Ultrasound micromolding of porous polylactide/hydroxyapatite scaffolds

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Abstract. Ultrasound micromolding (USM) preparation of hybrid scaffolds based on polylactide (PLA) and hydroxyapatite (HAp) particles has been evaluated. PLA was stable under the applied ultrasound source since a minimum degradation was detected. Porous materials were achieved using polyethylene glycol (PEG) and NaCl salts to the initial PLA and the subsequent leaching of the micromolded specimens. To avoid cavitation and decomposition problems during micromolding, it was necessary to use HAp free of typical synthesis impurities like carbonate and nitrate compounds. Compact PLA/HAp pieces allowed a maximum HAp load of 60 wt%, while porous specimens could be obtained with a maximum load of 38 wt%. Physical characterization of new scaffolds was performed by X-ray diffraction, spectroscopic and calorimetric techniques, stress-strain tests and contact angle measurements. Results indicated that a degree of porosity of 35% and relatively good mechanical properties could be achieved (*i.e.*, 580 MPa, 4%, and 15.6 MPa for the Young modulus, elongation at break, and tensile strength, respectively). Scaffolds showed the positive effect of HAp and porosity on cell proliferation; this latter was 40% higher than that detected for non-porous PLA specimens.

Keywords: processing technologies, ultrasound micromolding, micropieces, hydroxyapatite, porous scaffolds

1. Introduction

Hybrid materials based on biodegradable polymers are continuously being developed to cover a wide range of biomedical applications. Healing of skin and wounds, reconstruction of bone, cartilages, and muscles, reparation of the nervous system, regeneration of vasculature, and even drug delivery and gene therapy are examples. Probably, the development of new materials for tissue regeneration is one of the most promising areas since concentrates great research efforts. In tissue engineering, it is important to highlight the use of scaffolds to develop biological substitutes that restore or maintain the tissue function.

A biocompatible, biodegradable and cell colonizable material is required for the regeneration of the damaged tissue [1, 2]. Therefore, efforts are focused on developing biocompatible synthetic polymeric systems to build porous matrices that can act as templates for new tissue formation. Matrices can also be seeded with either cells or specific growth factors to enhance proliferation, and/or loaded with fibers and particles such as HAp to increase biocompatibility [3]. HAp is usually selected as a component of biomaterials for being the main inorganic constituent of bones, for enhancing cell proliferation and for having a high bioactivity. HAp is obviously found in nature, but it

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can alternatively be synthesized by precipitation or hydrolysis following well-standardized procedures [4–6]. The use of HAp alone as a ceramic implant is limited because it is extremely brittle with low mechanical strength and fatigue failure [7, 8]. Thus, HAp is usually employed in a wide variety of biomedical applications in the form of composites. PLA is an ideal polymer matrix due to its biocompatibility, relatively low degradation rate, and good mechanical properties. Furthermore, PLA biodegrades into non-toxic products and has been approved by the food and drug administration (FDA) [9–12].

PLA/HAp scaffolds have been widely used in bone repair applications, showing very good osteoconductive and osteoinductive behaviors [13, 14]. These scaffolds have been prepared by several processes such as induced phase separation [15, 16], *in situ* polymerization [17, 18], mechanical dispersion, melt blending [19], *in situ* precipitation, and ultrasound methods [20, 21].

USM is a relatively new processing technique that uses ultrasound as a heating source. Specifically, energy is transferred to the polymer through increased vibration of the molecular chains. This heating process is really accurate and efficient, just melting the amount of material needed. Moreover, ultrasound vibration reduces the polymer viscosity facilitating the material pumping and mold filling [22]. The combination of the accurate energy transfer and dosing systems has made USM suitable for large production of micro-sized specimens with high spatial resolution [23–27]. USM seems ideal for producing pieces for biomedical applications where the added value materials are expensive, and an inefficient cost cycle would bring great economic losses as it may be the case of other alternative processes such as microinjection molding [28].

The efficiency of USM has already been proved for the production of microspecimens based on commodities like polyethylene, polypropylene and polystyrene [29, 30] and biodegradable polymers such as polylactide, polycaprolactone and polybutylene succinate [24, 31]. All these previous studies have concluded that high-quality specimens can be produced after an accurate setting up of the wave amplitude, time, and pumping force molding parameters. USM has also been probed appropriately to produce nanocomposites (*e.g.*, polylactide or poly(ϵ -caprolactone) incorporating nanoclays [32] or carbon nanotubes

[33], respectively), and even specimens with an added pharmacological activity [34].

The present work is focused on the preparation of PLA/HAp composites by USM, taking into consideration the influence of the origin of the inorganic component and evaluating particle distribution and processing parameters. USM preparation of porous PLA/HAp composites is a second goal of the present work that appears fundamental for applications like tissue engineering where cell colonization is required.

