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In vitro degradation of PLCL/nHA biodegradable scaffolds

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ABSTRACT

We investigate the effect of bioactive nanoparticles on the *in-vitro* degradation of PLCL and PLCL/nHA composite scaffolds. The concentration of nanohydroxyapatite significantly affected the degradation rate. An increase in the crystallinity of the amorphous portion of the polymer was observed. This increased crystallinity was more pronounced in the pure PLCL samples than in those with more nHA. During the degradation process, we observed the appearance of multiple micropores on the scaffold walls as the hydrolysis process progressed and, by the sixth week, the remains of the degradation products were visible on the pore walls.

Keywords: biodegradable polymer, nanohydroxyapatite, scaffolds, pH, in vitro, degradation.

INTRODUCTION

Tissue engineering is an interdisciplinary research field that includes all processes involved in the design and the production of biological substitutes so that damaged and necrotic tissues may be replaced, regenerated and improved, in order to restore their normal biological functions.

Among some of the key factors in tissue engineering are the creation of suitable scaffolds that can provide initial mechanical support, with adequate surface properties to permit cell adhesion and three dimensional and interconnected porous structures that

can transport sufficient amounts of gases, and nutrients, as well as regulatory factors that promote cell proliferation and differentiation. The scaffold is supposed to mimic a natural extracellular matrix and should be biocompatible and biodegradable. Additionally, it should be elastic and capable of withstanding cyclic mechanical strain without cracking or suffering significant permanent deformation [1,2].

Scaffold biodegradability is critical and should ideally match the formation of the new tissues, which will assist with the progressive substitution of the scaffolding by the extracellular matrices, so that the overall structural integrity of the biological system is maintained, while the degree of inflammation or toxicity is reduced to a minimum. It is believed that the ideal in vivo degradation rate may be similar or slightly less than the tissue growth rate. At the end of the process, the scaffold should have been completely degraded and resorbed [3].

Research has been conducted into both natural and synthetic materials for use as tissue engineering scaffolds. Among these materials, polymers offer the unique properties of high surface-to-volume ratios, high porosity with very small pore sizes, controllable biodegradability and good mechanical behaviour. Aliphatic polyesters can be considered representative of synthetic biodegradable polymers, polycaprolactone (PCL), polyglicolide (PGA), polylactide (PLA), and their copolymers have attracted considerable attention, due to their degradability, biocompatibility and processability. They have also been approved by the US Food and Drug Administration (FDA) for a wide range of applications in the biomedical field. Poly(lactide-co-ε-caprolactone) (PLCL), has demonstrated not only good elastic and biodegradable characteristics but also good cellular interaction [4-6].

Besides, these polymers are considered non-osteoconductive. The addition of bioactive particles such as hydroxyapatite (HA) to form composite scaffolds has been reported to enhance mechanical and biological properties, reinforcing the scaffolds and making them osteoconductive. In addition, the use of nano-sized HA may have other special properties, due to their small size and huge specific surface area.

A proper understanding of polymer degradation kinetics is of major importance for the manufacture of any implantable medical device, but there are relatively few results on the effect of nanoparticules such as nHA on the ageing and degradation behaviour of PLCL[7-9]. In this study, thermal-induced phase-separation (lyophilisation) was employed to prepare PLCL scaffolds and PLCL/nHA composite scaffolds. The objective of this investigation is to study *in-vitro* degradation of porous PLCL/nHA composites for use as scaffolds in bone engineering. We set out to characterize the *in-vitro* degradation mechanism of this device in an aqueous media (PBS). For this purpose, we used Fourier Transform Infrared Spectroscopy (FTIR), Differential Scanning Calorimetry (DSC), Scanning Electron Microscopy (SEM) and Gel Permeation chromatography **(**GPC), which have previously demonstrated their utility in these kinds of studies.

