradiate photothermal agents, which then convert the optical energy to heat energy and lead to thermal ablation of surrounded cells or tissues [50]. Many inorganic nanomaterials, such as gold nanostructures, CuS NPs, carbon nanomaterials, copper chalcogenide semiconductors, Pd nanosheets, Bi_2Se_3 nanoplates and $\text{W}_{18}\text{O}_{49}$ nanowires, are widely explored as photothermal agents. Among them, gold nanostructures and CuS NPs represent two main streams.

CuS NPs are a new class of photothermal agents that provide an alternative to gold nanostructures. The NIR absorption of CuS NPs are derived from d-d energy band transition of Cu^{2+} ions [51], and thus their absorption wavelength is not affected by their shape and surrounding environment. In addition, CuS NPs are degradable and can be excreted from liver and kidneys, and thus are favorable for clinical applications. Recently, extensive research has been explored to use the photothermal ablation property of CuS NPs for medical applications, such as photothermal therapy, and transdermal delivery. The detailed summary is listed below [52].

20.3.3.1. Photothermal therapy

Several CuS nanostructures are reported as excellent transducer agents for photothermal therapy, including the CuS flower-like superstructures [53] with the mean size of $\sim 1\ \mu\text{m}$ and CuS plate-like nanocrystals [10] with a mean size of $\sim 70\ \text{nm} \times 13\ \text{nm}$ (Figure 5). These CuS NPs exhibit strong absorbance and conversion efficiency upon 980 nm laser irradiation, leading to a higher local temperature to kill the cancer cells.

Figure 5. (A) Photothermal effect of the aqueous dispersion of Cu₉S₅ NCs (40 ppm) with the NIR (980 nm, $0.51\ \text{W cm}^{-2}$) irradiation for 10 min. (B) Photothermal effect of the aqueous dispersion of the Au nanorods (40 ppm) irradiated with 980 laser (a power density of $0.51\ \text{W cm}^{-2}$) for 10 min. (C) TEM image of the Cu₉S₅ NCs. (D) TEM image of the Au nanorods. Images of (A) to (D) are adapted with permission from [10] © (2011) American Chemical Society

Some hollow CuS NPs with numerous mesoporous pores could efficiently load drug molecules, thus providing a combination of photothermal and chemotherapy. For example, hollow CuS NPs with 130 nm in diameter were loaded with hydrophobic drugs (Camptothecin). Upon NIR irradiation, drugs were demonstrated to be released from the NP systems for chemical cytotoxicity, and the converted heat by CuS NPs offers thermal killing of cells [54]. CuS NP-based photothermal therapy could also be combined with immunotherapy. In an interesting design [11], chitosan-coated hollow CuS NPs were assembled with immunoadjuvants (CpG motifs) to construct a

nanocomplex. Upon NIR irradiation, photothermal ablation-induced tumor cell death released tumor antigens into the surrounding milieu, and the nanocomplex was re-assembled to form chitosan-CpG complex; which altogether elicit and enhance the immune response. The results show that the combinational photothermal-immunotherapy could treat both primary tumor and distant metastasis tumor (Figure 6).

Figure 6. Diagram of HCuSNPs-CpG-mediated photothermal immunotherapy for the treatments of primary and distant tumors. This figure is adapted with permission from [11] © (2014) American Chemical Society

20.3.3.2. Transdermal delivery

Compared with other drug delivery routes, transdermal drug delivery provides several advantages, such as sustained and steady-state pharmacokinetics, avoidance of hepatic first-pass effects, and improvement of patient compliance [55]. Taken the advantage of photothermal ablation properties, CuS NPs could be covered on the skin surface to thermally deconstruct the stratum corneum, thus assisting transdermal delivery of drugs. In an interesting report, Ramadan *et al.* coated the skin surface with gel formations of drug-bearing CuS NPs. They then applied nanosecond-pulsed NIR laser ($1.3\text{--}2.6\text{ W cm}^{-2}$) to irradiate the skin. The pulsed irradiation could rapidly elevate the local temperature, resulting in the disorder of keratin networks and decompose of stratum corneum, thus facilitating the uptake of drug-bearing CuS NPs. Upon the end of

pulsed irradiation, the temperature was rapidly cooled down, and thus this method ensures the skin temperature will not exceeds 40–50 °C in the localized regions and will not produce any severe damage.

20.3.4. Theranostics

Theranostics, the integration of therapy and diagnostics in one delivery vehicle, is paving the way towards the goal of personalized medicine. Theranostic nanoconstructs allow for early recognition of diseased sites followed by triggered guidance of therapy. In addition, these theranostic agents employ imaging modalities to monitor the response to disease treatment. Many nanomaterials are explored for theranostic applications, such as iron oxide NPs, quantum dots, carbon nanotubes, gold NPs and silica NPs. Recent years, CuS NPs are also investigated for cancer theranostics because of their many intriguing properties discussed above.

In an interesting report, Zhang *et al.* designed Cy5.5-conjugated, CuS-loaded, hyaluronic acid (HA) NPs (Figure 7). In this system, Cy5.5 fluorescent signal is quenched by CuS inside the NPs, and HA is fabricated to target cluster determinant 44 (CD44) overexpressed on cancer cells and lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1). Once HA NPs are delivered into the tumor-bearing mice, they are accumulated in tumor effectively through the enhanced permeation and retention (EPR) effect and then specifically internalized into tumor cells through receptor-mediated endocytosis. Inside the tumor cells, HA NPs will be degraded into short saccharide units by hyaluronidases (mainly Hyal-1 and Hyal-2) and released CuS and Cy5.5. Due to the complete degradation of HA NPs, Cy5.5 will be separately from the quenching of CuS particles, yielding strong fluorescent signal for tumor imaging; meanwhile, released CuS particles serve as photoacoustic imaging agents to delineate the blood vessels and photothermal agents to destroy the tumor cells. Using this theranostic system, the tumor area could be clearly observed by fluorescence and photoacoustic signals over time, which peaked at the 6 h time point (tumor to normal tissue ratio of 3.25 ± 0.25 for fluorescence imaging and 3.8 ± 0.42 for photoacoustic imaging), and the tumor growth could be efficiently inhibited by photothermal ablation, with a tumor inhibition rate of 89.74 % on day 5.

Figure 7. (A) *In vivo* applications of HANPC for NIR fluorescence and PA image-guided photothermal therapy. (B) NIR fluorescent imaging of CD44-positive SCC7 tumor-bearing mice received HANPC *i.v.* treatment. Images were acquired at indicated time points, and fluorescent signals were normalized by the maximum average value. The color bar indicates radiant efficiency (low, 0; high, 0.209×10^6). White circles were used to indicate tumors location. (C) Photoacoustic tomography imaging of blood vessels in SCC7 tumor-bearing mice intravenously received HANPC or free CuS. (D) Thermal images of SCC7 tumor-bearing mice *i.v.* treated with PBS, HANP, CuS, and HANPC (left row, 5 mg kg⁻¹ of HANPC, illuminated at 6 h pi.) with 808 nm laser illumination taken at indicated time points. The injected amount of HANP and CuS were calculated according to the loading efficiency. The laser power density was 1.5 W cm⁻². Images of (A) to (D) are adapted with permission from [56] © (2014) American Chemical Society

20.4 SUMMARY

In this review, we introduce current strategies to synthesize CuS NPs with various morphologies, and their investigations in biomedical applications, including biosensors, molecular imaging, photothermal ablation and theranostics. As a new class of nanomaterials, CuS NPs have the advantages of consistent NIR absorption, low cost, low cytotoxicity and biodegradability, indicating great potential in this research field.

Notably, the development of CuS NPs for biomedical application is still in its infancy, and many challenges exist before translating current animal-based

research to the clinic. The photothermal conversion efficiency of CuS NPs needs to be improved, probably by optimizing their shape and size. Continuous efforts are needed to enhance their drug delivery capabilities, presumably by modifying the surface chemistry and morphology, or by designing hollow/porous/coreshell architectures with polymeric coating. The combination of chemotherapy, photothermal therapy and molecular imaging will offer numerous new avenues for the design of multifunctional delivery systems. Research on CuS NPs will continue to thrive over the next decade, and tremendous opportunities lie ahead for their potential biomedical applications.

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**POTENTIAL USE OF HYBRID
IRON OXIDE-GOLD NANOPARTICLES
AS DRUG CARRIERS**

Anthony D.M. Curtis and Clare Hoskins*

School of Pharmacy, Institute for Science and Technology in Medicine,
Keele University, Keele, ST5 5BG, UK

*Corresponding author: c.hoskins@keele.ac.uk

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21.1. INTRODUCTION

Hybrid nanoparticles are composed of more than one material fused together to increase functionality. These often consist of polymers coated onto the surface of metallic nanoparticles to render them useful in biomedicine [1,2]. In this chapter the term hybrid nanoparticles refers to an iron oxide core surrounded by a gold coat [3]. Iron oxide nanoparticles have been exploited clinically for their magnetic resonance (MR) properties and imaging capabilities. Traditionally iron oxide contrast agents used clinically for MR imaging (MRI) were composed of a dense metallic core surrounded by either a dextran or carboxydextran polymer shell (Feridex[®] and Resovist[®] respectively) (Figure 1). However, safety concerns over the degradation of the iron core into toxic free radicals led to their removal from clinical use in humans in the UK [4].

Figure 1. Structure of Feridex[®] and Resovist[®] MRI contrast agents

Many speculated that the degradation in physiological pH was achieved even after polymer coating due to the polymer motility. Hence, in order to improve the safety profile of these contrast agents rigid coatings were required. This was realised by coating the particles with gold; the rigid nature of the gold shell acts as a physical barrier ensuring core degradation is eliminated whilst harnessing additional functionality to the particles. Colloidal gold is non-toxic and possesses unique optical properties due to its surface plasmon resonance

(SPR) [5]. SPR is the phenomenon whereby light at a certain wavelength (unique to the particle due to size, shape *etc.*) can be both scattered and absorbed. This absorption results in a rapid conversion of light into heat and, hence, the nanoparticulates experience a thermal rise. This stimuli responsive nature can be exploited in applications such as tumour ablation and thermo-responsive drug delivery.

By gold coating the surface iron oxide nanoparticles a core-shell dual functional structure is observed which is referred to as a hybrid nanoparticle (HNP). The benefit of such particles, other than reduced toxicity is the combined properties conferred by the core and shell components. These particles retain the magnetic character inherent to iron oxide and the optical properties inherent to colloidal gold. Thus, HNPs have potential as theranostic agents. Theranostics are an emerging platform which offer simultaneous diagnosis and therapy, resulting in decreased treatment times. These agents allow for real time imaging after administration, enabling location mapping before initiating drug release. By coupling treatments to diagnostics and controlling drug release a rapid and localised clinical effect can be achieved.

21.2. SYNTHESIS OF HNPs

HNPs are synthesised by various methods. The iron oxide core can be synthesised *via* both physical and chemical routes [6-8]. The physical routes include gas phase deposition and electron beam lithography which are both highly complex and control over particle size is difficult. Chemical routes such as wet precipitation offer much simpler approaches with greater control over particle size and morphology dependant on reaction conditions (salts used, pH, time, temperature *etc.*). In order to form HNPs a gold coating surrounding the iron oxide core is required. This can also be achieved by various routes. Gold can be coated directly onto the iron oxide surface or a polymer intermediate can be used to act as a spacer between the two metallic surfaces (Figure 2) [9,10]. The later approach is more commonly used as some studies have shown that the direct coating approach leads to migration of gold atoms from the shell into the iron oxide core which dampens the magnetic potential and, hence, decreases effectiveness as an MRI contrast agent [11]. Synthesis using a polymer intermediate or spacer is commonly carried out based on electrostatic interaction and monitored through zeta potential measurement. The iron oxide core possesses a negative zeta potential due to the salt associations from reaction (usually SO_4^{2-}). Conjugation of long chain cationic polymers can be achieved by simple stirring or sonication due to the positive charges present on their backbone. It has been reported that iron oxide particles coated with poly(ethylenimine) enhanced the T2 relaxivity of the particles from $127.6 \text{ mM}^{-1} \text{ s}^{-1}$ to $260.2 \text{ mM}^{-1} \text{ s}^{-1}$ respectively. This enhancement was not

observed in identical particles coated with poly(allylamine) and poly(L-lysine) [3].

Figure 2. Differing structures of HNPs resultant from synthetic route both A) without and B) with the use of a polymer intermediate

In order to achieve gold coating, 2 nm gold seeds are conjugated electrostatically onto the cationic polymer intermediate and subsequent iterative reduction of chloroauric acid in solution attaches gold onto the nanoparticle surface and form the complete rigid coating. Coating thickness is controlled *via* iteration number. Initially a thin coat is formed (<5 nm) which becomes thicker upon increased iteration number. Upon further iterative increase in chloroauric acid reduction steps anchor points appear to initiate morphology change into spikey or star shaped particles. The presence of the gold coat of the HNP does not appear to effect relaxivity with iron oxide core T2 relaxivity being $127.6 \text{ mM}^{-1} \text{ s}^{-1}$ before coating and HNP relaxivity being $175.3 \text{ mM}^{-1} \text{ s}^{-1}$ respectively in one study [3]. Gold coating thickness not only provides the physical barrier to iron oxide degradation but also possesses SPR for nano-heating. Gold coating thickness is an important parameter in developing HNPs into effective heating sources. Studies have shown that increasing the thickness of the gold coating decreases its ability to absorb and scatter laser irradiation resulting in heating. One study reported a 4°C decrease in temperature change after 60 seconds of laser irradiation (95 nm). It is proposed that with decreased coating thickness greater surface area is available (internal surface and external surface of the shell) and thus a greater level of SPR is achieved resulting in greater temperature rises [3].

21.3. STABILITY AND BIOCOMPATIBILITY OF HNPs

In order for HNPs to be applicable as contrast agents, drug carriers or indeed theranostics they must possess both physical and chemical stability under physiological conditions. Nanoparticles in solution tend to aggregate together

over long periods of time due to a reduction in Gibbs' free energy. Additionally, iron oxide nanoparticles notoriously undergo aggregation arising from magnetic interactions which needs to be addressed before clinical usage can be considered. Although a wealth of data is available on iron oxide nanoparticles, limited stability studies have been carried out using HNPs. Studies focussed on the surface chemistry of the HNPs in relation to their fate at physiological pH have been reported. This work showed that the uncoated HNPs aggregated immediately upon introduction to phosphate buffer saline (PBS), however, stability could be achieved with the adsorption of bovine serum albumin or amphiphilic polymers such as Pluronic F127 onto the HNP surface resulting in lack of aggregation for more than 5 days in PBS [12]. Another study investigated the stability of uncoated HNPs stored in solution over a 6 month period (Table 1) [13]. According to this study no significant aggregation had occurred with hydrodynamic diameter and transmission electron microscopy studies being in agreement. Additionally the T2 relaxation and heating ability due to laser irradiation remained unchanged [13]. Often HNPs are further coated with polymer stabilisers which ensure long-term solution stability. Poly(ethylene glycol) (PEG) is commonly employed for this purpose due to its biocompatibility, stealth properties and long blood circulation times; other cationic polymers such as poly(ethylenimine) are used where further surface modification is required. These polymer stabilisers can be conjugated onto the HNP surface both *via* electrostatic interaction and the use of thiol ($-SH$) functional groups. Thiol functionalities are well documented to form strong dative covalent bonds with gold surfaces. This can be exploited for attachment of polymers, drugs or other functional ligands.