2. Experimental

2.1. Materials

Ecorene® NW30 (NatureWorks, Naarden, The Netherlands), a commercial-grade of PLA powder with a number average molecular weight of 43 000 g/mol and a high crystallization degree (*i.e.*, 35%) was employed. CHT ceramic hydroxyapatite type I was provided by Bio-Rad (Hercules, Cal, USA). This HAp was constituted by rather homogeneous particles with sizes smaller than 200 μm . Polyethylene glycol (PEG) (M_w : 100 000 g/mol), sodium chloride (NaCl), and NH_4OH were provided by Sigma-Aldrich (Saint Louis, MO, USA). NaCl was sieved in order to get particles with sizes smaller than 100 μm .

2.2. USM equipment and molding process

The main parts of the USM equipment (Sonorus®, Ultrason S.L., Barcelona, Spain) are schematized in Figure 1. The USM process requires a digital ultrasound generator (1000 W–30 kHz), a controller (3010 DG digital system, Mecasonic, Barcelona, Spain), a focused piezoelectric transducer (Mecasonic, Barcelona, Spain), which converts electrical energy into kinetic energy and creates undulatory movement, and an acoustic unit constituted by a booster and a sonotrode that operates in longitudinal vibration. The first element amplifies or reduces the wave amplitude (0–137.5 μm) while the second transfers vibration energy to the material and provides the pressure/force required to transfer it into the mold. The equipment is also provided with an electric servomotor control (Berneker and Rainer, Barcelona, Spain) fitted with software from Ultrason S.L. (Barcelona, Spain), a little plasticizing camera, and a mold capable of preparing 8 test specimens of dimensions 1.5 cm $\times$ 0.1 cm $\times$ 0.1 cm, and an automatic ejection system.

Figure 1. Scheme showing the main parts of the USM equipment (1: Transducer, 2: Controller and generator, 3: Booster, 4: Sonotrode, 5: Plasticizing camera and 6: Mold and ejectors). An example of a processed specimen is given in the upper left of the Figure and a representation of the molding process (I: Feeding, II: Molding and III: Ejection stages) is illustrated in the lower part.

Each micromolding experiment was performed with around 380 mg of powdered material that was loaded directly to the feeding/plasticizing camera of the USM equipment. When mixtures were employed (*e.g.*, those involving PLA, NaCl, PEG and/or HAp), the appropriate amount of each powdered component was externally mixed. Samples were processed with the optimized molding parameters (amplitude, force, and time) that were kept as close as possible to those already determined for neat PLA in previous works [46]. In the case of porous specimens (scaffolds), a subsequent wash for 24 h was needed to remove the added water-soluble NaCl and PEG components.

The complete molding process can be described by three steps. In the first one the mold is closed and the sonotrode in the feeding position (stage I in Figure 1). Then, the powdered sample (*i.e.*, PLA or the different mixtures with the added compounds) is loaded into the plasticizing camera. The second step is the main one and consists of three substages: a) the sonotrode firstly compacts the powdered mixture

applying the selected force and acting as a piston (stage II-a in Figure 1), b) the sonotrode begins to vibrate according to the selected amplitude that is regulated by the booster. The polymer begins to flow and fill the mold cavities (stage II-b), c) after the selected time set up, the sonotrode vibration stops, and the mold is cooled down, while the sample is still compacted by the sonotrode (stage II-c). Finally, the mold is opened, the sonotrode returns to the feeding position, and the sample is released from the mold by the ejection system (stage III).

Processed samples will be named, indicating the amount of each component if it is different from zero. Thus, PLA-40/PEG-10/NaCl-25/HAp-25 means an initial mixture having 40, 10, 25, and 25 wt% of PLA, PEG, NaCl, and HAp, respectively.

2.3. Synthesis of hydroxyapatite

HAp was synthesized by precipitation at room temperature following the procedure described by Zhang and Lu [5]. 500 mM of $\text{Ca}(\text{NO}_3)_2 \cdot \text{H}_2\text{O}$ in ethanol and 500 mM of NH_4PO_4 in water ($\text{pH} < 10$) were vigorously stirred at room temperature for 1 h. Reagent volumes were determined to get a Ca/P ratio of 1.67, which is the typical value for natural HAp [35]. The pH of the medium was adjusted by adding NH_4OH . The mixture was kept at 37 °C for 24 h, and after that, the product was washed with milli-Q water and centrifuged four times at 5000 rpm. The precipitated extract was frozen overnight at –80 °C, and then it was dried at low temperature for 48 h. The HAp obtained was ground and filtered.

2.4. Leaching of NaCl: mass extraction yield and porosity calculations

NaCl extraction was calculated by measuring the conductivity of the washing water with an SX-650 Sanxin electrical conductivity meter (0–50 mS/cm). Calibration was performed with standard NaCl solutions at 21 °C. Values were averaged from three measurements. The weight of the removed salt, W_r , was calculated according to Equation (1):

$$W_r = \frac{V \cdot (\sigma - b)}{a} \quad (1)$$

where V is the volume of washing water [ml], σ is the conductivity [S/m], and a and b calibration constants.

The yield of extracted salt, r_{NaCl} , was then determined as Equation (2):

$$r_{\text{NaCl}} [\%] = 100 \cdot \frac{W_i - W_r}{W_i} \quad (2)$$

where W_i is the theoretical weight of salt loaded in the specimen.

