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## **In-vitro Degradation of PLLA/nHA composite scaffolds**

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## **Abstract**

Porous PLLA scaffolds and PLLA/nHA composite scaffolds with interconnected pore networks and a porosity of over 90% can be fabricated with lyophilization techniques. In this study, the degradation behavior of PLLA and PLLA/nHA composite scaffolds is investigated over 8 weeks in PBS at 37 °C. Thermal analysis using differential scanning calorimetry was performed. The percentage crystallinity of all the samples in the study increased by approximately 10%, which represents a considerable increase in the glass transition temperature. The melting rang enthalpy of the scaffolds did not change to lower temperatures as would be expected. Spectroscopic analysis was performed by Fourier Transform InfraRed (FTIR) spectroscopy. Scaffold microstructure and morphology were investigated with scanning electron microscopy (SEM) and we observed a low content of nHA with no significant effect on solvent crystallization or pore structure. The compressive modulus and the yield strength of the scaffolds were investigated in conjunction with the study of their degradation rates. In comparison with the mechanical properties of the PLLA scaffolds, which remained largely unchanged, those of the PLLA/nHA composite scaffolds decreased as the degradation progressed.

**Keywords:** composite scaffold, nanohydroxyapatite, in vitro-degradation, DSC, SEM, mechanical properties.

## 1.Introduction

Poly-L-lactide (PLLA) is a thermoplastic, semicrystalline, biocompatible and biodegradable aliphatic polyester, widely used in a range of biomedical applications, such as implantable drug delivery systems, fixation of prosthetic joints and tissue engineering [1-2].

PLLA belongs to the poly ( $\alpha$ -hydroxy esters) family and is usually obtained by ring-opening polymerization of lactide, although its production from renewable resources is also possible. Certain grades of this polymer have also been approved by the FDA for *in vivo* applications in human beings [3].

The degradation mechanisms of this polymer in aqueous media occur by simple hydrolytic scission into lactic acid, which is its monomeric form and that is eliminated from the body by incorporation into the tricarboxylic acid cycle. Respiration is one of the main paths for the oxidization of lactic acid, which is finally broken down into CO<sub>2</sub> and eliminated through the lungs [4-5].

Understanding the kinetics of polymer degradation is of major importance for the manufacture of any implantable medical device. In tissue engineering, for instance, scaffold resorption should match the rate of tissue formation, so that the overall structural integrity of the system is maintained. Too rapid a degradation rate would not only jeopardize the mechanical support afforded by the scaffold, but would also cause accumulation of the acidic matter that could induce inflammation of the surrounding tissue [6-8].

The degradation rate of PLLA has been found to depend on a huge range of factors, such as molecular weight, purity, crystallinity, temperature, pH, and sample dimensions. The strong relationship of crystallinity with the previous thermal history of the polymer has been demonstrated. Different handling techniques will therefore influence those degradation properties too [9-10].

Degradation has been shown to be highly dependent on parameters such as size and also porosity, which enhance the effects of diffusion inside the polymer, so the degradation products (responsible for autocatalysis) can escape more easily from the polymer mass [11].

The addition of bioactive particles to form composite scaffolds, such as hydroxyapatite (HA), has been reported to improve osteoconductivity for bone tissue engineering purposes, although it also modifies the natural resorption behavior of the PLLA. There are conflicting results on this matter and some authors have observed increased degradation rates, while others consider that these bioceramics have the ability to slow down processes of polymer mass [12-13].

There are relatively few results on the effects of nanoparticles such as nHA on PLLA ageing and degradation behavior. Many articles have studied PLLA degradation, in relation to such aspects as pH, crystallinity, and the % of absorbed water, among others. However, the fabrication technique used in this article was thermally induced phase separation (lyophilisation), as well as the incorporation of nHA prior to the fabrication of the scaffold, a technique that permitted homogeneous dispersion of the nHA particles. In this paper, the effect of nHA content on the mechanical properties of the PLLA scaffolds was studied, as well as the evolution and the degradability of the PLLA/nHA composite scaffolds.