Table 1. Physical stability of HNPs over 6 months stored in solution at room temperature ($n = 3 \pm SD$) [13]

Physical property	0 months	6 months
Size using photon correlation spectroscopy, nm	250 (22)	255 (62)
Polydispersity index	0.112 (0.010)	0.312 (0.021)
Zeta potential, mV	+2.4 (0.00)	+2.4 (0.01)
Size from transmission electron microscopy, nm	70 (0.5)	70 (0.4)
Magnetic coercivity from SQUID measurement, $kA\ m^{-1}$	10.35 (5)	6.2 (0.2)
T2 relaxivity from MRI measurement, $mM^{-1}\ s^{-1}$	175.3 (1)	161.7 (7.3)
ΔT from laser irradiation of $10\ \mu g\ mL^{-1}$ suspended in 2 % agar, $^{\circ}C$	7.0 (0.5)	7.5 (0.8)

Degradation of metallic nanoparticles into toxic free radicals has been well documented especially in metal oxides [14,15]. The withdrawal of Feridex® in the UK has reformed many coating strategies and prompted more stringent toxicity studies. These were lacking previously primarily due to lack of universal understanding and agreement of testing procedures required. Nanosafety committees have formed worldwide to unite the nano research community and advise on best practice for the toxicity evaluation of particles. In HNPs, the toxicity arising from the iron oxide core is theoretically eliminated due to the rigid inert gold coating. However, in order for long term use studies are required to verify such hypotheses. Studies have been carried out *in vitro* to determine the chemical stability of HNPs (both uncoated and PEGylated) in solutions designed to mimic physiological (pH 7.2) and lysosomal (pH 4.6) conditions [13]. After two weeks levels of Au and Fe were monitored in the media. In uncoated and PEGylated HNPs particles at pH 7.2 & 4.6 negligible levels (< 0.01 %) of both metals were observed indicating that HNPs were resistant to chemical degradation over the duration of study [13].

The safety issues experienced with iron oxide nanoparticles and their degradation *in vivo* has led to great focus on metallic particle biosafety. Hence, a plethora of *in vitro* tests have been carried out on HNPs to ensure they are safe for clinical application [14]. HNPs have demonstrated increased levels of biocompatibility compared with polymer coated iron oxide nanoparticles. This further demonstrates that the rigid core protecting the iron does not degrade after cellular internalisation. Cellular response assays including the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, trypan blue exclusion, lactate dehydrogenase (LDH) cell membrane integrity, reactive oxygen species generation and lipid peroxidation studies have been reported in various cell lines [13,16]. All studies reviewed to date have been in agreement that HNPs do not cause significant cytotoxicity or trigger and other adverse cellular response. These studies show the potential of HNPs to be used clinically. When compared to the previously used MRI contrast agent Feridex®, HNPs magnetic ability is similar but, their safety profile is enhanced. Cellular uptake studies have also demonstrated that uncoated HNPs are capable of cellular internalisation and polymer coating increases transfection efficiency.

21.4. USE OF HNPs AS DRUG CARRIERS

HNPs can be exploited as drug carriers in addition to their imaging capabilities. Drugs can be conjugated both electrostatically *via* Van der Waals forces or physically *via* dative covalent bonding. Gold surfaces form permanent covalent bonds with thiol containing molecules. Addition of further polymer coats can also be used to further functionalise the surfaces with drug molecules, functional ligands, dyes *etc*. Studies have shown the conjugation of anticancer drugs such as cisplatin and 6-thioguanine (6-TG), as well as proteins such as

cytochrome c, photosensitizers such as thiolated heparin–phosphoramide a (PhA) and functional ligands such as cyclic RGD peptidomimetic targeting integrin $\alpha\beta 3$ [13,17–20].

Nanotechnologies are of growing interest in cancer therapy due to unique properties arising from their size. Cancerous tissue proliferates at such a rapid pace which results in poorly formed vasculature. Nanoparticles are capable of accumulation within the ‘leaky’ capillaries surrounding tumour tissue. These particles cannot permeate back into the bloodstream due to the poor lymphatic drainage. Thus, anticancer drugs can be passively targeted to their desired site once conjugated to or formulated within nanoparticulates. The phenomenon by which this passive targeting occurs is called the Enhanced Permeability and Retention (EPR) effect [21]. Studies have shown that once conjugated onto or into nanoparticles, drug molecules (or other cargo) exhibit greater cellular uptake [13,18,22]. Hence, a more rapid clinical effect can be achieved. When cisplatin was conjugated onto the surface of HNPs *via* a thiolated PEG linker, it improved the cytotoxicity of the drug 110-fold compared to the unbound drug in human ovarian cancer (A2780) cells [16]. Similarly, 6-thioguanine conjugated onto HNPs gave rise to an 80 % decrease in cell viability in human pancreatic adenocarcinoma (BxPC-3) cells after only 24 h incubation with $50 \mu\text{g mL}^{-1}$ (drug concentration) which was an improvement from the unbound drug which resulted in a 55 % reduction in viability. Here, the IC_{50} was reduced 10-fold (Figure 3) [13].

Figure 3. Investigation of biological significance of HNP-6TG formulations: A. Drug uptake after 4 h (12.5 & $50 \mu\text{g mL}^{-1}$) incubation and B. cell viability by trypan blue exclusion after 24 h incubation on BxPC-3 cell lines [13].

Anticancer therapeutic agent doxorubicin (Dox) has been conjugated onto the surface of HNPs along with a thiolated PEG [23]. Up to 100 mg DOX of attachment was achieved per milligram of the HNPs. In higher pH ranges the drug release occurred in greater quantity. The cytotoxicity of the novel formulations were investigated *in vitro* on breast cancer (MCF-7) cell lines. The studies showed that the HNP-Dox were more toxic compared with the free drug. Further work is ongoing in order to fully elucidate the use of these formulations *in vivo* [23].

These studies show the huge potential of HNPs as drug delivery vehicles. Efforts are now underway worldwide to further exploit this potential in cancer therapy as dual purpose theranostic agents.

21.5. INCORPORATION OF HNPs INTO SUPRAMOLECULAR STRUCTURES

HNPs can be incorporated into larger supramolecular structures which can further enhance their applicability in biomedicine. These include the development of magnetomicelles. Reports have successfully shown conjugation and potential of drug conjugated onto the surface of HNPs [22]. However, insoluble compounds are much more difficult to formulate onto their surface due to the potential to further decrease HNP stability resulting in precipitation. Hydrophobic drugs make up approximately 40 % of all new drugs under development. They pose huge problems for pharmaceutical companies because without careful formulation they are rendered useless. Traditional formulation strategies such as the use of surfactants and co-solvents have their own disadvantages and hence have been improved with the advancement in nanotechnology. Amphiphilic polymers have been used to solubilise hydrophobic compounds with greater stability and efficiency compared with surfactants. Addition of HNPs into the intrinsic structure of amphiphilic polymers provides them with added properties in imaging. A recent study showed incorporation of HNPs into the intrinsic structure of a graft amphiphilic polymer consisting of a poly(allylamine) (PAA) backbone grafted with 5 % molar ratio pendants of oxadiaxole (Ox) [22]. Amphiphilic polymers spontaneously aggregate into core-shell nano-aggregates in aqueous environments due to a reduction in Gibbs' free energy. Polymer derivatives of PAA have shown huge potential in hydrophobic drug solubilisation and delivery for drugs such as propofol, prednisolone, griseofulvin and novel anticancer drug bis-naphtal-amidopropyl-diamino-octane (BNIPDaoct) [24]. The inclusion of HNPs into these self-assemblies results in greater functionality which can be exploited for image guided or stimuli responsive drug delivery. In this work the HNPs were attached to the polymer backbone *via* dative covalent bonding between the gold surface and the thiol functional group in the Ox pendant group [22]. The resultant PAA-Ox5 aggregates were exploited as drug carriers using direct conjugation of drug molecules onto the HNP surface (6-TG) and hydrophobic encapsulation (BNIPDaoct). *In vitro* assays in human pancreatic adenocarcinoma (BxPC-3) cells showed that the polymer-HNP formulations (both conjugated and encapsulated) resulted in increased drug uptake compared with the free drug. MTT assays showed that a significant decrease in the IC₅₀ value of the formulations compared with the free drug was also observed [22].

Other work has highlighted the inclusion of HNPs into biodegradable scaffolds based on the thermally responsive polymer poly(*N*-isopropylacrylamide) (pNiPAM) (Figure 4) [25]. Here the HNPs provide a localized trigger for heat generation leading to structural conformation changes after laser irradiation with a Q-switched Nd:YAG laser. Studies were carried out on the pNiPAM-HNP composites to investigate their potential as heat triggered scaffolds for drug release. Methylene blue was used as a model compound which is indicative of their potential for a host of drug compounds or growth factors utilised in regenerative medicine. These studies show that after irradiation of the HNPs, scaffold deformation occurred due to internal structural heating resulting in the release of the methylene blue dye [25].

Figure 4. Physical properties of pNiPAM-HNP composite. A) Temperature change in pNiPAM-HNP composite exposed to laser irradiation using a Q-switch Nd:Yag laser at 10 pulses s⁻¹. FESEM images of composite (B&C) [25].

Other studies have reported inclusion of HNPs into graphene oxide. This system possessed increased relaxivity compared to the HNP alone as well as SPR due to the gold shell. Anticancer therapeutic doxorubicin was loaded into structure resulting in 6.05 mg mg⁻¹ (0.32 mg mL⁻¹). This system could improve treatment regimes in cancer therapy due to its theranostic potential arising from the high level of drug loading in addition to the magnetic properties [26].

These studies highlight the applicability of HNPs in nanomedicine; not only they are useful as sole platforms for drug delivery, but they also are of benefit to larger more established macromolecular systems.

21.6. SUMMARY

The use of HNPs is rapidly emerging in drug delivery. With growing intelligence regarding nanosafety more rigorous tests are being implemented. Although a relatively young area of research the studies to date show a progression in the understanding and development of these particles as suitable carrier vehicles. They have adequately demonstrated good physical and chemical stability properties and biocompatibility *in vitro* on various cell lines. However, *in vivo* studies are yet to be realised and will be the next step required before clinical application can be implemented.

FUTURE OUTLOOK

The advancement in novel nanomedicines and multifunctional treatment strategies looks set to revolutionise modern healthcare. HNPs and theranostic agents are at the forefront of this exciting era. The development of agents which provide a rapid diagnosis and treatment regime hold great potential for increased patient lifespan and quality of life especially in disease states such as cancer. Additionally, increased time between initial treatment and relapse due to the improved efficiency and penetration ability may occur. There are still hurdles to be crossed and areas where understanding is still lacking and the number of *in vivo* trials is small, however, interest in this field is growing exponentially and knowledge is rapidly being gained. Current clinical efforts in drug delivery are not swamped by nanomedicines, however, this may be due to their relative age. Many polymeric nanoparticulates are currently undergoing the rigorous clinical trials which are required before marketing. The widely held opinion is that HNPs are expected to follow suit and will advance into clinical trials. A lot of scepticism over nanoparticle toxicities is still at large especially with the use of metallic components, however, as nanosafety is a currently major strategy with funding agencies and charities the growth of nanotoxicity databases will emerge.

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**PLEIOTROPIC FUNCTIONS OF MAGNETIC
NANOPARTICLES FOR *EX VIVO* GENE
TRANSFER AND CELL TRANSPLANTATION
THERAPY**

**Daisuke Kami^{1*}, Masashi Toyoda²,
Masatoshi Watanabe³, and Satoshi Gojo⁴**

¹ Department of Regenerative Medicine, Kyoto Prefectural University of Medicine, Kyoto, Japan

² Department of Vascular Medicine, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan

³ Faculty of Engineering, Yokohama National University, Kanagawa, Japan

⁴ Department of Regenerative Medicine, Kyoto Prefectural University of Medicine, Kyoto, Japan

*Corresponding author: dkami@koto.kpu-m.ac.jp

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22.1. INTRODUCTION

Magnetic nanoparticles (MNPs) are widely used in research and medical applications and have attained new levels of versatility and functionality. In this study, we determined the feasibility of traceable *ex vivo* gene transfer using MNPs instead of a multiple-step approach involving *in vitro* gene transfer, isolation of cells, and marking for *in vivo* tracing. This is a breakthrough technology that introduces new possibilities and a novel concept for cell transplantation therapy. In this chapter, we describe the development of this concept and its future potential.

22.2. NANOTECHNOLOGY AND MAGNETIC NANOPARTICLES

Nanotechnology is the manipulation and application of nano-sized materials. These methods have already been applied in various fields. Compared to micro-sized particles, nanoparticles (approximately 100 nm) that exhibit magnetic characteristics display higher fluidity and surface area as well as improved reaction efficiency. These particles also express magnetic properties and show easily controllable behaviours; therefore, they produce a strong effect in a limited space [1].

Divalent or trivalent iron oxide is the major material used in the formulation of magnetic nanoparticles (MNPs). Iron oxide presents low cell toxicity and has been used in the clinical field as a contrast agent for magnetic resonance imaging (MRI), which will be described later. MNPs have also been prepared using cobalt, manganese, nickel, and neodymium metal oxides; however, these display stronger cytotoxicity than iron oxide MNPs, and thus need to be coated [2]. Coating substances are generally selected to reduce the cytotoxicity of magnetic particles. In many cases, dispersing agents are used as coating substances to provide additional functionality to the nanoparticles.

Researchers are currently attempting to add functionality to the surface of MNPs [2]. For example, particles coated with dispersants, which enhance dispersibility, could be unmobilised under a magnetic field. Antibodies can also be coupled to particle surfaces, allowing the effective acquisition of a target *via* interactions between the coupled nanoparticles and proteins, bacteria, or cells with epitopes for the antibody that exclusively express the specific protein (magnetic-activated cell sorting, immunoprecipitation, and magneto-microfluidics) [3]. In addition, pharmaceutical agents, such as anticancer drugs, could be coupled with MNPs and administered at a targeted site at the minimum required dosage. These complexes could then be localised under a

magnetic field, thereby reducing adverse effects to the body. MNPs could serve as an effective drug delivery system [4].

In addition, the fine vibrations caused by exposure of MNPs to an alternating magnetic field result in the generation of heat. This phenomenon, called hyperthermia, could be applied to the treatment of cancer. Recently, a system has been devised wherein MNPs are concentrated within the cancer *via* neoangiogenic blood vessels, and heat is generated using an alternating magnetic field [5].