The weight percentage of extracted sample (*i.e.*, salt plus PEG), W_e , was calculated from the theoretical weight of NaCl and PEG in the specimen, W_{it} , and the weights of the specimen before being washed, W_0 , and once the washed specimen was dried, W_d (Equation (3)):

$$W_e [\%] = 100 \cdot \frac{W_0 - W_d}{W_{it}} \quad (3)$$

The porosity of each specimen, P , was finally estimated, according to Equation (4):

$$P [\%] = 100 \cdot \frac{\frac{W_0 - W_w}{\delta_w}}{\sum \frac{W_i}{\delta_i}} \quad (4)$$

where W_w is the weight of the wet specimen after being washed, W_i is the loaded weight of each component (*i.e.*, PLA, PEG, and HAp), δ_w is the water density, and δ_i the density of each component.

2.5. Specimen characterization

Molecular weight was determined by gel permeation chromatography (GPC) using a liquid chromatograph (Shimadzu, model LC-8A, Tokyo, Japan) equipped with an Empower computer program (Waters, Milford, MA, USA). The polymer was dissolved and eluted in 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) containing CF_3COONa (0.05 M). The flow rate was 1 mol/min, the injected volume 20 μl , and the sample concentration 2 mg/ml. A PL HFIP gel column (Polymer Lab, Agilent Technologies Deutschland GmbH, Böblingen, Germany) and a refractive index detector (Shimadzu RID-10A, model LC-8A, Tokyo, Japan) were employed. The number and weight average molecular weights were determined using polymethyl methacrylate standards.

Infrared spectra (ATR-FTIR) were recorded at a resolution of 4 cm^{-1} , with a Fourier transform FTIR 4100 Jasco spectrometer (Tokyo, Japan) dotted with a Specac MKII Golden Gate Single Reflection Diamond ATR system (Jasco International, Tokyo, Japan).

Scanning electron microscopy (SEM) images of micromolded specimens were taken by using a Focused Ion Beam Zeiss Neon40 microscope (Zeiss,

Oberkochen, Germany) operating at 5 kV. Samples were mounted on a double-side adhesive carbon disc and sputter-coated with a thin layer of carbon by using a Mitec K950 Sputter Coater (Quorum Technologies Ltd., Ashford, UK).

Contact angles (CA) were measured at room temperature with sessile drops using an OCA-15 plus Contact Angle Microscope (Dataphysics, USA) and SCA20 software. Contact angle values of the right and left sides of distilled water drops were measured and averaged. Measurements were performed 10 s after the drop (0.5 μl) was deposited on the sample surface. All CA data were an average of six measurements on different surface locations.

Calorimetric data were obtained by differential scanning calorimetry with a TA Instruments Q100 series (TA instruments, New Castle, DE, USA) with Tzero technology and equipped with a refrigerated cooling system (RCS, TA instruments, New Castle, DE, USA). Experiments were conducted at a heating rate of 10 $^{\circ}\text{C}/\text{min}$ and under a flow of dry nitrogen. The sample weight was approximately 5 mg, and calibration was performed with indium.

Thermogravimetric analysis (TGA) and differential thermogravimetric analysis (DTGA) were performed with a Q50 thermogravimetric analyzer of TA Instruments (New Castle, DE, USA) under a flow of dry nitrogen with approximately 5 mg samples and at a heating rate of 10 $^{\circ}\text{C}/\text{min}$.

Real-time synchrotron studies at variable temperatures were carried out on beamline BL11-NCD at ALBA Synchrotron (Cerdanyola del Vallès, Barcelona, Spain) by using a wavelength of 0.100 nm and a WAXD LX255-HS detector from Rayonix. Polymer samples were confined between Kapton films and then held on a Linkam hot stage (Tadworth, UK) with temperature control within ± 0.1 $^{\circ}\text{C}$. WAXD profiles were acquired during the heating runs in time frames of 20 s and a rate of 10 $^{\circ}\text{C}/\text{min}$. WAXD diffraction patterns were calibrated by means of a geometrical calibration process of a well-known sample (standard Cr_2O_3). Diffraction profiles were normalized to the beam intensity and corrected, considering the empty sample background.

2.6. Cell proliferation studies

MDCK cells (Madin-Darby Canine Kidney epithelial-like cell line) (American Tissue Cell Culture, ATCC, USA) were cultured in Dulbecco's modified Eagle's medium (*i.e.*, DMEM with 4500 mg/l of

glucose, 110 mg/l of sodium pyruvate, and 2 mM of L-glutamine) supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin, 50 mg/ml streptomycin and 2 mM L-glutamine at 37 °C in a 10% humidified atmosphere of 5% CO₂ and 95% air. Culture media were changed every two days. For subculture, cell monolayers were rinsed with PBS and detached by incubating them with 0.25% trypsin/EDTA for 25 min at 37 °C. The incubation was stopped by resuspending in 5 ml of fresh medium. Cell concentration was determined by counting with a Neubauer camera (Sigma-Aldrich, Saint Louis, MO, USA) and using 4% trypan blue as dye vital.