## **2. Materials and methods:**

### **2.1 Raw materials**

Optically pure poly(L-lactide) containing less than 0.01% of residual solvent and less than 0.1% of residual monomer was supplied by Biomer L9000(Germany). Its specific rotation in chloroform at 20 °C was -157.3°. The weight-average relative molecular weight  $M_w = 141940.8$ ,  $M_n = 95680.8$  and polydispersity  $M_w/M_n = 1.4835$  of PLLA were determined using gel permeation chromatography (GPC, Perkin Elmer 200) in tetrahydrofuran (THF). GPC was performed with a THF solvent using a Perkin Elmer 200 reflective index detector. Calibration was done in accordance with polystyrene standards with a flow rate of 1ml/min. Nano hydroxyapatite (nHA) was supplied by Aldrich Chemistry (USA), with a particle size  $> 200$  nm and  $M_w = 502.31$  g/ml. 1,4 Dioxane purchased from Panreac p.a. (Barcelona, Spain) was used as a solvent. Phosphate Buffer Solution in Water (PBS), supplied by Fluka Analytical (Sigma Adrich, USA) at a pH of 7.2, was used as the degradation fluid.

## 2.2 Fabrication of porous scaffolds

Pure PLLA and PLLA/nHA composite scaffolds were fabricated by TIPS (thermally induced phase separation) followed by the application of a freeze-drying technique. Briefly, PLLA was dissolved in 1,4 dioxane in a proportion of 2.5% (w/v), by stirring for 2 hours at a temperature of 50°C. After its complete dissolution, the resultant solution was poured into aluminium moulds. At this step, nHA was blended by ultrasonic stirring for 5 minutes, in proportions of 10%, 30% and 50% of total polymer mass, to form the composite scaffolds. The solutions were frozen and freeze-dried for several days for complete extraction of the solvent. Porous foam scaffolds with porosity of up to 90% were obtained by this method.

## 2.3 In-vitro degradation

Samples for degradation were cut into a disk. The scaffold disk was cut to a diameter of 15 mm and weighed. After that, the specimens were placed in identical glass test tubes containing 10ml of PBS, totally immersed and incubated in a thermostated oven at 37°C and under static conditions. Following 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 pH meter PCE 228 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 any relevant weight loss.

## 2.3 Characterization

**2.3.1. Water absorption** and weight loss were evaluated by weighing.

The percentage of water absorption,  $W_a\%$ , was calculated in 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  $W_0$ .

### 2.3.2. Mercury pycnometry

The porosity of the scaffolds was quantified by mercury pycnometry. The scaffolds were dipped, one by one, into a container of mercury, placed on electronic scales with the help of a metal device. Knowing the density of mercury ( $\rho_{HG} = 13.57 \text{ g/cm}^3$ ) and the mass indicated by the scale, we may calculate the volume of the mercury ( $\text{Vol}_{Hg}$ ). The volume displaced by the mercury is equivalent to the volume of the sample in question. So, knowing the initial mass and  $\text{Vol}_{Hg}$  thereof ( $M_{sa}$ ), the bulk density ( $\rho_a$ ) may be calculated with the following equation:

$$\rho_a = M_{sa} / \text{Vol}_{Hg} \quad (3)$$

Using both bulk density and the density of the polymer ( $\rho_p$ ), measured by pycnometry on the pulverized material, the percentage porosity was calculated with the following equation:

$$\% P = (1 - \rho_a/\rho_p) \times 100 \quad (4)$$

where, P is the percentage porosity. Measurements were made for each material.