MRI contrast agents using iron oxide have been commercially available since the late 1990s. Various diagnostic utilities of MNPs have previously been reported, and these particles are highly reliable contrast agents for normal use. MRI, using iron oxide-based contrast agents, is generally the first choice as the most effective and non-invasive technique for the diagnosis of metastatic liver cancer. Moreover, recent advances in MRI technology have given rise to new prospects for MRI use (in combination with other analytical methods).

As mentioned above, MNPs can be applied in various fields and potential for use in the medical care industry in the near future.

In this chapter, we report a novel cell transplantation treatment strategy focusing on gene transfer using MNPs.

22.3. GENE TRANSFER USING MNPs

One of the main biological applications of MNPs is their use in a gene transfer method called magnetofection [6], in which nucleic acids such as DNA (plasmids) and RNA can be transferred into cells. Such nucleic acid transfer is an important tool that is routinely used in current life science research, such as for the control of target gene expression and cell labeling. In recent years, gene transfer technology has been successfully used in the production of induced pluripotent stem cells (iPSCs) [7,8] and in Cas nuclease RNA-guided genome editing (CRISPR) [9] or transcription activator-like effector nuclease (TALEN) [10] editing, thus suggesting a further increase in its importance in the near future.

Currently, there exist three principal methods for gene transfer: (1) viral vectors, such as recombinant retroviruses, lentiviruses, adenoviruses, and adeno-associated viruses, to deliver genomic materials into cells; (2) electroporation to disturb cell membranes by electrical stimulation and promote the passive transfer of genes of interest into cells; and (3) chemical reagents such as cationic polymers to encircle nucleic acids, fuse with cell membranes, and release them into the cytosol. Among these, the method utilising a chemical reagent is suitable for clinical applications owing to its relatively low cytotoxicity with negligible genomic incorporation. However, the gene transfer efficiency of chemical reagents is generally lower than that of

other methods. Therefore, the improvement of transfer efficiency using the reagents has been highly anticipated.

We attempted to improve the gene transfer efficiency of the transfection reagent by utilising MNPs as nucleic acid carrier agents. Divalent and trivalent iron, cobalt, manganese, nickel, and neodymium are currently available MNPs. Among these, iron oxide is used as an MRI contrast agent and is known to display a low cytotoxicity. Therefore, we used iron oxide as the nucleic acid carrier and the cationic polymer poly(ethyleneimine) (PEI) as the dispersing agent for iron oxide. PEI is a well-characterised polymer that has been used as a gene transfer reagent. Because of the commercial availability of PEI with various molecular structures, modifications, and molecular weights, the specific form of PEI can be selected according to the application. We chose deacylated PEI (PEI max, Polysciences, Inc., Warrington, PA, USA.), which displayed lower cytotoxicity and a linear form, to coat the iron oxide. This type of PEI was also more cationic than typical PEI. The MNPs composed of iron oxide and deacylated PEI display high dispersibility and cohesiveness under the magnetic field; this enables the construction of a superior magnetofection system (Figure 1, Tables 1 & 2). In addition, the low toxicity of these nanoparticles allow for the simultaneous introduction of multiple plasmids [11].

Figure 1. Diagrammatic illustration of magnetofection

Table 1. Reaction mixture of magnetofection for 6 well plate

Plasmids (1 µg/µl)	1.0 µl/well
PEI-coated MNPs (50 µg MNPs/ml)	7.5 µl/well
Deionised water or Opti-MEM*	41.5 µl/well
Total **	50.0 µl/well

* Life Technologies, Inc.

** Mixtures were reacted for 15 min at room temperature

Table 2. Transfection efficiency of our magnetofection method

Cell line	Species	Description	Transfection efficiency	References
P19CL6	Mouse	Embryonic carcinoma	81 %	[12]
MEF	Mouse	Embryonic fibroblast	9 %	[13]
HeLa	Human	Cervical cancer cell	40 %	[14]
TIG-1	Human	Fetal lung fibroblast	--	[11]

The gene transfer efficiency of this magnetofection system depends on the size of the magnetoplex, which is comprised of MNPs and plasmids. Cationic polymer PEI-coated MNPs (positive charge) and nucleic acids (negative charge) are electrodynamically coupled to form the magnetoplex complex [14]. The size of the magnetoplex complex varies according to the amount of MNPs used. A larger quantity of MNPs results in a larger number of nucleic acids that are coupled and a larger magnetoplex. The gene transfer of MNPs is dependent on endocytosis, the mechanism by which the magnetoplex enters the cell. The larger the size of a magnetoplex, the poorer the efficiency of gene transfer [14]. This trade-off is quite important to establish an optimal condition for magnetofection, which depends upon the host cells.

22.4. STRATEGIES FOR THE DEVELOPMENT OF A NEW METHOD FOR CELL TRANSPLANTATION THERAPY USING MNPs

Based on the properties described above, we reported a concept that could assist in the development of a new method for cell transplantation therapy using magnetofection in 2014 [13]. The transfection of genes into cells using magnetofection involves the capture of MNPs within the cells (Figure 1). A chronological quantitative measurement of the residual amounts of nanoparticles within the cells using an inductively coupled plasma mass spectrometer demonstrated no significant changes in the number of MNPs per cell over a two-week period. In addition, as the cells detached by trypsinisation

reacted to the magnetic force, it was possible to control their dynamics within the solution. Based on these findings, we hypothesised and attempted to demonstrate the separation and purification of magnetofection-treated gene transfected cells using magnetic force, and the tracing of these cells *in vivo* using MRI after grafting into mice. This strategy can be effectively applied to (1) highly efficient gene transfer, (2) the separation and purification of cells by magnetic force (*in vitro* cell separation), and (3) imaging of the transplanted cells *in vivo* using MRI (Figure 2). The pleiotropic roles of MNPs are easily applied to a single system for cell transplantation therapy.

Figure 2. Diagrammatic illustration of the new strategy for cell transplantation therapy using MNPs

Because magnetofection introduces a large amount of nucleic acids into cells, the cells are subjected to a high degree of cytotoxicity. However, it has been discovered that MNPs themselves exhibit almost no cytotoxicity, and that the level of cytotoxicity increases with the quantity of nucleic acids in a dose-dependent manner. Future research should aim to optimize the cytotoxicity and gene transfer efficiency of these nanoparticles. According to our current preliminary data, a high gene transfer efficiency and low cytotoxicity can be achieved using approximately half the conventional nucleic acid quantity. Because a high transfer efficiency can be achieved using a small quantity of

nucleic acids, there is a low chance of nucleic acid insertion into the genomic sequence of host cells, which is an advantage for cell transplantation. These results will be summarised in the future.

22.5. FUTURE DEVELOPMENT

Cell transplantation therapy is currently being carried out worldwide, with many research groups utilising autologous somatic stem cells [15]. A major reason for the transplantation of cells into a patient following *in vitro* cell culture and proliferation is to bypass ethical issues and immune rejection of cell transplantation [16]. Moreover, the secretion of paracrine cytokines has been suggested to be the major mechanism influencing the efficacy of the transplanted cells. However, transplanted cells display individual secretory properties with respect to cytokines, exosomes, microRNA, and so on [17,18]. There also exist cases with few targeted cytokines or low secretion of exosomes and microRNA, since it is quite difficult to verify the quality of donor cells. This would cause variation in the overall therapeutic effect. In addition, owing to the difficulties associated with cell tracing after transplantation, it would not be possible to determine the amount of time that cells would remain in a required site.

The novel concept of cell transplantation therapy reported in this chapter offers a solution to these problems. By introducing targeted nucleic acids into cells using MNPs, cells with stable characteristics can be produced. Furthermore, transfected cells can be purified using magnetic force. Since the dynamics of these purified cells can be observed non-invasively using MRI even after transplantation into the target organs, it would be possible to assess the resulting cell behaviour. This allows the standardisation and better management of cell transplantation therapy, a process for which quality control was previously thought to be difficult. This strategy is a good example of theranostics using nanotechnology.

It is likely that the modification of nanomaterials using various techniques can provide added value to cell transplantation therapy in the future. These techniques may represent a breakthrough, reviving the currently stagnant cell transplantation therapy field.

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**CHALLENGES IN DEVELOPMENT OF
ESSENTIAL OIL NANODELIVERY SYSTEMS
AND FUTURE PROSPECTS**

**Deborah Quintanilha Falcão*, Samanta Cardozo Mourão, Juliana
Lopes de Araujo, Patricia Alice Knupp Pereira, Anne Caroline
Andrade Cardoso, Kessiane Belshoff de Almeida, Fiorella Mollo
Zibetti, and Barbara Gomes Lima**

Department of Pharmaceutical Technology, Faculty of Pharmacy,
Universidade Federal Fluminense, Rio de Janeiro, Brazil

*Corresponding author: deborah@vm.uff.br

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23.1. INTRODUCTION

Nanotechnology can be defined as the science and engineering involved in the design, synthesis, characterisation, and application of materials and devices whose functional organisation is in the nanometer scale. Such substrates can be designed to display specific and controlled chemical and physical properties, which in medicine means to interact with cells and tissues in the subcellular level with a high degree of functional specificity, thereby providing the integration between technology and biological systems. Therefore, nanotechnology is not a natural emerging scientific discipline, but a multidisciplinary meeting of traditional sciences to provide the collective expertise necessary for the development of new technologies [1,2]. Despite the initial concept proposed by Feynman in 1959 to explore the possibility of a material handling scale of individual atoms and molecules, the term nanotechnology was not used until 1974, then being related to the electronics industry [3].

The application of nanotechnology in the pharmaceutical field is a topic that every day arouses more attention in the research and development (R & D) field and has been slowly transforming the landscape related to treatment, prevention, and diagnosis of many diseases, leading to the concept of "nanomedicine" in 2005, which was introduced by the National Institutes of Health in the United States. Nanomedicine is thus a wide field of study that includes nanoparticles (NPs) that mimic biological actions, "nanomachines", nanofiber and polymeric nanoconstructors of potential use as biomaterials and nanoscale devices (*e.g.*, silicon microchips for drug delivery), sensors, and laboratory diagnostics [2,4]. In this context, several innovative techniques exploit the unique characteristics of "nano" by applying them to obtain new pharmaceutical forms of controlled release and drug delivery systems that may increase the action of the active agents [3,5].

The concept of drug delivery systems was introduced in the early twentieth century by Paul Ehrlich with the "magic pill", in which the drug is released precisely in its exact site of action, thereby increasing its effectiveness. In this regard, the application of nanotechnology tends to make this more feasible due to the ideal small size of the particles, releasing the substance in the correct location and time [2,6]. Among the different systems used as drug carriers (nanocarriers) are the submicron emulsions (nanoemulsions, microemulsions, liquid crystals), polymeric micelles, cyclodextrins, liposomes, and NPs (polymeric, lipid, and metallic) [4].

These nanosystems are able to offer a therapeutic protection system to the drug against possible instability in the body, promoting maintenance of plasma levels at constant concentration; increased therapeutic efficacy; gradual and controlled release of the drug by conditioning the environmental stimulation

(sensitive to changes in pH or temperature); significant decrease in toxicity by reducing plasma levels of maximum concentration; reduced instability and decomposition of sensitive drugs; possibility of directing to specific targets; incorporation of both hydrophilic and lipophilic substances in the same formulation; and reduction of the therapeutic dose and number of administrations, leading to a consequent improvement in patient compliance and convenience [5]. Depending on the particle charge, surface properties, and relative hydrophobicity of the nanosystems, they can be designed to be adsorbed in organs or tissues preferentially, releasing the drug in high concentration in the desired location. The main disadvantages of nanoscaled particles are difficulties in production, storage, and administration due to physical instability phenomena such as aggregation. The choice of wall material and production method is therefore of paramount importance and combined with the ability to modify drug release makes such nanosystems ideal candidates for therapeutic purposes presenting a wide range of applications, such as cancer therapy, administration of vaccines, contraceptives, delivery of anti-microbial and anti-viral agents, *etc.* [6].

23.1.1. Essential oils (EOs)

Essential oils (EOs) can be defined as a “product obtained from a natural raw material of plant origin, by steam distillation, hydrodistillation or by mechanical processes from the epicarp of citrus fruits” [7]. They are complex mixtures of volatile substances, usually aromatic. Therefore, they are also known as volatile oils, ethereal oils, or essences. The term oil is related to the oily liquid appearance at room temperature. EOs differ from fixed oils due to their main characteristic of high volatility. Another important point is the pleasant and intense aroma, hence their being called essences [8].

Spices have been used since ancient times for a large number of purposes, such as medicine, perfume, preservatives, and to add aroma and flavour to food. The first distillation of EOs appeared in the Orient (Egypt, India, and Persia) more than 2000 years ago and was improved in the ninth century by the Arabs. However, only in the thirteenth century were EOs made by pharmacies and their pharmacological effects described in pharmacopoeias. Paracelsus von Hohenheim used the term “essential oil” for the first time in the sixteenth century when naming the effective component of a drug as “Quinta essential” [9]. Nowadays, EOs are widely used in perfumes and make-up products, as food preservers and additives, in sanitary products, in agriculture, and as natural products. Moreover, EOs are used in massages or baths, most frequently in aromatherapy. They still represent an important part of the traditional medicine in pharmaceutical preparations, and several monographs are reported in the official pharmacopoeias. In fact, more than three thousand EOs are known, and about 10 % of them have commercial importance for industries [10,11].

EOs are synthesised by all plant organs and stored in secretory cell cavities, canals, epidermic cells, or glandular trichomes. It seems that they play important roles in self-defense against bacterial and fungal infections and in pollination, as well as in intraspecific communication [12]. They are generally complex mixtures of volatile organic substances produced as plants' secondary metabolites composed by hydrocarbons (terpenes, sesquiterpenes, and phenylpropanoids) and oxygenated compounds (alcohols, esters, ethers, aldehydes, ketones, lactones, phenols, and phenol ethers), characterised by low molecular weight. Despite the complex content of EOs, compounds are present at quite different concentrations. Generally, two or three are major components at fairly high concentrations compared to others present in trace amounts. These major components are usually used as a quality parameter of the EO and are frequently related to its biological properties. However, the assumption that only active phytochemicals are involved in the biological mechanism is already overcome. Interaction among compounds in the EO can involve the protection of an active substance from degradation by enzymes from the pathogen or play a synergistic role [13]. For example, the geometric isomers neral (39.50 %) and geranial (51.44 %) (known together as citral) are the major components of the *Cymbopogon citratus* EO, popularly known as lemongrass [14]. The quality parameter of lemongrass EO is related to citral content over 75 % [15].