PLA/HAp scaffold, PLA scaffold, and PLA non-porous sample were placed in polyester plates, sterilized, exposed to UV light for 15 min, and finally treated for cell culture. 100 µl containing 5 · 10⁴ cells were deposited in the plate and incubated for 60 minutes. Then, 1 ml of culture media was added to each plate. Quantification of proliferated cells was performed after one week.

Percentages of proliferated cells were calculated through the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [36]. This determines the ability of the mitochondrial dehydrogenase enzyme of viable cells to cleave the tetrazolium rings of MTT and form formazan crystals that are accumulated in healthy cells. This process is detected and can be quantified through a color change: the characteristic pale yellow of MTT transforms into the dark blue of formazan crystals. Specifically, 50 µl of MTT (3 mg/ml) was added after a week to the culture plates, which were further incubated for 4 h. Samples were subsequently washed twice with Phosphate-Buffered Saline (PBS; 0.1 M, pH 7.4: 80 g NaCl, 2.0 g KCl, 21.7 g Na₂HPO₄ · 7H₂O, 2.59 g KH₂PO₄) and deposited in a new plate. Then, 1 ml of dimethyl sulfoxide (DMSO) was added, and the absorbance was measured at 540 nm in Elisa EZ-Read 400 (Biochrom, Cambridge, UK) after 15 min of gently stirring. Values were averaged according to three measured replicas. Controls were conducted by cell culture on the wells without any material. The statistical analysis was performed by one-way ANOVA, followed by Tukey test with a confidence level of 95% ($p < 0.05$).

To obtain representative fluorescence images of samples coming from the proliferation assay, samples were fixed in glutaraldehyde (2.5% in PBS) overnight at 4 °C, and then dehydrated by extensive washing in

an alcohol battery. Finally, samples were dried in air and sequentially stained with green-fluorescent Alexa Fluor Atto-488 phalloidin dye and 4',6-diamidino-2-phenylindole (DAPI) for labeling actin and nucleus, respectively. The images were obtained with a confocal laser scanning microscope (LSM 900 Zeiss) controlled by ZEN 2.6 software (blue edition) (Carl-Zeiss Microscopy GmbH, Jena, Germany).

3. Results and discussion

3.1. Ultrasound micromolding of PLA/HAp composites

The ability to prepare uniform PLA/HAp composites by means of the USM technique has been explored considering the HAp type (*i.e.*, commercial or synthesized), the particle size, and the maximum allowed load.

The starting USM processing parameters were those previously determined for the commercial PLA powder sample: amplitude, force, and time of 24 µm, 300 N, and 1.2 s. Basically, previous studies indicated that lower amplitudes were not enough to get an appropriate PLA flow and that 300 N was the minimum force that allowed to fill the mold cavities completely. Forces higher than 500 N caused an overloading of the sonotrode (*i.e.*, it could not vibrate as a consequence of the high compaction of PLA). Processing time of only 1.2 s was enough and therefore was selected for all experiments since a slight degradation was noticed for times higher than 3 s. Table 1 summarizes representative GPC data for the assays performed with PLA. Note that a minimum variation of M_n from 43 000 to 38 800 g/mol (*i.e.*, less than 10%) was determined when PLA was processed under the indicated optimal conditions.

Table 1. Influence of USM processing parameters on the molecular weight of polylactide specimens.

Molding parameters ^a	Mold filling ^b	M_n [g/mol]	M_w [g/mol]
Commercial sample	–	43 000	69 100
24-200-1.5	40%	35 400	69 700
24-300-1.2	100%	38 800	72 200
24-300-3.0	100%	32 200	64 300
24-400-1.2	100%	37 700	71 300
24-500-1.2	–Ovl–	33 900	67 500
24-600-1.2	–Ovl–	–	–

^aAmplitude [µm]-Force [N]-Time [s].

^bQualitative evaluation (*e.g.*, 8 half specimens or 4 complete specimens correspond to 50%). Sonotrode overload is indicated as –Ovl–.

Figure 2. Molded specimens, with synthesized (a, b) and commercial-grade HAp (c). HAp contents were 20 and 60 wt% for synthesized and commercial particles, respectively. Images of processed specimens incorporating synthesized HAp were obtained with sieved granules in the 100–80 μm size range. Proximal and distal parts of a given specimen are indicated in (c).

PLA composites were not able to be well-molded when the synthesized HAp was employed. For a load of 50 wt% the equipment only compacted the powdered mixture, which therefore could not properly flow and fill the mold. Results progressively improved when the load decreased down to 30 wt%. The fluidization of the material was achieved when the HAp amount was decreased to 20 wt%, but the whole mold could still not be completely filled, and some of the final specimens resulted in being uncompleted despite the fact that the force was increased up to 400 N (Figure 2).

Synthesized HAp corresponds to nanometric crystals that, unfortunately, become agglomerated (Figure 3), giving rise to granules with a broad size distribution. These granules could be sieved into fractions with sizes of 250–125, 100–80, 80–45, and <45 μm , it is, therefore, possible to evaluate the effect of the particle size on the micromolding effectivity. Note that the high dimensions of aggregates may difficult the correct flow of the mixture towards the mold when the content of the inorganic component is high.