MATERIALS AND METHODS

Raw materials

Medical grade Poly L-lactide-co-ε-Caprolactone (PLCL) with a comonomer ratio of 70/30 was purchased from Purac Biomaterials (PURASORB PLC 7015, Netherlands), and purified by dissolution in chloroform. The weight-average relative molecular weight Mw=130489.8, Mn=79759.7, and polydispersity Mw/Mn =1.63 of PLCL were determined with GPC (GPC, Perkin Elmer 200) in tetrahydrofuran (THF). GPC was performed with a THF solvent using a Perkin Elmer series 200 reflective index detector. Calibration in accordance with polystyrene standards was done at a flow rate of 1ml/min. Nanohydroxyapatite (nHA) was supplied by Aldrich Chemistry (USA), with a particle size > 200 nm and the solvent was Mw= 502.31 g/ml. 1,4 Dioxane purchased from Panreac p.a. (Barcelona, Spain). Phosphate Buffer Saline (PBS) solution, supplied by Fluka Analytical (Sigma Aldrich, USA) at a pH of 7.2, was used as the degradation fluid.

Fabrication of porous scaffolds

Pure PLCL and PLCL/nHA composite scaffolds were fabricated by Thermally Induced Phase Separation (TIPS) followed by a freeze-drying technique. Briefly, PLCL was dissolved in 1,4 dioxane in a proportion of 2.5% (w/v), by stirring for 2 hours at a temperature of 50ºC. After its complete dissolution, the solution was poured into aluminium moulds. At this stage, the nHA was blended in proportions of 10%, 30% and 50% of total polymer mass by ultrasonic stirring for 5 minutes, to form the composite scaffolds. The solutions were frozen and freeze-dried for several days to extract the

solvent completely. Foams that could serve as porous scaffolds with a porosity of up to 90% were obtained with this method.

In-vitro degradation

Degradation tests were conducted in vitro at 37ºC and under static conditions. First of all, the samples were cut into rectangular pieces and weighed and they were then totally immersed in identical glass test tubes containing 10ml of PBS. The tubes were put into an incubator at 37ºC. After selected degradation times (1, 2, 4, 6 and 8 weeks), the specimens were recovered, carefully wiped to remove surface water and weighed to determine water absorption. The pH change in the degradation medium was determined using a PCE 228 pH meter supplied by PCE Instruments (Spain) and corrected by temperature. Finally, the samples were dried over 2 weeks to a constant weight that was recorded to determine overall weight loss.

Characterization

Water absorption and weight loss were evaluated by weighing.

The percentage of water absorption W_a% was calculated by the following equation:

$$W_a\% = \frac{W_w - W_r}{W_r} \times 100 \quad (1)$$

where, W_w is the weight of the wet/swallow specimen after removing surface water and W_r is the residual weight of a completely dry sample after degradation. Weight loss percentage (W_L%) was estimated with the following equation:

$$W_L\% = \frac{W_0 - W_r}{W_0} \times 100 \quad (2)$$

the original mass of the sample was designated as W_0.

Mercury pycnometry.

The porosity of the scaffolds was quantified as in our previous reference [10]. Briefly, the bulk density of the polymer (ρ_a) was calculated by Mercury pycnometry, measurements of each material. The percentage porosity (%P) was calculated by the following equation:

$$\% P = (1- \rho_a/\rho_p) \times 100 \quad (3)$$

where, ρ_p is the bulk density of the polymer.

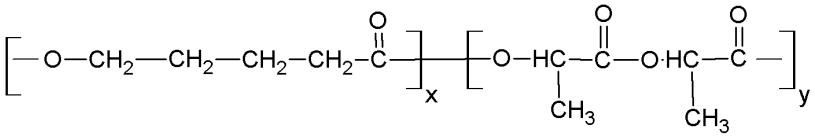
SEM analysis. The bulk morphology of the scaffolds was examined using Scanning Electron Microscopy (SEM) (HITACHI S-3400N, Tokyo, Japan). Prior to analysis, the samples were coated with a layer of gold, in a JEL Ion Sputter JFC-1100 at 1200 V and 5 mA., to avoid sample charging under the electron beam.