Different factors such as climate, soil composition, plant organ, age, and vegetative cycle stage can interfere in the chemical profile of the EO, as observed with any other vegetal material. Furthermore, the extraction method is closely related to the quality of the EO. An inappropriate extraction procedure can damage or alter the action of the chemical signature of the EO, which can result in change in bioactivity and natural characteristics. EOs can be obtained by different methods, such as distillation (steam distillation, hydrodistillation, and hydrodiffusion), solvent extraction, supercritical carbon dioxide, pressurised hot water, expression (cold pressing), or microwaves. Most of the commercialised EOs are chemotyped by gas chromatography and mass spectrometry analysis. Analytical monographs have been published to ensure the good quality of EOs [10,11,16].

Besides their high volatility, EOs can easily decompose. Such a characteristic is especially related to heat, humidity, light, or oxygen exposure. Degradation of EO constituents is due to oxidation, isomerisation, cyclisation, or dehydrogenation reactions, activated either enzymatically or chemically, and normally influenced by the conditions during the processing and storage of the plant material, upon distillation, and in the course of subsequent handling of the oil itself. The degraded product may result in a loss of quality and pharmacological properties [17]. In addition, some aged EOs as well as oxidised terpenoids are known for their skin-sensitising capacities [18].

At present, promising approaches have been reported using EOs or their isolated compounds in medicinal products for human or veterinary [17] use as

well as in medical devices [12]. They have been widely used for pharmaceutical, sanitary, cosmetic, agricultural, and food applications [10]. Traditionally, EOs have been used for many biological properties which have been extensively described in literature, as shown in Table 1.

Table 1. Some biological properties of EOs

Biological property	Essential oil
anti-microbial	lemongrass [14], peppermint [19], clove, tea tree, thyme, geranium, marjoram, palmarosa, rosewood, sage, mint [20], salvia [21], peppermint [22], patchouli, vanilla, ylang-ylang [23], melissa [24]
antioxidant	clove, cinnamon, nutmeg, basil, oregano, thyme [10], rosemary [25]
anti-diabetic	rosemary [25]
anti-viral	star-anise [25], peppermint [19]
hypotensive	lemongrass [14]
insecticidal	star-anise [25]
sedative	lemongrass [14], peppermint [19]

In order to overcome the intrinsic limitations of the EOs described above and explore their biological properties, a significantly large part of the current scientific work in this area applies nanotechnology on the encapsulation of EOs. Nanosystems are often designed to protect the active compounds against environmental factors (*e.g.*, oxygen, light, moisture, and pH), decrease oil volatility, transform the oil into a powder, and control the release of active compounds. Additionally, due to the subcellular size, nanocarriers may increase the cellular absorption mechanisms, reducing toxicity and increasing bioefficacy.

The aim of this chapter is to offer an overview on nanoencapsulation of EOs that covers the present status of the field and development perspectives. Different nanosystems were developed intending to be EOs delivery systems. This review focuses on inclusion complex with cyclodextrins, submicron emulsions, NPs (polymeric, lipid, and magnetic) and liposomes.

The nanotechnology combined to EOs could bring an important contribution to the development of new delivery systems. These new delivery systems carrying EOs are promising candidates as future and new products for health improvement.

23.2. EOs NANODELIVERY SYSTEMS

23.2.1. Challenges in development

At this point, it is well established that the encapsulation of EOs in nanocarriers has many advantages, especially in providing stabilisation against volatilisation and degradation as well as controlling release. However, many aspects must be observed when designing nanodelivery systems for such a complex mixture with delicate characteristics as EOs. As previously mentioned, nanocarriers can be structured by a great variety of material and processes. The choice of the type of nanocarrier (*e.g.*, NP, nanoemulsion) to be developed and the wall material to be employed must be strongly related to its target. The choice of the method is equally important, since the difficulty of scaling up for production and particle aggregation which can occur due to physical instability during storage and administration. In the particular case of encapsulating EOs, the selected method should avoid any physical treatment that could increase the loss of its compounds by volatilisation or degradation, such as heat, humidity, light, or oxygen exposure and high pressure. When designing these nanocarriers it is important to keep in mind that the primary goal is to obtain spherical particles with a smooth surface, low mean diameter, homogeneous distribution (polydispersity index), high encapsulation efficiency (EE), and low aggregation potential, which is directly related to the zeta potential (ZP).

Particle size, shape, and surface properties of the NPs play a crucial role in the uptake of nanosized delivery systems across the mucosal membrane. NPs between 50–300 nm, positive ZP, and hydrophobic surface were found to have a preferential uptake as compared to their counterparts [11].

In a previous work, Falcão *et al.* had compared the encapsulation of *Cymbopogon citratus* EO (lemongrass) in β -cyclodextrins by precipitation method and poly(caprolactone) (PCL) polymeric nanoparticles (PNPs) by oil in water (o/w) emulsion method using ultrasonic emulsification. The EO : β -cyclodextrin inclusion complex obtained was irregular in shape and had a larger mean diameter (441.2 nm) and lower inclusion efficiency (9.46 %), and the NPs (240.0 nm) were spherical in shape and demonstrated a higher EE (36.51 %). Moreover, according to the chemical comparison of the oil composition before and after the encapsulation processes, it was demonstrated that the NPs incorporated more compounds than the cyclodextrin complex. In the PCL NPs, the chemical profile indicated the loss of most volatile compounds, probably during the emulsification step due to slight heating resulting from the ultrasound treatment. The major compounds (neral and geranial) were almost completely incorporated, showing that these substances have an affinity for the polymer used as the wall material. On the other hand, the inclusion profile of EO compounds in the inclusion complex was different. With the exception of *trans*-caryophyllene, the sesquiterpenes present in the original oil were not identified in the complex. It is likely that

this type of molecule was not included in the central cavity of the host molecule due to steric effects. The authors concluded that, in the employed methods, encapsulating with PCL is a better choice to develop *Cymbopogon citratus* EO delivery systems than the inclusion complex using β -cyclodextrin as a host molecule [14].

23.2.2. Inclusion complex of cyclodextrins with EO

A molecular complex refers to the physical association between a host and a guest (active ingredient) molecule, and in the case of EOs the complexes are reported as cyclodextrins. Cyclodextrins in hydrophobic molecules such as EOs can form inclusion complexes that can enhance their aqueous solubility. This is possible due to their hydrophobic internal cavity and hydrophilic exterior, which compose cyclodextrins' structure.

Ciobanu *et al.* studied the complexation and retention capacities of α -cyclodextrin, β -cyclodextrin, γ -cyclodextrin, hydroxypropyl- β -cyclodextrin, randomly methylated- β -cyclodextrin, a low methylated- β -cyclodextrin, and cross-linked β -cyclodextrin polymers for the four major components of peppermint (*Mentha piperita*) EO (menthol, menthone, pulegone, and eucalyptol). The controlled release of aroma compounds from cyclodextrins was evaluated by multiple headspace extraction experiments. The obtained results indicated the formation of a 1 : 1 inclusion complex for all the studied compounds. β -cyclodextrins presented the greater aqueous phase formation constant and retention ability. Furthermore, it was observed that β -cyclodextrin confers protection against evaporation of the aroma, so it is possible to have a better release efficiency by using cyclodextrin polymers [19].

Haloci *et al.* produced inclusion complexes between the *Satureja montana* EO and β -cyclodextrin by co-precipitation method with different oil : β -cyclodextrin ratios. The chemical composition and biological properties of the EO before and after inclusion in β -cyclodextrin were studied. The best inclusion efficiency was achieved at the ratio of 20 : 80, showing 94.07 % of powder recovery. The qualitative and quantitative chemical composition of the EO showed no significant change after the inclusion process, which is related to the maintenance of the biological properties of the EOs. However, the complex obtained with an oil : β -cyclodextrin ratio of 10 : 90 showed an increase in anti-fungal and antioxidant activities when compared to the free oil [26].

EOs from species like *Eugenia caryophyllata* and *Piper nigrum* usually present in their composition a major compound known as β -Caryophyllene, which has interesting biological properties. Employing the co-precipitation method, Liu *et al.* developed an inclusion complex of β -caryophyllene and β -cyclodextrin with inclusion efficiency of 62.04 %. The study compared the pharmacokinetic characteristics after oral administration between the free

β -caryophyllene and the complex in rat plasma. An increase in oral bioavailability for β -caryophyllene was observed in the inclusion complex. It was demonstrated after 12 h of administration of the β -caryophyllene/ β -cyclodextrin complex compared to the free β -caryophyllene. Furthermore, other pharmacokinetic parameters corroborate this result [27].

Lippia sidoides EO was encapsulated using β -cyclodextrin by suspension method, followed by the removal of water by employing a spray drying technique. Particles of 10–12 nm were obtained and an inclusion efficiency of 70 % in the EO : β -cyclodextrin ratio of 1 : 10 water in water (w/w). The mentioned oil is a potent anti-microbial of local use, and by the described process an important reduction in volatilisation was observed that enhanced thermal stability [28].

Carvacrol, a phenolic monoterpene usually present in the EO of oregano (*Origanum* spp.), was encapsulated with β -cyclodextrin to increase its stability and solubility by slurry complex method and compared with a physical mixture (PM). Particle size (PS) of the developed inclusion complex (7.5 μ m) were much smaller than those of PM (80.2 μ m) and β -cyclodextrin alone (14.8 μ m). The complex showed longer hyperalgesic effects (about 24 h) than the free substance that possesses durability of approximately 9 h. Furthermore, without complex formation, significant changes in nociceptive responses were not observed. Another factor that should be mentioned is the use of minor amounts of active compound to achieve the desired effect, which also is advantageous [29].

23.2.3. Submicron emulsions containing EO as delivery systems

Emulsions can be classified by the particle size as macroemulsion, nanoemulsion, and microemulsion. When the droplet size is in the nanometer range, they are also called submicron emulsions [30].

The encapsulation by emulsification promotes protection from volatilisation of EOs [31–33]. Due to their intrinsic properties, nanoemulsions may present several advantages for encapsulating functional lipophilic compounds over macro and microemulsions [34]. Salvia-Trujillo *et al.* studied the characteristics of microfluidised EO nanoemulsions. Microfluidisation involves the application of high pressure on a coarse emulsion for the production of nanoemulsions. The macroemulsions containing EOs (lemongrass, clove, tea tree, thyme, geranium, marjoram, palmarosa, rosewood, sage, or mint) were prepared by high shear homogenisation and passed through a microfluidisation system, obtaining nanoemulsions in the range 2.22–20.88 nm of the average droplet size and low polydispersity index. Moreover, the results showed that nanoemulsions containing lemongrass, clove, thyme, or palmarosa EOs demonstrated the strongest anti-microbial activity. Only in the case of nanoemulsions with lemongrass or clove EOs could an enhanced activity against *Escherichia coli* (*E. coli*) be observed [20].

Dias *et al.* compared nanoemulsions containing Copaiba oil prepared with high pressure homogenisation and spontaneous emulsification. The results indicated that the advantages are greater when using high pressure homogenisation because of the higher β -caryophyllene contents and physical stability of the formulation. These nanoemulsions showed reduced loss of volatile fraction within 90 days of storage at 4 °C [35].

Severino *et al.* evaluated the anti-bacterial activity of modified chitosan-based coatings containing nanoemulsion of EOs against *E. coli* O157:H7 and *Salmonella Typhimurium*. The nanoemulsions were obtained by high pressure homogenisation. The droplet size of the four different nanoemulsions, made of carvacrol, mandarin, bergamot, and lemon EOs, respectively, were 133 nm, 161 nm, 164 nm, and 177 nm. Also, the nanoemulsions were compared in terms of minimum inhibitory concentration (MIC) against the two bacteria evaluated *in vitro* using the micro-broth dilution assay. Carvacrol nanoemulsion proved to be the most effective anti-bacterial agent and was therefore selected to be incorporated into modified chitosan to form a bioactive coating [36].

A study conducted by Kim *et al.* developed nanoemulsions of lemongrass oil by homogenisation using a vortex mixer, high shear probe mixer, or dynamic hot pressure (DHP) treatment. The nanoemulsions formed by DHP processing presented mean droplet sizes of 56.4 and 87.9 nm following the oil concentration of 0.5 and 4.0 g / 100 g, respectively. Also, the stability of the emulsions was improved when employing DHP, making this method a better choice than the others used in the study. In addition, the nanoemulsion containing lemongrass oil showed anti-microbial affects against *Salmonella typhimurium* and *E. coli* [37].

EOs formulated in submicronic emulsions are a great tool for the development of products that can be applied in the pharmaceutical, cosmetic, and food industries. The encapsulation in nanoemulsions increases the effectiveness of EOs' action because of the extremely small particle size, improving the stability of formulations and interaction with microorganisms, cells, and receptors and protecting the EOs from volatilisation. Also, the surfactant and method employed influence the physical stability and biological activity through the particle size, interaction between surfactant and EOs, and bioavailability.

23.2.4. EO loaded in polymeric nanoparticles

PNPs are classified as nanocapsules and nanospheres. Nanospheres are matrix systems. Nanocapsules have two compartments: a polymeric wall and a core, which is typically oily. The EO may be conjugated with the polymer (matrix or wall) or solubilised in the oily core. The polymers, whether of natural or synthetic origin, must be biocompatible and biodegradable.

PCL NPs were prepared by a solvent emulsification-diffusion technique for encapsulating eugenol, a volatile constituent of many EOs, showing the

effectiveness of the polymeric shell on improving its stability during storage and protecting against oxidative reactions caused by light [38]. Gomes *et al.* encapsulated eugenol also in NPs, but by using poly(D,L-lactide-co-glycolide) (PLGA) as a wall material by emulsion evaporation method. An EE of 98.27 % and PS of 179.3 nm were observed. Eugenol-loaded NPs showed anti-microbial properties inhibiting the growth of *Salmonella* spp. and *Listeria* spp. The authors related improvement on efficacy on the increased hydrophilicity of the active compound, sustained release, and small particle size [39].

To increase the stability of the oregano EO (*Origanum vulgare*), Hosseini *et al.* encapsulated in biodegradable NPs of chitosan by a two-step process: o/w emulsification and gelation of chitosan with sodium tripolyphosphate (TPP). Chitosan NPs showed PS between 40–80 nm and EE and loading capacity of about 21–47 % and 3–8 %, respectively, when the initial oil content was 0.1–0.8 g g⁻¹ chitosan. Furthermore, the nanoencapsulation of oregano oil into chitosan NPs improved its thermal stability and provided release control. It is important to emphasise that the increase of the initial content of oregano EO resulted in an increase in PS and loading capacity as well as reduction in the EE [40]. These results were consistent with those reported by Keawchaoon and Yoksan when carrying out the loading of carvacrol into chitosan-TPP NPs by the same method. Carvacrol-loaded NPs showed anti-microbial activity against *Staphylococcus aureus*, *Bacillus cereus*, and *E. coli* and a sustained release that reached a plateau level after 30 days [41].