The indicated fractions of synthetic HAp were subsequently used in USM experiments that were carried out with a HAp load of 20 wt% and processing parameters of 24 μm , 400 N, and 1.2 s. The main observed effect was a lower capacity of HAp to load the mold when the particle size increased. Namely, HAp preferably remained in the sprue for increasing sizes. This heterogeneous distribution could be easily evaluated through density measurements of representative parts (*i.e.*, sprue and specimen). Thus, densities determined by the flotation method using

Figure 3. SEM micrographs, showing typical morphologies of synthesized (a) and commercial (b) HAp crystals.

water and a KBr saturated aqueous solution were 1.81 g/ml (sprue) and 1.41 g/ml (specimen) when the 250–125 μm fraction was employed, 1.72 g/ml (sprue) and 1.52 g/ml (specimen) for the 100–80 μm fraction, 1.69 g/ml (sprue) and 1.55 g/ml (specimen) for the 80–45 μm fraction, and 1.66 and 1.58 g/ml

for the $<45\ \mu\text{m}$ fraction. Note that a value close to 1.62 g/ml should be expected for a sample with a HAp load of 20 wt% considering theoretical densities of 1.24 and 3.16 g/ml for PLA and HAp, respectively.

The inspection of processed samples (Figure 2) indicated that the sprue was plenty of bubbles; the material had a slight brown coloration and was very brittle. Observations pointed out the possibility of gas formation, cavitation phenomena, and degradation. Therefore, the use of synthetic particles was discarded for the rest of the experiments.

USM of mixtures with commercial HAp was completely different since good results were attained even for a high load of 60 wt%. In all cases, the applied force should be 400 N in order to get a perfect specimen (using amplitude and time of 24 μm , and 1.2 s, respectively). Lower forces were inefficient to fill the mold completely even when the HAp content was reduced to 10 wt%. Figure 2 shows that eight perfect specimens were obtained for the maximum load. Furthermore, no coloration and degradation evidence could be observed. In addition, cavitation phenomena were not detected since bubbles were absent.

The morphology of commercial HAp particles was completely different from that observed for the synthesized sample, as shown in Figure 3. In this case, HAp crystals had a regular sheet-like morphology with its larger dimension close to 10 μm . The brittle character of HAp caused some breakage of crystals during preparation, as detected in Figure 3. Nevertheless, the commercial HAp sample was rather homogeneous in size according to the weight of the different sieved fractions, and consequently, the effect of the particle dimensions on the distribution inside the molded specimen was not evaluated. Density measurements pointed out again a slight difference between sprue and specimen, which become more significant as the HAp load increased. Thus, densities of 2.46 g/ml (sprue) and 2.27 g/ml (specimen) and 1.64 g/ml (sprue) and 1.60 g/ml (specimen) were found for loads of 60 and 20 wt%, respectively. Samples with an intermediate load of 40 wt% could already be considered practically homogeneous with densities of 2.23 g/ml (sprue) and 2.12 g/ml (specimen). Homogeneity inside the specimen was also evaluated by analyzing two half parts of each specimen: proximal (P) and distal (D) parts along the flow direction (Figure 2c). In this case, minimum

differences were found, and for example, density values of 2.10 g/ml (distal) and 2.14 g/ml (proximal) were determined for the sample having 40 wt% of HAp. Finally, it can be pointed out that similar particle distribution was found for samples having a 20 wt% load of synthetic (*i.e.*, densities of 1.66 g/ml (sprue) and 1.58 g/ml (specimen) for the $<45\ \mu\text{m}$ fraction) and commercial (*i.e.*, densities of 1.64 g/ml (sprue) and 1.60 g/ml (specimen)) particles. Therefore, USM seems a suitable process to get a homogeneous particle dispersion even for high loads and particle sizes in the micrometer range.

SEM micrographs of processed specimens (Figure 4) clearly revealed the presence of HAp particles inside the PLA continuous matrix with a rather homogeneous distribution in the inner cross-sections and an irregular distribution on the specimen surface.

FTIR analysis allowed explaining the negative effect of synthesized HAp in comparison with the commercial-grade sample. Clear differences can be observed between both spectra, as shown in Figure 5.

Thus, the commercial-grade sample had a high purity, and only typical bands for the phosphate compound at 1090, 1020, 960 cm^{-1} were detected [35, 37, 38]. By contrast, some other minor bands (see blue ellipsoids) were observed in the synthesized sample. Note the bands around 1740–1600 cm^{-1} that can be associated with carbonyl groups of carbonated HAp [39]. Furthermore, the band at 1360 cm^{-1} can be related to residual nitrates. The CO_2 formation caused by the degradation of carbonate by ultrasound heating leads to internal cracks, enclosed bubbles, and poor heat conductivity. The presence of nitrates also negatively affected the characteristics of the molded specimen since when heated, burned and colored the sample.