DSC analysis. The thermal characteristics of the polymer were determined using differential scanning calorimeter (DSC TA Instruments) equipped with an intracooler. Approximately 10 mg of polymer was placed in a crimp-sealed DSC hermetic aluminium pan. A nitrogen purge gas was used to prevent oxidation of the samples during the experiments, which were subjected to temperature scans ranging between -20 ºC and 200 ºC at temperature/time ratios of 10 ºC/min.

RESULTS

The degradation of biodegradable polyesters is usually due to chemical hydrolysis of the ester bonds in its backbone, which results in the formation of carboxylic acid end groups that act as catalysts in the reaction, producing 6-hydroxycaproic acid, lactic acid and oligomers[10]. The hydrolytic degradation mechanism can be classified into surface degradation and bulk degradation[11-12]. It is only the polymer surfaces that are degraded

in the surface degradation mechanism, which decreases their size and results in a loss of weight. In contrast, bulk degradation proceeds uniformly in the vertical direction to the surface, which results in a decrease of molecular weight. Acidic degradation products might accumulate during bulk degradation, if the degradation products cannot diffuse from the polymer matrix. Therefore, the internal (core) part of the polymer matrix can degrade faster than the outer part. This type of degradation mechanism is known as an autocatalyzed bulk degradation mechanism[13-14].



Poly L-lactide-co-ε-Caprolactone (PLCL)

Weight Loss and Molar masses

The weight loss, calculated with equation (2), shown in the previous section, indicated that there was no significant variation in weight for all samples in the degradation period under study. These results are in agreement with the degradation studies of other researchers, which also showed no appreciable loss until 8 or 9 weeks after degradation [15].

The standard technique for the characterization of polymer degradation is GPC, used in degradation assays to determine the reduced molecular weight of the copolymer. This technique allows us to establish the values of Mw, Mn and I, which are directly related to the length of the polymer chains and the dispersity of their molecular weight [10]. In Figure 1, we can see how the viscous average molecular weight decreases from the first week of degradation for all the samples in this study. The concentration of nanohydroxyapatite significantly affected the degradation rate, as certain samples with

varying contents of nHA reached lower molecular weights. Thus, after degradation over eight weeks, the samples with the highest molecular weights were PLCL / 30 wt % NHA and of PLCL / 50 wt % (Mw).

In Table I, we can see that the samples in the degradation process with less nanohydroxyapatite showed higher polydispersity indices, indicating that there were more macromolecular chains with different molecular weights, due to the increase in bond cleavage.

The polydispersity index of the copolymer increased as a result of the hydrolysis process[14, 16]. Composite degradation was much slower, especially for compounds with a higher content of nHA. This may be due to the high water absorption capacity of the bioactive particles. Bear in mind that the water molecules that penetrate into the polymer matrix are responsible for the hydrolytic decomposition of the polyester chains. Therefore, the hydroxyapatite nanoparticles were able to absorb the water molecules that penetrated into the solid phase, thereby reducing degradation. In all likelihood, the nHA particles were able to absorb the degradation products, momomers and oligomers, which further reduced the autocatalytic degradation of the polymer phase.

Water Uptake

Water absorption is the first event that occurs, when a scaffold enters into contact with a Phosphate Buffer Saline (PBS) solution. It causes and also reflects hydrolytic degradation. In Figure 2, we can see that all the scaffolds show significant water absorption rates until the fourth week of immersion in PBS, from which point this stabilizes at the end of the degradation process[17]. The water absorption process is a balance between the dissolution of oligomers held in solution and the material

consumption of PBS residue. The increase in water uptake reflects the degradation rate in the initial state. The accumulation of hydrophilic degradation products within the scaffold leads to an increase in water absorption during the degradation process. When the absorption of water reaches a certain value, the speed of absorption is reduced as a result of the dissolution of degradation products[18].

PCL is a polymer with low water uptake values and degradation times[14], while PLLA has an important water absorption rate (about 180 % after eight weeks of degradation)[18], although its copolymer experiences more significant levels of water uptake at around 250-450, depending on the concentration of nHA in the composite scaffolds. This level of water uptake may be due to the different morphology and pore sizes of these polymer composites.