Another approach to encapsulate an EO was studied by Lertsutthiwong *et al.* In order to reverse problems regarding volatility and low water solubility, they incorporated turmeric oil into alginate nanocapsules by a three-step procedure using o/w emulsification, gelification with calcium chloride, and solvent removal. The results showed that particle size can be affected by the solvent employed in oil solubilisation, and the presence of a surfactant and the sonication step is crucial to the size homogeneity. However, during the process of preparing alginate nanocapsules, about 42 % of the turmeric oil was lost and the good physical stability in long-term storage was observed only at 4 °C. Better physical stability at a higher temperature (25 °C) and less oil loss was obtained by adding chitosan during preparation of the nanocapsules [42,43].

Decreased volatilisation and stronger aroma of tea tree oil (*Melaleuca alternifolia* EO) were achieved by Flores *et al.* through its incorporation into PCL nanocapsules. Employing the interfacial polymeric deposition method, it was possible to obtain PS of 212 nm, negative ZP (–13.5 mV), and oil content of 95.7 %. Tea-tree-loaded nanocapsule suspension showed high anti-fungal activity against *Trichophyton rubrum* in different *in vitro* models of dermatophyte nail infection. It was considered by the authors as a promising topical therapy of onychomycosis [44,45].

Lippia sidoides oil, rich in thymol, was encapsulated in PNPs formed by a matrix of chitosan and angico gum and evaluated with respect to the *in vitro*

release profile and activity against *Stegomyia aegypti* larvae [46]. The polymeric nanocarriers were obtained by spray drying an emulsified coacervate using the same proportions of chitosan : angico gum (1 : 10) but varying the proportion of EO : angico gum-chitosan (1 : 2, 1 : 4, 1 : 10, and 1 : 20). Among the tested ratios, higher loading (6.7 %), EE (77 %), and an EO sustained release were obtained for the EO : angico gum-chitosan (1 : 10) sample. Moreover, this release profile provided increased bioavailability, enhancing its larvicidal effect with 85 and 92 % mortality after 24 and 48 h, respectively. Subsequent studies published regarding *L. sidoides* oil encapsulation in NPs presented complex biopolymers as wall materials such as chitosan/cashew gum [47,48] and alginate/cashew gum [49], thus showing the potential use of these nanosystems as a larvicide for fighting dengue.

23.2.5. Solid lipid nanoparticles and nanostructured lipid carriers for delivery of EO

A good way to improve the use of EOs is by encapsulating them in lipid NPs – more specifically, the solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) – due to the many advantages that utilising this kind of system can provide to volatile oils. Firstly, the EOs are mostly lipophilic, presenting a good miscibility with the lipid matrix, improving the entrapment efficiency [50]. Lipids in a solid form protect the EOs, decreasing the sensitivity to air, light, and temperature and reducing the volatilisation [51,52] and degradation of the active components.

During the first decade of the current century, studies involving the encapsulation of EOs in SLN and NLC began to emerge. The most widely used method is high-pressure homogenisation by the hot homogenisation technique [51-53], where the aqueous phase (around 5 to 10 °C above the lipid melting point) is added into the oil phase, under stirring. The reduction of droplet size is obtained employing a high pressure homogeniser. Another tool also quite explored to reduce the size of the pre-formed emulsion is the ultrasound probe [54,55]. However, in some cases, it is possible to produce particles in nanometric range even with vigorous stirring [56], but the right choice of surfactants is imperative, otherwise, only micrometer particles are obtained [57,58].

SLN were produced by a high-pressure homogenisation method containing frankincense and myrrh oil (FMO) in a ratio of 1 : 1, and the *in vivo* anti-tumour activity was evaluated. The average diameter was 113.3 nm and the EE was 80.60 %. The stability of the entrapped oil in SLN was higher when compared to the free FMO during the 6 days of storage. The anti-tumour efficacy of the free FMO was compared with SLN-FMO and an inclusion complex with β -cyclodextrin (FMO- β -CD). SLN-FMO showed higher activity, 43.66 % of the inhibition rate, compared to the other formulations FMO suspension (31.23 %) and FMO- β -CD (34.81 %) [52].

Zedoary turmeric oil (ZTO) (*Curcuma zedoaria*) was encapsulated in NLC by melt-emulsification technique. NPs were formulated in pure Crodamol® SS or with Miglyol® 812N using soybean phosphatidylcholine as a surfactant. When comparing pure Crodamol® SLN (PS: 399 nm; EE: 81.1 %) and Crodamol® with Miglyol® NLC, a decrease was observed in the average diameter and an increase in EE as the proportion of Miglyol®/Crodamol® increased (*e.g.*, with 30 % [wt] of Miglyol®, PS: 128.2 nm; EE: 94.2 %). The greater complexity of the matrix decreases the surface tension, leading to a reduction of particle size and larger space for insertion of the drug molecules in the lipid matrix, increasing the EO entrapment. *In vitro* drug release experiments indicated that NLC could enhance the drug release rate over the SLN, and the drug release rate could be adjusted by the liquid lipid content in lipid NPs. NLC showed a burst release for the first 8 h, explained by the oil release from the outer surface, followed by a sustained release until 90 h due to the matrix erosion. In SLN, the EO was released more slowly because oil diffusion through the solid lipid is more difficult than through the liquid lipid in NLC. When the percentage of the liquid lipid increases, the release of the oil is accelerated. These results were corroborated by *in vivo* study. ZTO-NLC also showed a prolonged acting time when compared to free ZTO after intravenous administration [54].

β -elemene, an isolated compound from the EO from the same species (*C. zedoaria*), was formulated into SLN by the method combining probe sonication and membrane extrusion. Glycerol palmitostearate, monostearin, or a combination of 70 and 30 % of both, respectively, were employed as a lipid. The surfactant (poloxamer 188) was added in the oil or aqueous phase. A smaller mean diameter (26.5 nm) and a bigger entrapment (99.9 %) were obtained using only lipid monostearin with the addition of the surfactant in the oil phase. This could be explained by the presence of more hydrophilic groups from the monostearin, which facilitates the emulsification process. A premix of surfactant with lipids may also facilitate the breakdown of the lipid phase into smaller droplets. The most stable formulation concerning size and β -elemene content parameters, after 8 months of storage, was obtained employing a combination of 70 % glycerol palmitostearate and 30 % monostearin with the surfactant added in the lipid phase. The *in vitro* release profile showed a steady release with the SLN depletion after 80 h, which may suggest a model of solid solution [50,55]. Although a significant difference was not observed in the pharmacokinetic parameters between SLN- β -elemene and commercial emulsion after intravenous administration *in vivo*, the tissue distribution in the liver, spleen, and kidney was higher using SLN- β -elemene, whereas the β -elemene concentration was lower in the heart and lung [55], corroborating the already reported opsonisation and uptake from NPs by the reticuloendothelial system [59].

The *Artemisia arborescens* EO was encapsulated in SLN by the hot pressure homogenisation technique utilising Compritol® 888 ATO as a lipid matrix and Poloxamer 188 (SLN-1) or sodium cocoamphoacetate (SLN-2) as a surfactant.

SLN-2 showed no difference in particle size (207 nm) between SLN-oil and SLN-placebo as well as a greater EO entrapment (92 %). Since the formulations have been developed for use as a pesticide, they were sprayed. There was no variation in particle size in SLN-2 before or after spraying, whereas for SLN-1 there was a small increase; when stored at 40 °C, the SLN-1 gelled, preventing spraying. However, SLN-1 showed a better ability to avoid oil volatilisation. After 48 h at 35 °C, SLN-1 lost 37.07 % of the oil, whereas SLN-2 evaporated 45.51 % [51]. The same NPs were also evaluated for skin permeation in Franz diffusion cells. With SLN the oil accumulated in the outer layers of skin, whereas with the pure oil the permeation was larger and with a low accumulation [60]. These results suggest that SLN can reduce pesticide toxicity.

23.2.6. Functionalised magnetic nanoparticles loading EO

Magnetic nanoparticles (MNPs) are widely studied for their potential biomedical applications, such as diagnostic imaging, drug targeting, drug delivery, stabilisation of EOs and inhibition of microbial biofilm development, improved surfaces with anti-adherent properties, hyperthermia, and cancer treatment. Fluorescent magnetic nanoparticles (FMNPs) are being used in an increasing number of medical applications, offering chemical groups designed to permit the specific attachment of drugs and to improve their biocompatibility. The magnetic component of the MNPs in general is magnetite, Fe_3O_4 , a proven biocompatible iron oxide [12,21,61].

Due to the significant increase of actual anti-microbial drug resistance, new alternative strategies for combating microbial infections have been studied to explore the widely known properties of EOs. The combination of the stabilising carrier properties of MNPs with EOs (or isolated compounds from EOs) could improve stability and represent a successful approach for the development of novel materials and surfaces, refractory to microbial biofilms formation [12,21,61].

Anghel *et al.* developed and characterised a novel nanostructured phytoactive coated rayon/polyester wound dressing surface, refractory to *Candida albicans* adhesion, colonisation, and biofilm formation, based on FMNPs and *Anethum graveolens* and *Salvia officinalis* EOs with potential application for wound care. The FMNPs were prepared by wet chemical precipitation from aqueous iron salt solutions by means of alkaline media and showed a spherical shape with an average diameter of 15 nm, estimated by transmission electron microscope (TEM). The amount of EOs entrapped in the FMNPs evaluated by thermogravimetry (TG) analysis was 1.22 % for *S. officinalis* and 6.65 % for *A. graveolens*. The NPs were able to stabilise and control the release of EOs, significantly enhancing the anti-biofilm effect for at least 72 h. The nanobiocoatings preferentially inhibit the early stages of biofilm formation (after 24 h), but also reduce the formation and development of mature

biofilms. While the inhibitory effect of *A. graveolens* reached the maximum intensity on 48 h biofilms, the effect of *S. officinalis* gradually increased with the biofilm age, exhibiting a maximum efficiency at 72 h. According to the authors, the difference on activity from both systems is related to the different release rate of the two phytochemicals from the NP carrier [21]. In a similar study, *Satureja hortensis* EO inhibited *C. albicans* adhesion and biofilm formation for up to 72 h of incubation. FMNPs were developed with an average size of 10 nm and 14.37 % of EO content [62].

In other studies, a core/shell/EO nanofluid was used to create a coated shell on a prosthetic medical device. The catheter pieces were coated with suspended core/shell by applying a magnetic field on nanofluid, and the EO was applied by adsorption in a secondary covering treatment. FMNPs were synthesised by precipitation method. The microbial adherence ability and biofilm development were investigated up to 72 h in catheter pieces with and without a hybrid NPs/EO nanosystem [22,61,63].

R. officinalis EO coated NPs of up to 20 nm in size were fabricated by Chifiriuc *et al.* by employing oleic acid as a surfactant. The nanobiosystem strongly inhibited the adherence ability and biofilm development of the *Candida albicans* and *Candida tropicalis* tested strains to the device surface [63].

Core/shell/*Eugenia carryophyllata* EO nanostructures of up to 20 nm in size were obtained, and the activity was evaluated against different strains of *Candida* spp. The nanosystem drastically decreased the number of biofilm embedded cells, especially after 24 and 48 h of incubation. For some of the tested strains, the fungal adherence and biofilm development was totally inhibited at 24 h [61].

Anghel and Grumezescu reported the development of a 5 nm core/shell magnetic nanostructure combined with *Mentha piperita* EO. Magnetite coated with lauric acid was prepared and characterised. The *M. piperita* EO content was estimated by TG analysis as approximately 17.3 %. An improvement was observed on resistance to *S. aureus* adherence and development of biofilm for up to 72 h [22].

More recently, iron oxide NPs functionalised with myristic acid exhibiting a size below 20 nm were fabricated with the EOs of ylang-ylang (*Cananga odorata* subsp. *genuina*), patchouli (*Pogostemon cablin* syn. *P. patchouli*, *P. heineanus*), and vanilla (*Vanilla planifolia*) to be further used as coating agents for medical device surfaces. NPs were prepared by co-precipitation of Fe^{+2} and Fe^{+3} in basic aqueous dispersion of myristic acid and then characterised. The amount of patchouli, vanilla, and ylang-ylang was estimated by TG analysis as 7.74 %, 9.94 %, and 15.58 %, respectively. According to Bilcu *et al.*, the content of EOs adsorbed on the surface of NPs seems to be related to the polarity of EOs. Vanilla EO loaded NPs strongly inhibited both the initial adherence of *S. aureus* cells after 24 h and the development of the mature biofilm at 48 h. Patchouli and ylang-ylang EOs mostly inhibited the initial

adherence phase of *S. aureus* biofilm development. In the case of *K. pneumoniae*, all tested nanosystems exhibited similar efficiency, being active mostly against the adherence to the tested surface. The tested oils did not exhibit any significant influence on the biofilms after 72 h, indicating that the bactericidal effect is related to the release rate from the iron based nanocarrier attached to the catheter surface [23].

Magnetic microspheres consisting of magnetite NPs functionalised with poly(lactic acid)—chitosan polymers and *Melissa officinalis* EO were described by Grumezescu *et al.* MNPs/EO not exceeding 20 nm were prepared by co-precipitation of Fe^{+2} and Fe^{+3} in a basic aqueous solution. Microspheres with diameters ranging between 350 and 530 nm were pelliculised by Matrix-Assisted Pulsed Laser Evaporation (MAPLE) and characterised. The obtained MAPLE-deposited thin-films showed excellent anti-adherence and anti-*S. aureus* biofilm formation. The microspheres exhibited a great impact on microbial colonisation, impairing the normal formation and maturation of the biofilms. This effect was probably mediated by the release profile of the EO from the polymeric nanosystem, which remained significant up to 72 h of incubation [24].

23.2.7. Liposomes as EO nanocarriers

Liposomes or (phospho)lipid vesicles are spherical self-assembled colloidal particles formed spontaneously by a curved lipid bilayer which entraps part of the solvent [64]. Liposomes are interesting carriers of drug systems, especially due to their biocompatibility, intracellular delivery, targeting and entrapment of hydrophylic molecules in an aqueous core and lipophylic molecules into a lipid bilayer [65].

Considering EOs' delivery, the main reasons to use a liposome as a nanocarrier for EOs are for the improvement of solubility and absorption of lipophylic drugs and to enhance cellular membrane interactions, thereby increasing therapeutic effectiveness [66-70].

Depending on the size and number of bilayers, liposomes can be classified as multilamellar vesicles (MLVs), large unilamellar vesicles (LUVs), and small unilamellar vesicles (SUVs), which present specific characteristics regarding loading capacity and different preparation methods [71]. The PS, bilayer fluidity, and stability as well as the EE are important parameters for therapeutic efficiency and could be affected by physical chemical properties of EO or isolated compounds and by the composition of liposomes [64].

It was observed that EE is slightly lower when comparing SUVs to MLVs for *Zanthoxylum tinogassuiba* [72] and *Artemisia arborescens* [73] EOs, which can be attributed to a smaller bilayer area for SUV. These results were not observed with *Santolina insularis* EO; the entrapment efficiency was not considered different for SUVs and MLVs, but it was related that MLVs had a more irregular size distribution than SUVs [74].