In summary, the use of synthesized HAp requires exhaustive cleaning and purification steps in order to avoid the presence of carbonated or nitrates byproducts. Despite the fact that HAp nanocrystals are highly interesting for biomedical applications, the rest of the study is performed with the commercial sample to avoid the effect of impurities. Finally, a careful drying is essential to reduce the moisture absorbed when hygroscopic salts are added, as will be mentioned later in the section concerning the preparation of porous scaffolds. Moisture can render bubbles and lead to a cavitation phenomenon as also observed for some samples (not shown).

Figure 4. SEM micrographs showing the surface (a, c) and inner cross-sections (b, d) of a PLA micromolded specimen having 40 wt% of commercial HAp. Low magnification images allowed to determine the type of distribution (homogeneous or irregular), while the platelet-like morphology of the incorporated HAp particles could be clearly observed in the high magnification images.

Figure 5. FTIR spectrum of synthesized HAp (red line) and commercial HAp (black line).

3.2. Characterization of PLA/HAp micromolded specimens

Micromolded composite specimens were characterized considering the GPC molecular weight, thermal

properties, crystalline structure, and mechanical properties.

Figure 6 compares the GPC curves of the commercial PLA powder, the processed PLA specimen, and the PLA-HAp composite specimen having the maximum load (*i.e.*, 60 wt%). Specimens were processed using the above indicated optimal parameters (*i.e.*, 24 μm , 300 N, and 1.2 s for PLA and 24 μm , 400 N, and 1.2 s for the composite). Processing of PLA leads to a small increase of the tail of the curve corresponding to the smallest molecular weight fraction, and therefore, minor evidence of degradation could be detected through the M_n values. Specifically, M_w and M_n molecular weights were 69 100 and 43 000 g/mol for the commercial sample and 72,200 and 38 800 g/mol for the processed PLA with a force of 300 N. The incorporation of the commercial and carefully dried HAp lead also to a scarce degradation, which could be mainly attributed to the increase of the applied force [34]. In this case, M_w and M_n

Figure 6. GPC chromatograms of commercial PLA powder, micromolded PLA and PLA-40/HAp-60. Processing parameters were 24 μm , 1.2 s and 300 N (PLA) and 24 μm , 1.2 s and 400 N (PLA/HAp).

values decreased to 69 700 and 35 900 g/mol. It should also be indicated that for PLA alone, M_n decreased to 37 700 g/mol when the processing force was increased to 400 N. The incorporation of a ratio of HAp so high as 60 wt% had, therefore, a minimum influence on degradation.

The incorporation of HAp had a clear nucleation effect; thus, the cold crystallization exothermic peak of PLA/HAp composite shifted to a lower temperature (Figure 7). This behavior is not proportional to the HAp amount, at least in the high value range between 20 and 60 wt%, where all PLA/HAp nanocomposites showed a cold crystallization peak 10 °C lower than that observed for neat PLA (*i.e.*, 103.8 °C with respect to 113.8 °C). In addition, the melting enthalpy of the composite increased if the real amount of PLA is taken into account (*i.e.*, 25.9 J/g become

Figure 7. DSC heating runs of USM specimens of PLA and the representative PLA-60/HAp-40 composite.

43.2 J/g for a sample containing only 60 wt% of PLA, a higher value than the enthalpy of 39.1 J/g determined for the neat PLA). Samples showed minimum differences between their T_g s and the enthalpic relaxation peaks. It is interesting to note that after processing PLA and PLA/HAp-40 remained practically amorphous since cold crystallization and melting (169 °C) peaks had a similar enthalpy (*i.e.*, 39 J/g for neat PLA and 26 J/g for PLA/HAp-40).

Dynamic WAXD profiles were also recorded during the heating run of USM specimens (Figures 8a and 8b). An amorphous profile was recorded for the PLA specimen at room temperature, but around 104 °C, the cold crystallization of PLA started as evidenced by the apparition of α -form characteristic peaks [40, 41] at scattering vector ($q = 2\pi/d$, d being the Bragg spacing) values of 11.5, 13.3 and 15.5 nm^{-1} (*i.e.*, Bragg spacings of 0.546, 0.472 and 0.405 nm that correspond to (200)+(110), (203) and (015) reflections, respectively). These peaks started to reach the maximum intensity around 125 °C (see Figure 8c). Then a partial melting of defective lamellae took place before a complete fusion that ended around 180 °C. The behavior of the composite was completely similar except by the presence of the crystalline HAp peak at 18.15 nm^{-1} (Bragg spacing of 0.34 nm) [4, 37]. The main difference between PLA reflections of the processed specimens concern to the relative intensity of diffraction peaks and even to their broadness (Figure 8d), which suggests the development of smaller PLA crystals in the composite as presumably from its higher nucleation density.