### 2.3.3 Differential scanning calorimetry (DSC)

The thermal properties of the polymers were investigated using a DSC (TA Instruments) under a nitrogen atmosphere. Samples were heated from -20 to 250 at a rate of 10°C.min<sup>-1</sup>. The first scan was used to record the melting temperature and heat of fusion. The melting temperature,  $T_m$ , was noted as the maximum value of the melting peaks, and the midpoint temperature of the glass transition was determined as the glass transition temperature,  $T_g$ . Crystallinity,  $X_c$ , was calculated according to equation:

$$X_c (\%) = 100 \times (\Delta H_{\text{melt}} - \Delta H_{\text{crystallization}}) / \Delta H_{100\%} \quad (5)$$

where,  $\Delta H_{\text{melt}}$  (J.g<sup>-1</sup> of the crystalline polymer) is the enthalpy of fusion of the specimen and  $\Delta H_{100\%}$  is the enthalpy of fusion of a 100% crystalline polymer, which for PLLA is 93 J.g<sup>-1</sup> [14].

### **2.3.4 Infrared Spectroscopy (FTIR).**

Infrared spectra of the samples were recorded on a Thermo Nicolet Avatar 370 Fourier Transform Infrared (FTIR) spectrophotometer equipped with an Attenuated Total Reflectance (ATR) attachment with ZnSe crystal. Spectra were repeatedly scanned 32 times between 4000 and 650 cm<sup>-1</sup> at a resolution of 4 cm<sup>-1</sup>.

### **2.3.5 Mechanical property**

Mechanical testing of the pore scaffolds was performed using a Universal Testing Machine (Instron, Model: 4502 UK). The preparation procedure for the PLLA and PLLA/nHA composite scaffolds was the same as described for the fabrication methods. The diameter of the scaffold disk was 15 mm and its thickness was 2 mm. The compressive modulus was defined as the initial linear modulus and the yield strength was determined from the intersection of the two tangents on the stress-strain curve around the yield point. Five scaffolds were mechanically tested for each sample.

**2.3.6 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.

### **3. Results and discussion**

PLLA is a hydrolysable polymer that follows a bulk degradation mechanism. The ester bonds in the backbone chain break randomly to generate carboxyl groups, which in turn autocatalytically accelerate the degradation reaction. In general, there are four steps in the degradation of a PLLA scaffold: 1) swelling and hydration of the polymer; 2) breakage of the ester bonds; 3) diffusion of the soluble degradation products; and, 4) disappearance of the polymer scaffold chips.

#### **3.1 DSC**

Degradation of semicrystalline PLLA occurs through random bulk hydrolysis of the ester bonds in the polymer chains and follows a general stage degradation profile. The first stage begins with water diffusion into the amorphous regions initiating random hydrolytic scission of the ester bonds. The second stage commences when most of the amorphous regions are exhausted and the crystalline regions are attacked [14]. The more loosely packed macromolecule segments are more vulnerable to water molecule attacks in the amorphous regions. Consequently, the amorphous regions are preferentially removed, leading to the relative increase of the crystallinity in the remaining material and to a reorganization of loose chain ends. This behavior was observed in our composite scaffolds over the 8 weeks of degradation (see Table Ia and Ib), where the percentage of crystallinity increased by approximately 10% for all samples studied [1, 15]. The breakage of the segments leads to a decrease in molecular entanglement and an increase in chain mobility. The chains could then reorganize themselves into a more orderly macromolecular arrangement and this increased mobility was able to compete for nHA that favored the rigidity of the polymer chains and produced a considerable rise in the glass transition temperature [16] see Table Ic. As



expected, therefore, there were no changes to melting enthalpy of the scaffolds (see Table Id)

### **3.2 FTIR**

The nHA, PLLA and PLLA/nHA scaffolds were initially analyzed by IR (Figure 1). Looking at the spectra, we can see that no absorption band has altered its position or intensity. There was therefore no uniform interaction of the polymer with the nanohydroxyapatite on the PLLA, which was dispersed in the mixture, as was later confirmed in the SEM observation. This is contrary to the results obtained by other authors with PLLA/nHA composite scaffolds using the manufacturing process of electrospinning [16]. The peak corresponding to  $1750\text{ cm}^{-1}$  (C = O stretching) in samples of scaffolds and PLLA scaffolds (Figure 2) and composite scaffolds (Figure 3) at different weeks of degradation dimmed when degradation broke up the chains over longer periods of time.