Regarding the liposome characterisation, it was observed that the loading of EO could reduce [69,73-75] or slightly increase the PS [68,72]. This indicates that the effect of oil incorporated into the packaging and accommodated in the lipid bilayer depends on the type of substance and the employed process [72].

Considering the liposome composition, the inclusion of a non-ionic surfactant in the phospholipid bilayer reduced the EE of *Artemisia arborescens* EO [73]. Yoshida *et al.* evaluated the effects of different cryoprotectors on *Eugenia uniflora* EO liposomes. They observed that threhalose is a more effective agent cryoprotector, although other agents, such as sucrose, can be used in specific situations [75].

Liposomes have been studied for many diseases, including cancer. It can be due the passive targeting to the tumoural tissues and other possibilities as increase the circulation time by using hydrophilic polymers or active targeting by coating liposome with antibodies [76].

Celia *et al.* studied anti-cancer activity in an *in vitro* cell model of free bergamote EO and the same EO encapsulated in liposomes. The liposomes were prepared by hydration of lipid thin-film composed by dipalmitoyl-*sn*-glycero-3-phosphocholine monohydrate (DPPC), cholesterol, and 1,2-Distearoyl-phosphatidylethanolamine-methyl-poly(ethylene glycol) conjugate-2000 (DSPE-mPEG2000). Then the MLVs were extruded through a stainless-steel extrusion device. It was observed that the liposomal forms presented a greater reduction in cell viability and a greater increase in cell mortality than free EO, indicating an improvement in activity, which can be attributed to the increase of cellular uptake of the liposomes [68].

A similar study was carried out with free *Zanthoxylum tingoassuiba* EO entrapped in liposomes using human glioblastoma G-15 cells. The MLVs were prepared by thin-film hydration method and the SUVs were prepared by sonication of MLVs. The free EO was separated from liposomal EO by centrifugation. The liposomes, especially SUVs, are more efficient in reducing the cell viability than free EO. In addition, an increase was observed in thermal-oxidative stability for the liposomal form [72].

The capability of liposomes to enhance intracellular drug delivery is useful to intracellular therapy such as viral infection. The anti-viral effect against HSV-1 was improved when EO from *Artemisia arborescens* was encapsulated in liposomes. As in other studies, the MLVs were prepared by thin-film hydration following sonication to form SUV. The entrapped oil was separated from free oil by ultracentrifugation. Three formulations were tested considering different surfactants. The effect was dependent on the composition and liposome size, with MLVs showing better results than SUVs due to the higher drug leakage from SUVs [73].

It is common for EOs have anti-microbial activity. This can be explained by their lipophilic characteristic that perturbs and interacts with membrane

cells [77]. Thus, the use of liposomes could improve anti-microbial activity by furthering the membrane interaction. This was observed with tea tree oil loaded in liposomes prepared by reversed phase evaporation, with or without silver ions, in comparison with free EO against *Candida albicans*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* [78].

A similar effect was expected with *Zanthoxylum tingoassuiba* EO entrapped on MLVs prepared by thin-film hydration. Despite the vesicle size (greater than 1 μm), an incomplete release was observed after 15 h, suggesting a possible increase in EO penetration through the cytoplasmatic membrane, being enhanced by reduced particle size [69].

It is evident that, due to their hydrophobic compartment, liposomes are useful nanocarriers to EO delivery, especially for intracellular therapy and any kind of treatment that depends on the interaction between biomolecules and membrane cells.

23.3. OUTLOOK

Nanotechnology is an innovative, multidisciplinary approach that has potential applications in medicinal and health research. Nanocarriers can display specific and controlled chemical and physical properties, interacting with cells and tissues with a high degree of functional specificity. When applied to natural products, more specifically EOs, nanotechnology can be an important tool in the development of new formulations.

EOs are widely known for their biological properties and are extensively used in folk medicine for promoting health, as well as preventing and treating different diseases. However, their physicochemical characteristics, such as high volatility, low water solubility and stability, together with the side effects associated with their use have limited their application in medicine. They are more commonly used for external routes, such as topicals, gargles, and mouthwashes, or for inhalation. Oral administration is rarer and requires previous dilution. Processing such oils in delivery systems is an alternative for overcoming these intrinsic issues and increasing their medical application, since it can also expand the range of administration routes to be explored, such as oral and parenteral.

Several studies have reported the development of nanocarriers for EO delivery, and an improvement in stability and controlled release are normally described. However, the challenge in designing this kind of system is even greater when encapsulating such a complex mixture. The EE, particle size distribution, surface characteristics, and release profile must be carefully evaluated. A high content of EO seems to be the most challenging of all, since many fabrication processes employ high-energy methods to achieve the nanometer range that can accelerate the volatilisation and degradation of the oils, such as ultrasound

and high pressure homogenisers. The affinity between oil and encapsulating material seems to play an important role in optimising the entrapment and can modulate the release profile, acting as a useful tool according to the intended purpose.

Despite great challenges, a well-designed nanosystem can make an important contribution to the development of promising candidates for new products for the improvement of human or veterinary health.

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**CELLULAR MAGNETIC TARGETING
AND CORONARY EMBOLISM**

**Zheyong Huang^{1*}, Yunli Shen², Ning Pei³,
Juying Qian¹, and Junbo Ge¹**

¹ Shanghai Institute of Cardiovascular Diseases, Zhongshan Hospital,
Fudan University, 180 Feng Lin Road, Shanghai 200032, China

² Department of Cardiology, Shanghai East Hospital, Tongji University,
150 Jimo Road, Shanghai, 200120, China

³ College of Science, Shanghai University, 99 Shangda Road, Shanghai
200444, China

*Corresponding author: zheyonghuang@126.com

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24.1. INTRODUCTION

Given the adult heart's minimal capacity for endogenous regeneration, cell therapy has emerged as a promising approach for the regeneration of damaged vascular and cardiac tissue after acute myocardial infarction and heart failure [1]. Intracoronary (IC) injection permits the relatively homogeneous dissemination of donor stem cells to the target area in a more physiological manner with less myocardial injury than an intramyocardial injection [2]. IC has become the most commonly used approach in clinical studies, particularly when small cells, such as bone marrow mononuclear cells (MNCs), are used.

However, systematic review suggests only a mild improvement in global heart function, and a high degree of heterogeneity among clinical trials [3]. The first prerequisite for cell therapy success is the engraftment and thus homing of transplanted cells to the target area. Poor cell homing, retention and engraftment are major obstacles to achieving a significant functional benefit, irrespective of the cell type or delivery route used. Data showed that only 1–3 % of the delivered cells were recruited at the infarct sites *via* IC administration. The retention of cells in the heart is extremely low, even undetectable, after a few weeks when administered by the intravenous route [4–7]. The predominant number of cells was found in non-targeting organs such as the liver, spleen and lungs.

To induce the migration and homing of transplanted cells for optimisation of the efficacy of cell-based therapies, much effort has been made in identifying chemokines and their receptors (CXCR4 / SDF-1 axis, *et al.*) in the last decades [8,9]. However, due to the extreme complicity of the “cell-extracellular matrix-cytokine” network and the homing molecular mechanisms, the chemoattractant molecule-targeted method remains far from able to precisely and effectively regulate stem cell migration into the target tissue [10,11].

Magnetic targeting strategy, traditionally used in chemotherapy for tumours [12], was introduced to localise magnetic nanoparticle-loaded cell delivery to target lesions *in vivo* in recent years [13–19]. The accumulation and retention of the magnetic responsive cells can be enhanced by using an external magnetic field produced by an electromagnet, which is focused on the area of interest [20]. We previously demonstrated in an *in vitro* study that the cell capture efficiency reached 89.3 % in a deep magnetic field with a magnetic flux density of 640 mT, a magnetic intensity gradient of 38.4 T m⁻¹, and a flow velocity of 0.8 mm s⁻¹ [21]. Cheng *et al.* [22] were the first to introduce a magnetic targeting strategy to attract transplanted cells to the heart. Using a 1.3 Tesla magnet applied above the rat apex during the intramyocardial injection of magnetic responsive cardiosphere-derived cells, they found that cell retention and engraftment in the recipient hearts increased by approximately 3-fold compared to non-targeted cells. Chauderge *et al.* [23]

adopted the subcutaneous insertion of a magnet over the chest cavity during therapeutic intracavitary stem cell infusion, and found that the average number of engrafted cells was 10 times higher with than without magnetic targeting. This magnetically-enhanced IC cell delivery was confirmed by another study [24]. Thus, magnetic targeting is proven to enhance cell retention and engraftment, and this novel method to improve cell therapy outcomes offers the potential for clinical applications.

A significant disadvantage of IC cell injection is the risk of myocardial damage resulting from coronary embolism [25,26], and this risk is most important if large cells are injected, particularly into diseased, narrowed coronary arteries. Considering the powerful attraction of the magnetic field, however, whether the magnetic accumulation of cells in the vascular lumen increases the risk of coronary embolism when IC injections are given, especially when relatively large cells such as mesenchymal stem cells (MSCs; approximately 20–25 μm in diameter) were used becomes a great concern.

To clarify whether the magnetic accumulation of cells in the vascular lumen increases the risk of coronary embolism when IC injections are given, we explored the safety and effectiveness of magnetically targeting IC-delivering bone marrow-derived MSCs in a rat model of ischaemia/reperfusion (I/R) [27]. Additionally, the possible mechanisms and the solution of cellular coronary embolisation were discussed.

24.2. MAGNETIC TARGETING OF IC DELIVERING MESENCHYMAL STEM CELLS IN A RAT I/R MODEL: A PARADOXICAL DISASSOCIATION OF FUNCTIONAL BENEFIT WITH CELL RETENTION

24.2.1. Study protocol

An I/R model was developed in female Sprague Dawley (SD) rats (150–200 g). Rats underwent left thoracotomy in the 4th intercostal space under general anaesthesia. The heart was exposed and a myocardial infarction was produced by ligation of the left anterior descending (LAD) coronary artery for 90 min. After that, the suture was released to allow coronary reperfusion.

A total of 108 survival rats were randomly divided into three treatment groups. The Mag group received 1×10^6 Mag-DiR-MSCs with magnetic guidance ($n = 36$). The NonMag group received 1×10^6 Mag-DiR-MSCs without magnetic guidance ($n = 36$), and the PBS group received phosphate buffer saline (PBS) alone ($n = 36$). In the Mag group, 20 min after establishment of the I/R model, 1×10^6 Mag-DiR-MSCs resuspended in 1 mL PBS were infused into the left ventricle cavity with 5 s of temporary aorta and pulmonary artery occlusion, as previously described [28,29]. IC delivery was confirmed by the temporary

bradycardia and epicardial blanching. For magnetic targeting, a Neodymium-iron-boron (NdFeB) permanent magnetic cylinder with a diameter of 8 mm (Shanghai Yahao Instrument Equipment Co., China) was used. The magnetic flux density (B) of the magnet surface is up to 600 mT, which was measured using a model 51,662 Leybold Tesla meter. The magnet was close to the injured myocardium (0–1 mm) during and after the cell injection for 10 min (Figure 1), which had been suggested to be the optimised time [22,30]. The surgical wounds were repaired and the rats were extubated and returned to their cages to recover.

Figure 1. Magnetic targeting with the cylindrical NdFeB magnet

Bone marrow MSCs were isolated from 4-week-old male SD rats as described previously [21,31]. MSCs were cultured in media containing SPIO (Resovist; Schering AG, Berlin, Germany) and poly(L-lysine) (PLL, 0.15 mg mL⁻¹) for 24 h, with the concentration of iron of 50 mg mL⁻¹ and PLL of 0.15 mg mL⁻¹ [21]. The average cellular iron content was 21.77 ± 3.62 pg *per* cell after 24 h of incubation. The magnetic SPIO-labelled MSCs (MagMSCs) were then incubated with 1 μ mol L⁻¹ ethyl indotricarbocyanine iodide (DiR, ABD Bioquest, Inc.) for 20 min at 37 °C according to the manufacturer's protocol.

Resovist was a kind of superparamagnetic iron oxide (SPIO) nanoparticles (magnetite – Fe₃O₄; maghemite – γ -Fe₂O₃) coated with carboxydextran and an overall hydrodynamic diameter of 62 nm, as measured with photon-correlation spectroscopy. It has good stability and good magnetism [32,33]. The polycrystalline iron oxide core consists of multiple single crystals, each being 4.2 nm in diameter, as measured with electron microscopy. Resovist contains 0.5 mmol L⁻¹ of iron *per* litre, including 40 mg mL⁻¹ mannitol and 2 mg mL⁻¹ of lactic acid, adjusted to a pH of 6.5 at 37 °C. The solution has an osmolality of 0.319 osmol kg⁻¹ H₂O and a viscosity of 1.031 MPas.

24.2.2. Magnetically enhanced MagMSCs retention in the rat heart

To determine the efficacy of magnetic targeting, 6 animals from each group were euthanised for magnetic resonance imaging (MRI) at 24 h after the injection of cells or PBS. MRI was performed in a 3.0 T clinical whole-body MRI scanner using a 12.5×10 cm small-diameter 4-element phased-array coil (Philips). Partial cardiac gating was used to optimise image acquisition, resulting in synchronisation of about one-half of heart beats. Images were acquired by T_2 *fl2d* sequence with a slice thickness of 2 mm, a flip angle of 20°, a field of view of 90 mm, a matrix of 400×400 , 26 cardiac phases, a repetition time of 7.5 ms, and an echo time of 2.8 ms. Consecutive short-axis slices were acquired to analyse the signal intensity in the myocardium of the left ventricle with *ImageJ* software. The relative signal intensity of the anterior wall was calculated as the signal intensity in the anterior wall divided by the signal intensity in the interventricular septum.

Due to the limited number of cells infused, no signal void was detected in any animal on T_2^* -weighted contrast-enhanced images. However, relative hypointensities representing labelled MSCs were observed at the anterior walls of left ventricular (LV) in cell-treated groups. Semi-quantitative analysis showed that the relative signal intensities of anterior wall in the Mag group were lower than those of the NonMag group (0.62 ± 0.06 vs. 0.80 ± 0.06 %, $P < 0.01$) (Figure 2). The results indicated that the application of NdFeB magnet enhanced cell retention at the target site.

Figure 2. Magnetically enhanced MagMSC retention and distribution in the rat heart. *In vivo* MR T_2 *fl2d* cardiac images at 24 h post-injection. The anterior walls of LV of cell-treated animals showed relative hypointensity, which was especially obvious in the Mag group.

Following cardiac MRI, the hearts were explanted for fluorescence imaging. Extensive PBS washing was performed to remove any cells adhering to the epicardium. The hearts were placed in a Carestream *in vivo* Multispectral Imaging System FX PRO to detect deep red fluorescence (DiR) under 748 nm excitation and 780 nm emission. The exposure time was set at 3 s and was maintained during the entire imaging session. Hearts from the PBS group were also imaged as controls for background noise.