Incorporation of HAp improved mechanical properties of the composite in terms on the Young modulus, which logically increased due to the higher global crystallinity of the sample and the higher modulus of the HAp component. Thus, values of 847, 800, 780 and 754 MPa were evaluated for specimens with HAp loads of 60, 40, 35 and 20 wt%, respectively, while a modulus of 707 MPa was measured for USM PLA. On the contrary, the elongation at break slightly decreased as a consequence of the lower cohesion between PLA and HAp (*i.e.*, from 11.4% for neat PLA to 11.0, 10 and 9.5% for composites with HAp content of 20, 40, and 60 wt%, respectively). The combination of the two factors led to a practically constant maximum tensile strength (*i.e.*, 58 MPa for PLA and 62, 60, and 59 MPa for composites with HAp contents of 60, 40, and 20 wt%, respectively). For the sake of clarity, Figure 9 compares the stress-strain

Figure 8. Three-dimensional representation of WAXD profiles of USM PLA (a) and PLA-60/HAp-40 (b) specimens. c) Intensity evolution of the main PLA peak during heating (10 °C/min) from room temperature to fusion. d) Comparison of WAXD profiles recorded at 125 °C of PLA and PLA-60/HAp-40 specimens. Arrow points out the reflection corresponding to the crystalline HAp.

Figure 9. Stress-strain plots obtained from the representative indicated samples. PLA-40/PEG-10/HAp-25/NaCl-25 (PLA/HAp scaffold) is also added for comparison purposes.

profiles of micromolded PLA and PLA-60/HAp-40 specimens.

The incorporation of HAp slightly increased the hydrophilicity of the processed specimen., which is a positive effect to promote cell growth. Thus, the contact angle determined for the USM PLA specimen decreased to 63° and 69° for composites having HAp contents of 60 and 20 wt%, respectively (Figure 10).

3.3. Preparation of porous PLA/HAp scaffolds by ultrasound micromolding

The salt leaching technique has been employed in order to get porous PLA/HAp specimens. To this end, NaCl and PEG have been incorporated in the initial mixture before processing. This procedure has recently been reported as a highly effective method to provide porosity in USM specimens [42]. Therefore, NaCl particles were sieved to get a homogeneous distribution with a small average size that facilitated the solubilization process, and that was appropriate for the subsequent cell colonization. A certain ratio of water-soluble PEG was necessary to increase the hydrophilicity of the specimen and make feasible

Figure 10. From left to right: water drop contact angle on PLA, PLA-80/HAp-20, PLA-40/HAp-60 and PLA-40/PEG-10/HAp-25/NaCl-25 surfaces.

both the entrance of water inside the processed specimen and the salt removal. To get a significant porosity, USM was carried out using a mixture of PLA, PEG, NaCl, and HAp powdered samples of 40, 10, 25, and 25 wt%, respectively. Note that the final scaffolds should have a HAp content close to a 40 wt% (*i.e.*, 38 wt%).

Processing parameters could be kept at the above-selected values of 24 μm , 400 N and 1.2 s since molds were completely filled, and specimens without coloration or other apparent degradation signals were obtained. In fact, GPC curves (not shown) of the processed specimens and after NaCl and PEG solubilization allowed estimating M_n and M_w values of 67 100 and 39 200 g/mol, respectively. SEM

Figure 11. SEM micrographs showing the inner cross sections of porous PLA/HAP scaffolds obtained by USM and subsequent washing (24 h) of PLA-50/NaCl-25/HAp-25 (a) and PLA-40/PEG-10/NaCl-25/HAp-25 (b) specimens. Blue circles pointed out the presence of some NaCl particles.

micrographs (Figure 11) of specimen cross-sections revealed the high porosity of the final scaffold and the absence of white and geometrically regular salt particles, which by contrast, were still detectable when PEG was not incorporated (Figure 11). In this case, perfect porous interconnectivity was not attained, and therefore, some salt particles were not accessible to water.

3.4. Characterization of PLA/HAP micromolded porous scaffolds

Table 2 summarizes the results attained about weight percentages of total mass extraction (*i.e.*, NaCl + PEG), NaCl extraction, and porosity of representative scaffolds after the leaching process in water for 24 h. For comparison purposes, values of PLA/NaCl-40 and PLA/PEG-10/NaCl-40 specimens from previous works were also added [42]. These two samples revealed the essential role of PEG to enhance NaCl extraction. Note that the extraction of NaCl was only 40% when PEG was not present, whereas the addition of a small PEG percentage allowed getting a NaCl extraction close to 100%. Moreover, as a consequence of this successful extraction, the porosity degree could be duplicated and attained a value close to 40%.

Looking at the homologous samples loaded with HAp, a similar trend was observed: Incorporation of PEG increased the NaCl extraction yield as well as the porosity percentage.

Mechanical properties of the porous scaffold obviously decreased with respect to those determined for the solid PLA/HAP specimen having a similar composition (*i.e.*, 38.4–40 wt% of HAp). Specifically,

Table 2. Mass and NaCl extraction percentages and degree of porosity of representative USM specimens.

Sample ^a	Mass extraction [%]	NaCl extraction [%]	Porosity [%]
PLA-60/NaCl-40	40 $\pm$ 8	38 $\pm$ 3	20 $\pm$ 5
PLA-50/PEG-10/NaCl-40 ^b	98 $\pm$ 6	98 $\pm$ 7	39 $\pm$ 3
PLA-50/PEG-10/HAp-40 ^b	100 $\pm$ 2	—	16 $\pm$ 6
PLA-50/NaCl-25/HAp-25	53 $\pm$ 6	41 $\pm$ 3	18 $\pm$ 4
PLA-40/PEG-10/NaCl-25/HAp-25	96 $\pm$ 13	92 $\pm$ 11	35 $\pm$ 4

^aThe number after each component corresponds to the loaded wt% in the feeding camera.