Our results here are in agreement with values obtained from pH, which stabilized after the fourth week of degradation.

pH variations

Changes in the pH of the aqueous media with ageing time were determined for the aqueous ageing media of the various scaffolds, to check whether the release of different amounts of acidic residues from the matrices could explain the different degradation rates. This analysis also provided information on total acid production. In Figure 3, we can see that the pH of the buffer solution for the PLCL scaffolds decreased during the first week for all samples, after which it started to increase. The samples with PLCL and PLCL/nHA 10 % wt had the lowest pH; in other words, those with the lowest content of nHA. For these samples, the pH remained constant from the 4rd week until the end of the degradation period. The scaffolds with a higher content of nHA during the degradation period, reached higher pH values, of around 7.24. Most probably, the nHA

particles were able to absorb the degradation products, momomers and oligomers, which further reduced the autocatalytic degradation of the polymer phase and they also formed an alkaline solution, which acted as a physical barrier that decreased the degradation rate even further. Accordingly, the reduction in the pH may be related to a loss of molecular weight[17, 18].

Thermal analysis

DSC characterization allowed us to observe changes in the thermal properties of Tg, Tm and Hf in the polymer during degradation. Scans were performed from 50 to 200 ° C with a heating and cooling rate of 10 ° C / min.

A preliminary characterization showed that the PLCL was a semi-crystalline material with a small endothermic melting peak at 124 ° C. However, the cooling was too rapid to allow crystallization, so that no exothermic peak appeared during the cooling cycle. In the second scan, the material behaved as an amorphous material with a glass transition temperature of 22.5 ° C, although a small perturbation was appreciable at 124°, corresponding to the melting of a small crystalline domain. Glass transition temperatures and other thermal properties of the material can be seen in Table 2, The Tg in the PLCL and nHA PLCL/10 wt% samples began to decrease at the beginning of the in vitro tests and reached the lowest values in the eighth week, when it dropped to 19 and 20° C, respectively. The cause of this reduction in the Tg may be due to a decrease in molecular weight, which would correspond to the beginning of the autocatalytic degradation mechanism in block[16,19], adding further support to our theoretical proposal.

The scaffolds with a higher nHA content experienced fewer oscillations in their Tg. This behaviour can be explained by the degradation speed, which was much higher in the samples with minor quantities of nanoparticles or none at all. These results coincided with those obtained for the molecular weight of those two samples, which rapidly decreased and affected the Tg.

As may be appreciated in Figures 4a and 4b, the scaffolds showed fusion peaks that were more evident as degradation progressed, which may be directly related to an increase in the degree of crystallinity. These endothermic fusion peaks appeared in the first scan, but not in the second, probably because our swift cooling period did not allow the polymer to crystallize. The values of Tm and ΔHm, which are included in table 2, respectively, were obtained in this way from the first scan.

The first thing we see is a slight but constant increase in the Tm from the start of degradation. This increase may be due to an increase in crystallinity. The samples with no degradation hardly presented differences between each other, but the PLCL without nHA underwent a constant and somewhat greater increase in Tm. The samples with nHA, however, appeared to stabilize their Tm as from week 6. There was also an increase in ΔHm, especially in the samples with lower quantities of nHA, while it remained more or less constant in those with more nHA. The difference between the samples was evident at the end of the degradation period. These differences may once again be due to the increase in the degree of PLCL crystallinity[16, 17, 19].

The absolute crystallinity values of the samples can not be calculated, because the theoretical value of the PLCL copolymer (70 /30) 100 % lens is not available in the literature. However from the thermograms, we may say that all porous substrates showed a degree of crystallinity in the first scan, although this peak increased

throughout degradation, all the more so in pure PLCL samples, which were the most widely degraded (Figures 4 and 5). On the second sweep, it also confirmed an increase in crystallinity that can be seen in Figures 5 and 6. This increased crystallinity was in the amorphous part of the copolymer, before it began to degrade, into which the water molecules that are the cause of hydrolysis can easily penetrate.