The fluorescence signals in the Mag group were approximately 3.4 times higher than those of the NonMag group (38508 ± 6754 vs. 11487 ± 2426 photon/S, $P < 0.001$), while the signal was 6155 ± 681 photon / S in the PBS group as a negative control. The results also reflected that the application of the NdFeB magnet enhanced cell retention at the target site.

X.2.3. Functional change of cellular magnetic targeting: a paradoxical disassociation of functional benefit with cell retention

Cardiac remodelling and LV function were assessed by transthoracic echocardiography 3 weeks after MI using a Vevo 770 high-resolution imaging system (Visual Sonics) with a 17.5 MHz probe.

The LV ejection fraction (LVEF) at baseline did not differ among the 3 groups, indicating a comparable degree of initial injury. At 3 weeks after surgery, the echocardiographic parameters, including LVEF, LV fractional shortening (LVFS), LVEDd and LVEDs, deteriorated more severely in the PBS group than in either the Mag or the NonMag MSC-treated groups. In contrast, the cell retention in the Mag group was comparable to that in the NonMag group ($P < 0.05$).

This result was also observed using Masson's Trichrome staining morphometry at 3 weeks, which showed less of a risk area in the cell treatment groups than in the PBS group, and interestingly, the Mag group did not show a smaller risk area than the NonMag group. These results indicated that cellular magnetic targeting did not have any additional functional benefits in the rat model of I/R, although the magnetic field could effectively attract cells to the injured site (Figure 3).

Figure 3. *In vitro* fluorescence signal of extracted heart at 24 h post-injection and echocardiographic assessment of LV at 3 weeks post-injection. A significantly escalated cell retention (fluorescence signals) with no functional benefit. LVEF was observed in the Mag group compared with the NonMag group, suggesting a paradoxical disassociation between the functional benefit and the cell retention.

24.2.3. Coronary embolism in cellular magnetic targeting

Because vascular-delivered cells reportedly translocate into the parenchyma after 48–72 h [25], the cellular embolisms within the hearts were examined at 72 h after the injection of cells or PBS. Six hearts from each group were stained with Prussian blue, and another 6 hearts from each group were frozen in optical coherence tomography (OCT) for fluorescence imaging.

Clear evidence of cellular embolisation was observed in the Mag group, as many blood vessels were filled with Prussian blue-positive multi-cellular clusters. Cellular emboli were mainly found in the micro-vessels. However, it is surprising that the emboli also obstructed the medium- or large-sized coronary arteries, which was observed in 3 of the 6 rats at 72 h, and in 2 of the 6 rats at 3 weeks. In contrast, the majority of delivered cells were located in the parenchyma, and only a small number of vessels were detected containing donor cells were detected at 72 h in the NonMag group (Figure 4).

Therefore, we hypothesised that magnetic targeting does enhance cell retention, however, magnetic strength can also undermine the therapeutic effects of cell transplantation *via* embolic injury.

Figure 4. Histopathological evidence of coronary emboli. (A) through (F), Representative images of Prussian-blue staining at 72 h after infusion. There were no emboli in the coronary vessels of the NonMag group (A). Blood vessels containing cells were readily detected and some vessels were totally occluded in the Mag group at 72 h (B-E) and 3 weeks (F) after infusion. MSCs appeared in multi-cellular clusters. (H) through (J), Fluorescence imaging of coronary emboli at 72 h post-injection. H and I are representative images of the Mag group and the NonMag group, respectively. Blood vessels were stained for CD31 antibody (green). MagMSCs were visualised by DiR red fluorescence, and the cell nuclei were counter-stained with 4',6-diamidino-2-phenylindole (DAPI). The quantification chart shows that more blood vessels were blocked by cell clumps in the Mag group than that in the NonMag group. Bar = 50 μ m.

24.3. MECHANISMS BY WHICH CELLULAR MAGNETIC TARGETING INDUCES CORONARY EMBOLISATION

It is generally believed that IC is a safe approach for cellular therapy, and the risk of cell-based coronary embolism is widely believed to be trivial. To date, there have been no reported coronary embolisms or subsequent myocardial infarction in clinical trials using IC injection of bone marrow and progenitor cells [34-36]. However, this concept is not necessarily relevant in a magnetic targeting setting. Here, we found that magnetic targeting increased the risk of coronary embolism in an ischaemic rat model subjected to intracavitary MSCs

injection. Although an external magnetic field improved short-term cell retention, it failed to translate enhanced cell retention into any additional therapeutic benefit. These findings are obviously contrary to the original intention of magnetic targeting strategies. Therefore, it is necessary to look at the possible mechanisms by which cellular magnetic targeting induces coronary embolisation.

24.3.1. Cell size

Cell size is generally considered to be a major factor in micro-embolism after intra-arterial cell injection. Larger stem cells, such as MSCs, are easily entrapped in the microcirculation after intravascular delivery. Upon intra-arterial injection, most *in vitro*-expanded MSCs became entrapped in the pre-capillary vessels and microvascular vessels, resulting in the cessation of blood flow in the feeding artery of skeletal muscle in the rat cremaster [25] and myocardial infarction in dogs [37]. However, there was no reported coronary embolism or subsequent myocardial infarction in clinical trial subjects exposed to IC arterial injection of bone marrow and progenitor cells [34-36]. The explanation for the differences in the results was that the mean diameter of the cells used in the human studies was most likely smaller than that of the cells used in animal experiments [25]. In our study, the size of Mag-DiR-MSCs ranged between 6–36 μm (average = 19.3 μm) in suspension, which is comparable to the iron-free MSCs, and larger than freshly prepared MNCs (Figure 5).

Figure 5. Cell size. (A), MagMSCs under microscopy. (B), MagMSCs under fluorescence microscopy. (C), The distribution of MagMSCs diameters measured by Scepter 2.0 Handheld Cell Counter. (D), The size of freshly prepared MNCs, MSCs, and MagMSCs.

However, the single cell size is no longer a determinant of vascular embolisation in the setting of cellular magnetic targeting. Magnetic attraction can attenuate cell loss *via* venous drainage, increase the intravascular stay, and enhance the formation of multi-cellular clusters [22,38], which was considered the theoretical basis for cellular magnetic targeting.

24.3.2. Magnetic characteristics of the NdFeB magnetic cylinder

The distribution of magnetic flux density of NdFeB permanent magnet can be calculated by finite element analysis. Here, the magnetic cylinder with a diameter of 8 mm used in this study will be used as an example.

The magnetic density decreases as the distance from the surface of the magnet increases; it increases as the distance from the centre of the magnet increases, and it reaches its maximum at its flank. Compared with the centre point of the magnet surface, the flank point of the magnet was 1.9 times higher in the magnetic flux density, and 27.5 times higher in the magnetic flux gradient, respectively. Compared with the point 1.5 mm vertically distant from the flank point of the magnet, the flank point of the magnet has a magnetic flux density that is 4.0 times greater and a magnetic flux gradient that is 1028.3 times greater. That is, the magnetic field of a permanent magnet is not distributed evenly, and such non-uniformity was characterised by the attenuation along the vertical axis and the polarisation along the horizontal axis. Interestingly, the polarisation trend was particularly evident at the plane closest to the surface of the magnet (Figure 6).

Figure 6. The magnetic field of the NdFeB magnetic cylinder. (A), The system of coordinates and flux density plot of the NdFeB magnetic cylinder. *O* denotes the centre point of the surface of the magnet pole. *r* and *z* denote the horizontal and vertical distance from the *O* point, respectively. (B), The distribution of magnetic flux density. (C), The distribution of magnetic flux gradient. Calculations reveal that *B* is *z*-axial rotational symmetric. When *z* remains unchanged close to the surface of the magnet, *B* increases with increasing *r* from the centre (the site *O*) to the flank, reaching a maximum at the flank before decreasing dramatically along the *r* axis. When *z* remains unchanged distant from the surface of the magnet, *B* decreases gradually with increasing *r*. *B*, Magnetic flux density; $(\frac{\partial B}{\partial r})$, Magnetic field gradient in the *r* direction.

24.3.3. Spherical shell-like distribution of SPIO nanoparticles within cell

A complex of PLL with the SPIO through electrostatic interactions allows the efficient incorporation of iron oxide nanoparticles into endosomes for

magnetic cell labelling. Labelling efficiency was reproducible in approximately 100 % with use of a very low concentration of iron oxide ($50 \mu\text{g mL}^{-1}$). Prussian Blue staining of SPIO-loaded MSCs showed intracytoplasmic iron inclusions as dense blue-stained vesicles, and the magnetic nanoparticles distributed evenly around the nucleus of MSCs as a spherical shell (Figure 7A).

Due to the higher magnetic permeability of SPIO, the magnetic field lines concentrated at the spherical shell developed by roundly distributed SPIO nanoparticles within the MagMSCs, which strikingly magnified the magnetic flux density in the spherical shell (Figure 7B) [39]. This finding is an important reason why SPIO-labelled cells have good magnetic responsiveness [21]. More importantly, the magnetised cells can further serve as a novel magnetic source to bring more cells together, and create an "aggregation begets aggregation" cascading waterfall effect.

Figure 7. Spherical shell-like distribution of SPIO nanoparticles within a cell. (A), Prussian blue staining of MagMSCs on cover glass. The magnified image shows blue magnetic particles distributed around the nucleus of cells as a spherical shell. (B), Finite element analysis of magnetic flux density of SPIO.

24.3.4. Magnetically guided distribution of MagMSCs in static state and theoretic analysis

Due to the non-uniformity of the magnetic field, it will definitely influence the distribution of MagMSCs either in static or flowing state. Firstly, we analysed the static state.

The MagMSCs suspension, at a concentration of 5×10^4 cells mL^{-1} , was replaced in a culture plate that was 34.8 mm in diameter and had a bottom thickness of 1.3 mm. The cell cultures, with or without an NdFeB magnetic cylinder beneath the bottom glass, were kept for 24 h with 5 % CO_2 at 37 °C. The MagMSC distributions were observed by inverted microscopy and cellular MRI. Cellular MRI was performed using a 1.5-T clinical MR scanner (CV/i, GE Medical

Systems) applying a T₂WI-flash sequence. The imaging parameters were as follows: repetition time (TR) = 800 ms; echo delay time (TE) = 26 ms, flip angle = 25°; 256 × 160 matrix and slice thickness = 2 mm, with no gap and a 90 cm² field of view (FOV).

Analysis of the distribution of the MagMSCs was theoretically based on measures such as iron content, SPIO thickness, cell size, and distribution of the magnetic flux density.

The magnetic field lines permeate non-labelled cells evenly. Due to the greater magnetic permeability of SPIO, the magnetic field lines concentrated at the spherical shell developed by rounded-distributed SPIO nanoparticles within MagMSCs, which strikingly magnified the magnetic flux density in the spherical shell.

We assumed that SPIO were rounded-distributed evenly within MagMSCs and formed a spherical shell. The thickness of ferric oxide particles is expressed as d , and the radius of MagMSCs is expressed as R . The volume of ferric oxide particles within spherical shell can be calculated by

$$V = 4\pi R^2 d = m/\rho \quad (1)$$

where m is the mass of Fe₃O₄ and Fe₂O₃ within cell, ρ is the mean density of Fe₃O₄ and Fe₂O₃ particles. If the mean iron content *per* cell is taken as 21.77 pg, then the mean mass of the Fe₃O₄ and Fe₂O₃ particle within a cell (m) equals 3.05×10^{-14} kg.

When $\rho = 5.21 \times 10^3$ kg m⁻³, $R = 9.65$ μm, and $m = 3.05 \times 10^{-14}$ kg, were put into the formula, we obtained $V = 5.86 \times 10^{-18}$ m³, and the thickness of ferric oxide particles within spherical shell was

$d = 5.01 \times 10^{-9}$ m. Thereby, we were able to approximate the ratio of magnetic flux density within spherical shell (B_{SPIO}) to the external magnetic flux density (B_{ex}) by the equation:

$$\frac{B_{\text{spio}}}{B_{\text{ex}}} = \frac{\pi R^2}{2\pi R d} \quad (2)$$

When $R = 9.65$ μm, $d = 5.01 \times 10^{-9}$ m, we calculated the spherical shell to be 963 times the magnetic flux density of the external magnetic field by the Equation (2). In the case of $B_{\text{ex}} = 10$ mT, we obtained $B_{\text{SPIO}} = 9.63$ T. Considering the saturation characteristic, the magnetic flux density within spherical shell (B_{SPIO}) should be equal to the saturation magnetic flux density of SPIO ($B_s \approx 0.7$ T).

The distribution of the magnetic flux density B , created by the permanent NdFeB magnetic cylinder, was calculated by finite element analysis. Calculations revealed that B is z -axial rotational symmetry. When r remained unchanged, B decreased with increasing z . When z remained unchanged close to the surface of the magnet, B increased with increasing r from the centre (the site O) to the flank, and reached a maximal result at the flank, before reducing dramatically along the r -axis. When z remained unchanged distant from the surface of magnet, B decreased gradually with increasing r .

Firstly, MagMSCs were evenly distributed in the plane of culture plate, where this was 1.3 mm from r direction. The distributions of magnetic flux density (B) in r direction at this level ($z = 1.3$ mm) are shown in Figure 2B. B - r curve was related to the value of z . B was minimal at the site O ($r = 0$ mm), and increased with increasing r , before reaching the maximal at one site.

The magnetic attraction force on the SPIO-loaded cells can be approximated by the formula:

$$F_M = V \nabla (M \cdot B) \quad (3)$$

where vector M is the magnetisation density of the cellular SPIO particle, vector B is the magnetic flux density of the external magnetic field and V is the volume of the SPIO particle within the cell.

The magnetic attraction force on MagMSCs, in r direction and in z direction, can be approximated by:

$$\mathbf{F}_M = VM \frac{\partial B_r}{\partial r} \hat{\mathbf{r}} + VM \frac{\partial B_z}{\partial z} \hat{\mathbf{z}} = F_r \hat{\mathbf{r}} + F_z \hat{\mathbf{z}} \quad (4)$$

The redistribution of cells in r direction depended on the transverse magnetic force on cells in r direction (F_r). F_r equals the product of V , M and the magnetic gradient ($\frac{\partial B}{\partial r}$). It was assumed that the content of SPIO in each cell was the same, and that M was the same. The magnetic attraction force on cell (F_r) is directly proportional to $\frac{\partial B}{\partial r}$. When $z = 0$ mm, $z = 1.0$ mm, $z = 1.3$ mm, and

$z = 1.5$ mm, the magnetic field gradient in r direction ($\frac{\partial B}{\partial r}$) was calculated by finite element analysis (Figure 2C).