^bFrom reference [42].

Young modulus, elongation at break, and tensile strength decreased to 580 MPa, 4%, and 15.6 MPa, respectively (Figure 9).

Contact angle measurements showed a change from a value of 30° determined for the processed specimen before the leaching process (Figure 10) to 60° after extraction of PEG and NaCl. Note that this angle is slightly lower than measured for the PLA/HAp scaffold having 40 wt% of HAp (*i.e.*, 70°). The difference suggests the presence of some residual NaCl since probably the increase of the specimen surface roughness and even the presence of voids should lead to a slight contact angle increase.

Figure 12 compares the TGA and DTGA curves of the USM PLA and PLA-40/PEG-10/NaCl-25/HAp-25 specimens after PEG and salt extraction. HAp provided a slight thermal stabilization and increased the onset decomposition temperature (*i.e.*, from 345 to 362 °C) as well the DTGA peak temperature (*i.e.*, from 368 to 384 °C). The residue detected at 550 °C was 29.2%, a value in relatively good agreement with the loaded amount of HAp (25 wt%). Nevertheless, the extraction of NaCl was uncomplete, being the NaCl extraction percentage (84%) slightly worse than that evaluated by conductivity measurements.

Figure 12. TGA (solid lines) and DTGA (dashed lines) profiles of neat PLA and the porous PLA/HAp scaffold prepared by USM.

The incorporation of HAp in materials used for different biomedical applications has an interest in order to improve biocompatibility and favor cell viability [1–3]. Particular attention merits the enhancement of the growth of bone tissue cells by biomineralization [13, 14]. The advantages of HAp incorporation are additive to the porous character of the final scaffold.

Figure 13 compares the growth of MDCK cells onto specimens of neat PLA and representative scaffolds

Figure 13. a) Bar graphic showing the relative cell proliferation determined at 7 days of culture for USM specimens of the indicated representative specimens. Asterisks indicate $p < 0.05$ vs PLA scaffold (ANOVA-Tukey test). Yellow box indicates mean $\pm$ SD for the control. Fluorescence micrographs showing the cell grown are also given in the right part for non-porous PLA (b) and porous PLA/HAp (*i.e.*, from PLA-40/PEG-10/NaCl-25/HAp-25) (c). The cells were stained for nuclei (DAPI, blue) and actin (phalloidin, green).

with and without HAp. Results pointed out the absence of cytotoxicity and the good biocompatibility of PLA since cell viability was like the control (yellow line). However, a significative higher relative cell viability ($p < 0.05$) was found for the porous samples as a consequence of their higher adhesion area. Despite the greatest improvement observed for porous specimens, it is also clear that the addition of HAp was beneficial, and a small increase in cell viability was detected. Therefore, cell proliferation increased by 45 and 30% with respect to the control for PLA scaffolds with and without HAp. Figure 13 also compares the relative viability on the specimen and onto the well once the specimen was removed. Results attained with the well were similar, as expected, to those found with the control. Fluorescence micrographs showed the formation of a uniform cell monolayer onto the surface of the PLA specimen. However, cells in the porous scaffold can penetrate the porous surface and fill the voids of the sample, as evidenced by their growth in different planes.

4. Conclusions

Hybrid scaffolds of polylactide and hydroxyapatite can successfully be obtained through a new micro-molding technology based on the application of ultrasound as the energy source. Specimens showed a minimum degradation and were obtained with a processing time so low as 1.2 s and a minimum material loss. Micropieces showed a relatively homogeneous distribution of HAp particles except for their surfaces. The maximum amount of HAp that could be loaded keeping a correct fluency in the feeding channels was 60 wt%, a content that rendered materials with higher mechanical properties than non-loaded specimens (e.g., Young modulus of 847 MPa in front of 707 MPa). PLA was practically amorphous after processing, although micro pieces could subsequently undergo cold crystallization. The quality of HAp was fundamental to get specimens without degradation and coloration as a consequence of cavitation phenomena induced by the decomposition of synthesis byproducts such as carbonate and nitrate salts. Porous scaffolds of PLA/HAp could also be prepared by adding water-soluble compounds (e.g., polyethylene glycol and NaCl salts) in the feeding mixture and performing a subsequent leaching process. The HAp content should be reduced to 38 wt% to

get materials with a porosity of 35%. Young modulus and tensile strength logically decreased with respect to the non-porous PLA samples, but acceptable values of 580 and 15.6 MPa were attained. The presence of HAp provided thermal stability and allowed to increase the hydrophilicity, an interesting feature for biological applications. In the same way, it was found that both porosity and presence of HAp particles favored cell colonization and specifically, an increase of the proliferation of assayed MDCK cells around 40% with respect to the control was found.

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