Thus, the crystallinity of the amorphous portion of the polymer, in our case the caprolactone, increased. Some authors say that a lactide chain length of 14 is required for a sequence to crystallize. From among the copolymers of PLCL, only L-lactide is capable of forming crystals[17].

The strand breaks and the chain ends increased at a degradation temperature (37 ° C) that is higher than the Tg (22.5 ° C), which facilitates molecular rearrangement and increases crystallinity. This behaviour agrees with the observations of other researchers[16, 17, 20].

Similarly, if we compare the thermograms of the different samples at the end of the degradation, we can see that the increase in crystallinity was more pronounced in the pure PLCL samples than in those with more nHA, both during the first and second scan (Figures 5a and 5b). This would once again prove that the nHA slowed the degradation of the scaffold, forming a physical barrier that prevented the entrance of PBS and that neutralized the acidity of the oligomers created by polymer degradation. These results contrast with those observed in other studies on compounds of PLCL and nHA[20-21], which may be due to both the internal scaffold structure and to the method used for the production and dispersion of nanoparticles.

FTIR

The Fourier Transform Infrared (FTIR) tests were completed with Attenuated Total Reflectance (ATR), in order not to have to dissolve and thereby modify the sample, which would have caused a precipitate of nanohydroxyapatite, as this substance is not soluble in the majority of organic dissolvents. The preliminary study indicated that the nanoparticles had not interacted with the functional groups of the PLCL, and were simply found dispersed in the polymer structure (Figure 6).

The spectra of the degraded samples presented some small variations[18, 20]. The first was the appearance of a wide but hardly intense band, as from the fourth week of degradation, of between 3000 and 3600 cm^{-1} that could be attributed to the stretching vibration of the OH bands from the COOH and OH groups (Figure 7). In addition, a very clear absorption band appeared in the region of 1756 cm^{-1} corresponding to the carbonyl group. This band is typical of the random copolymers of PLCL.

Another change may be observed in the region of 1450-1500 cm^{-1}, where the intensity of two bands is greatly diminished; this transformation may be explained by the contribution of the CO band. All of these changes may be due to the split of the ester bond in the hydrolysis that produces a carboxylic acid and an alcohol group, as a consequence of caprolactone degradation. The carboxylic acid is caproic and not lactic acid, because, as explained earlier, it is the amorphous part in copolymers such as PLCA that degrades first of all, as it is easier for the water molecules to penetrate into its structure.

SEM

Analysis by scanning electron microscopy (SEM) enabled us to observe the effect of the addition of bioactive nHA particles and their influence on the degree of scaffold degradation[17-18].

The micrographs of Figure 8 show a structure of interconnected pores. The pore walls are composed of both PLCL and nHA. The addition of NHA particles affected the morphology of the porous scaffolds, which became more irregular as the nHA content increased. These changes are due to the interference of nHA particles in the phase separation process. The separation of solid - liquid phases, typical of the observed morphology, is attributed to the crystallization of the solvent.

As regards the degradation process, we observed the appearance of many micropores on the scaffold walls, as the hydrolysis process progressed (figures 10a and 10b). The microporosity of the walls was less noticeable when the amount of nHa composites increased, as may be seen in Figures 10c and 10d. Moreover, in the sixth week, the remains of degradation products were visible on the walls of the pores. These residues were observed with greater frequency in samples with less nHA.

Conclusions

Degradation led to larger increases in the polydispersity of the copolymer. Degradation of the composites was much slower, especially for the composites with more nHA contents. This may be due to the high water absorption capability of the nHA particles. It should be noted that the water molecules, which penetrated within the polymeric matrix, are responsible for the hydrolytic decomposition of the polyester chains.

Therefore, the nHA nanoparticles were able to absorb the water molecules penetrating the solid phase which reduced the degradation. Moreover, the nHA particles were able to absorb the degradation products in the form of momomers and oligomers, which further reduced the autocatalytic degradation of the polymer phase.

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