If the mean iron content *per* cell is taken to be 21.77 pg, then the mean volume of the Fe_3O_4 and Fe_2O_3 particles within cell (V) equals $5.86 \times 10^{-18} \text{ m}^3$. According

to the analysis mentioned above, $M \approx 5.57 \times 10^5 \text{ Am}^{-1}$; from this, F_r can be calculated.

Firstly, F_r should be analysed along the r direction, when $z = 1.3 \text{ mm}$. When $0 \text{ mm} \leq r < 4 \text{ mm}$, the value of F_r is positive, and the direction of F_r is outward along the r axis. F_r increases first and then decreases with increasing r , and $F_r = 0$ when $r = 4 \text{ mm}$. Therefore, the cell at site $0 \text{ mm} \leq r < 4 \text{ mm}$ will move outside and finally stop at the site $r = 4 \text{ mm}$, where $F_r = 0$.

Inversely, when $r > 4 \text{ mm}$, the value of F_r is negative, and the direction of F_r is inward along the r axis. The absolute value of F_r increases first and then decreases with increasing r when $r > 4 \text{ mm}$, and $F_r = 0$ when $r = 15 \text{ mm}$. Therefore, the cell at site $4 \text{ mm} < r < 15 \text{ mm}$ will move inside and finally stop at the site $r = 4 \text{ mm}$, where $F_r = 0$.

In the same way, we can further determine that the cell at site $r \leq 0$ will also move to the site $r = 4 \text{ mm}$. Due to the symmetry of a magnetic field, the majority of cells would move and aggregate at the site $r = 4 \text{ mm}$, and form a round ring (Figure 8C).

According to the above theoretical analysis of cell movement in the magnetic field, the cell equilibrium position is close to the flank (site $r = 4 \text{ mm}$), suggesting that the MagMSCs may be captured and accumulated in the vicinity of $r = 4 \text{ mm}$ (Figures 8A–B).

The experimental findings were consistent with the analysis of cell dynamics based on electrodynamic and magnetomechanic principles. MagMSCs were evenly distributed in cell culture plates at the very beginning of the re-culture. Then, they were moved and redistributed as directed by the magnetic field generated by an NdFeB magnetic cylinder. 24 h later, a brown ring was formed in gross appearance (Figure 8C) and a signal void ring was observed by cellular MRI (Figure 8D). Surprisingly, the rings at the fringe of the magnet were so obvious that they could be observed by the naked eye. Inverted microscopy revealed that each ring consisted of numerous MagMSCs, which accumulated predominantly in layers at the edge of the magnet, rather than in the centre surface of the magnet. A small number of cells were sparsely dispersed elsewhere on the culture plate, and the density of MagMSCs was decreased as the distance from the edge of the magnet increased (Figure 8E). In contrast, MagMSCs were evenly distributed in the cell culture plate without a magnet. This polarised distribution of MSCs was also confirmed by the plot profile of grey value analysis using *ImageJ* software (Figure 8F).

Figure 8. Magnetically-guided distribution of the MSCs in the static state. (A), Schematic diagram of experimental procedure and the system of coordinates (red line). (B), The magnetic attraction force on MagMSCs in the r direction (F_r), when z is 1.3 mm (at the inner surface of the plate bottom). When $0 \text{ mm} \leq r < 4 \text{ mm}$, the value of F_r is positive, and the direction of F_r is outward along the r axis. F_r increases first and then decreases with increasing r , and $F_r = 0$ when $r = 4 \text{ mm}$. Inversely, when $r > 4 \text{ mm}$, the value of F_r is negative, and the direction of F_r is inward along the r axis. The absolute value of F_r first increases and then decreases as r increases when $r > 4 \text{ mm}$, and $F_r = 0$ when $r = 15 \text{ mm}$. Therefore, the cell equilibrium position is close to the site $r = 4 \text{ mm}$, suggesting that MagMSCs may be captured and accumulated in the vicinity of $r = 4 \text{ mm}$. (C) through (F), distribution of MSCs. The magnetic cylinder attracted MagMSCs to form a brown ring which was gross in appearance (C) and a signal void ring in cellular MR imaging (D), at the edge of the magnetic cylinder. This ring consisted of numerous cells in layers, and fewer cells were dispersed elsewhere on the culture plate (E). This polarisation of the MSCs was also confirmed by grey value analysis using *ImageJ* software (F).

These results showed that, when guided by a non-uniform magnetic field, SPIO-loaded cells in a static state were effectively attracted and distributed extremely unevenly. The next step was to examine how flowing cells were distributed.

24.3.5. Magnetically guided distribution of MagMSCs in flowing state

Now, it is necessary to turn to the flowing state. A total of 20 ml MagMSCs suspension, at a concentration of 5×10^4 cells mL⁻¹, was placed in a 50 ml syringe and flowed through a quartz tube (ID 2.3 mm, OD 4.3 mm, length 20 mm). This tube was positioned vertically, and the aforementioned magnetic cylinder was placed tightly at the mid-segment of the tube. The flow velocity was set at 1, 10, 30, and 50 mm s⁻¹, controlled by a syringe pump. The capture efficiency (CE) was calculated as previously described [21]. All experiments were performed in triplicate for each condition.

To roughly analyse the distribution of MagMSCs in the tube, the middle part of the tube that was influenced by the magnetic force was imaged digitally and assessed using NIH *ImageJ* software 1.37v (NIH, Bethesda, Md). Briefly, the image was inverted and transformed into a grey picture, and then the signal intensity distribution along the axis of the tube was presented as a plot profile. Signal intensity was calculated as the actual signal intensity in the position of interest minus the noise signal intensity in a remote position 2 cm from the magnet.

As observed in the static cells, the flowing MagMSCs were substantially attracted to the area where the magnetic pole was positioned. Control experiments performed in the absence of an applied magnetic field demonstrated minimal accumulation (data not shown). The accumulation of cells decreased as the average velocity ($\bar{v}$) increased ($r = -0.819$; $P < 0.001$). CE was 91.27 ± 5.93 %, 79.67 ± 4.51 %, 62.07 ± 5.90 % and 44.17 ± 3.40 % when the flow velocity was 1, 10, 30, and 50 mm s⁻¹, respectively (Figures 9A–B).

However, the MagMSCs predominantly accumulated at the edge of the magnetic cylinder, rather than being evenly distributed within the magnetic field. Furthermore, the edge of the magnet at the inflow direction attracted more cells than that at the outflow direction, and the distribution pattern of the MagMSCs was influenced by the flow velocity. This pattern appeared as a single-peak distribution with the peak value at the inflow edge of the magnet when the flow rate was low; a double-peak distribution appeared to have peak values at both the inflow and the outflow edges of the magnet when the flow rate was high (Figures 9C–F).

Figure 9. Magnetically-guided distribution of the MSCs in the flowing state. (A) A schematic illustration of the microfluidic system. The MagMSCs were injected into the tube, and the flow rate was controlled using a syringe pump. Cells were captured by use of an NdFeB magnet located beside the tube. (B) The efficiency of magnetically capturing flowing MagMSCs with different flow velocities. There was a negative relationship between the flowing velocity and the cell capture efficiency. CE denotes capture efficiency. (C) through (E), Representative images and plot profiles of cell accumulation in the photographed segment of the tube. Cells were unevenly distributed at the different velocities of 1 mm s^{-1} (C), 10 mm s^{-1} (D), 30 mm s^{-1} (E) and 50 mm s^{-1} (F).

The distribution pattern of the iron-labelled cells mainly depends on the characteristics of the external magnet applied, and the capturing capacity was

positively related to the magnetic flux density [21]. Influenced by the polarisation phenomenon of the magnet, the cells were accumulated predominantly in densified multi-cellular clusters at the edge of the magnetic cylinder in *in vitro* studies, presenting a “ring” shape and a “single or double-peak” pattern in the static state and in the flow state, respectively. Although three-dimensional multicellular clusters were considered more resistant to hostile environments, the polarisation phenomenon of cell distribution was obviously not what we might wish for. An excessive number of cells concentrated in a very limited space, even in a local point, would increase the risk of vascular occlusion. The more cells that accumulated, the more the risk of embolisation increased. This forms the basis for our concern regarding the risk of vascular embolism *in vivo*.

From our *in vitro* experiment, we can conclude that when the flow is slower, more flowing cells are attracted, which leads to a concentration at the inflow edge of the magnet (a single-peak distribution). Coronary blood flow velocity in the I/R injured myocardium was far lower than that in the normal myocardium, because the former offers conditions that are favourable for cell aggregation and emboli formation.

24.3.6. Proposed mechanisms of cellular magnetic targeting induced-coronary embolisation

Based on these findings, it is logical to assume that the combination of all of these factors (including high working magnetic force, polarisation phenomenon, low coronary velocity, and the magnetically responsive and magnetised cells) promoted excessive cell aggregation and eventually led to the formation of coronary emboli. In addition, it has been reported that both MSCs [40] and iron oxide nanoparticles [41] exhibited a possible pro-coagulant activity. Here, we propose the possible mechanisms by which cellular magnetic targeting induces coronary embolisation (Figure 10).

Figure 10. Proposed mechanisms by which cellular magnetic targeting induces coronary embolisation. (1) Relative large size of MSCs. (2) the iron-labelled MSCs (MagMSCs) is transiently magnetized under an external magnetic field. The inter-attractions among MSCs create an "aggregation begets aggregation" cascading waterfall effect. (3) Directly attracted by a potent magnetic field, the magnetically responsive MSCs are accumulated in vessels in a multi-cellular clusters. Excessive cell aggregation is very likely to develop coronary emboli, especially when a high and polarised magnetic force was applied, and when the coronary flow velocity is low in injured myocardium. (4) The possible pro-coagulation of the iron-labelled MSCs and the free iron released from the MSCs.

24.4. SOLUTIONS TO THE CELLULAR MAGNETIC TARGETING INDUCED-CORONARY EMBOLISATION

24.4.1. Optimise the working magnetic intensity

Our data were inconsistent with those of recent reports, in which the magnetically-enhanced IC delivery of cardiosphere derived cells (CDC) [30] or endothelial progenitor cells (EPC) [36] was safe and improved cell therapy outcomes in a rat model of I/R. No obvious vascular occlusion was noted, although cells in the vessels were detected 24 h after IC delivery in the study by Cheng [30]. It should be noted that intravascular cells may actually be in a pre-translocation state or a coherent state within 24 h of infusion, and may not form cellular emboli, because vascularly-delivered cells were translocated into the parenchyma after 48–72 h [25]. Differences in the size of the donor cells may provide an explanation for this finding. However, we believe that the main reason is the difference in the actual working magnetic intensity. In the study by Cheng [30], the actual working intensity was theoretically estimated to be

less than 0.3 T for a 1.3 T magnet that was placed ~ 1 cm above heart. In the study by Chaudergeur [36], a 0.1 T magnet (7 mm in diameter) was implanted into the subcutaneous layer, leading to an actual working strength that was far less than 0.1 T (The chest wall thickness of adult 150–200 g rats was measured at approximately 3 mm). In our study, the actual working strength was nearly equal to 0.6 T for a 0.6 T magnet that was placed close to the heart.

Recently, we explored the detailed relationship between differing magnetic intensity and targeting efficacy in a rat I/R model [42]. Rat MSCs labelled with SPIOs were injected into the LV cavity of rats during a brief aorta and pulmonary artery occlusion. The 0.15, 0.3, and 0.6 T magnets were placed 0–1 mm above the injured myocardium during and after the injection of 1×10^6 MSCs. Fluorescence imaging and quantitative polymerase chain reaction (PCR) revealed that magnetic targeting enhanced cell retention in the heart at 24 h in a magnetic field strength-dependent manner. Compared with the 0 T group, three magnetic targeting groups enhanced varying cell engraftment at 3 weeks, at which time LV remodelling was maximally attenuated in the 0.3 T groups. Interestingly, due to the low MSCs engraftment resulting from micro-vascular embolisms, the 0.6 T group failed to translate into additional therapeutic outcomes, although it had the highest cell retention. Magnetic targeting enhances cell retention in a magnetic field strength-dependent manner. However, too high a magnetic intensity may result in micro-embolisation and consequently undermine the functional benefits of cell transplantation.

24.4.2. Novel magnetic targeting systems

It is important to develop a homogeneous (*e.g.*, relatively homogeneous magnetic field with optimised magnetic intensity) and conformal (*e.g.*, the similar three-dimensional shape as the targeted area) magnetic targeting system. We designed a very intriguing magnetic pole for magnetic attenuation of cells [21,43]. By arranging the two cylindrical magnetic poles that had holes in the middle, we generated the magnetic flux, as shown in Figure 11, which was a magnetic field with z-axial symmetry in the cylindrical space between the poles. Using this apparatus and flowing the cells slowly through a tube inserted into the hole, MSCs loaded with MNPs were enriched at a controlled distance from the magnetic pole. The amount of cells at the focal point of the magnetic flux depended on the flow velocity and the magnetic flux density. If this spatially-focused feature can be scaled up, it promises a new strategy with which to promote the use of a magnetic field for homing and engraftment in cell therapy. However, the effect of the spatially focused magnetic capture system on cellular coronary embolisation still needs to be investigated.

Figure 11. Experimental apparatus: (A) Magnetic apparatus, (B) its sectional drawing, (C) system of coordinates, and (D) distribution of magnetic intensity

24.4.3. Retrograde coronary venous delivery

With unique access to the ischaemic myocardium, retrograde coronary venous delivery has been demonstrated to provide efficient cell dissemination in the setting of occluded or diffusely narrowed coronary arteries and has subsequently shown functional benefits in both animal and clinical studies [1-6]. Most investigations of magnetically-targeted cell delivery for cardiovascular applications are limited to intramyocardial and antegrade IC routes [15-17]. However, the magnetically-guided cell mass formation may increase the risk of coronary embolism after antegrade delivery [31], especially when relatively large cells such as MSCs (approximately 20–25 μm in diameter) are used. On the contrary, retrograde IC delivery could avoid this complication while allowing safer access to ischaemic myocardium. We introduced magnetically-targeted cellular cardiomyoplasty to the retrograde coronary delivery strategy [44] (Figure 12), and speculated that retrograde coronary administration rather than antegrade coronary infusion may serve as an optimal candidate for cellular magnetic targeting. However, further studies are required to verify our reasoning.

Figure 12. Transjugular cardiac vein retroinfusion in rats. After the ligation of left anterior descending coronary artery, a wire with a properly-bent end (A), and a tube were advanced to the left cardiac vein (B,C).

In conclusion, although magnetic targeting enhanced cell retention in an animal model of I/R, the inherent non-uniformity of a permanent magnetic field, *i.e.*, attenuation along its vertical axis and polarisation along its horizontal axis, could generate unfavourable vascular embolisation of donor cells, limiting its potential to improve therapeutic outcomes. The potential complication should be thoroughly investigated and overcome before clinical application. There is still a long way to go in the translation of cellular magnetic targeting into clinical applications.

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