### **3.3 Mechanical properties.**

In previous works, a great variety of scaffolds have been developed using thermally induced phase separation, in which pore structure and pore wall morphology can be controlled by phase separation parameters. When 1,4 dioxane was used, the porous structure resulted from a solid-liquid phase separation of the polymer solution. Pore characteristics were determined by the morphologies of solvent crystals under such quenching conditions. All the scaffolds in our study had a porosity of above 90%. The addition of the highest proportion of nHA particles to the polymer solution perturbed the solvent crystallization, making the pore more irregular and isotropic and decreasing the pore size of scaffold. This has been observed above all for equal proportions of PLLA/50 wt% nHA 50 (Figure 6 SEM). The compressive modulus increased with the nHA proportion added to all the scaffolds in the study (Figure 4a and 4b). The increase in mechanical properties was not only due to the presence of nHA, but was also due to the higher nHA concentration that decreased the pore size. In a previous article we have seen how the compressive modulus and the yield stress were heavily dependent on pore size, both for PLLA porous structures and for the PCL. The lower mechanical properties

of the scaffolds with larger pore sizes could be consistent with the reduced polymer content [17-19].

Greater irregularity in the pores provoked by a larger amount of nHA for the samples of PLLA/ 50 wt% nHA might imply a less uniform distribution and an agglomeration of nHA particles in the polymeric matrix. Agglomeration formed bigger particles with smaller surface areas, which led to a pronounced reduction in the mechanical properties, as a result of the lower interfacial contact with the polymer matrix (see Figure 4).

The mechanical properties of the samples (compressive modulus and yield strength) as a function of degradation time can be seen in Figure 5a and b. These mechanical properties decreased continuously as from the fourth week, during the degradation time, in such a way that the samples with a higher nHA wt% had reached very low values after 8 weeks. While the pure PLLA samples were found to retain their mechanical properties over time, a decrease was observed in the compressive modulus and yield strength of all the PLLA/nHA compositions as the degradation process progressed, the most drastically affected being the samples containing nHA.

Degradation in the amorphous regions led to the breakage of the molecular chains connecting between the crystalline domains, resulting in defects of scaffolds in PBS. In spite of the neutralization effect of the buffer, the concentration of the carboxylic groups produced by degradation was to some degree higher in the scaffold interior, leading to faster wall degradation and the mechanical properties decreased at an equally rapid rate [1, 20].

### **3.4 SEM**

In Figure 6, we can see typical SEM micrographs of PLLA scaffolds and PLLA/nHA composite scaffolds prepared with 2.5% (w/v) PLLA/1,4 dioxane solution at a quenching temperature of -70 °C. The micrographs show a continuous structure of interconnected pores and a PLLA/nHA composite skeleton. The walls of the pores were composed of both PLLA and nHA. The nHA nanoparticles were homogeneously distributed in the polymer matrix. The morphology of the PLLA and the PLLA/nHA composite scaffolds were similar for the samples prepared with the same procedure of fabrication, and they had a highly anisotropic tubular morphology with an internal

ladder-like structure. This structure was a characteristic morphology of the scaffolds formed by solid-liquid phase separation of a polymer solution, where the channels were parallel to the direction of solidification (heat transfer direction). Each channel had repeating partitions with uniform spacing perpendicular to the direction of solidification. The solid-liquid separation was attributed to the crystallization of the solvent. When the temperature of the polymer solution was lower than the freezing point of the solvent, solvent crystallization took place and the polymer phase was expelled from the crystallization front as impurities. After the solvent crystals had been sublimated, foam formed in the pores, similar to the geometry of the solvent crystals. The temperature gradient along the solidification direction (from sample surface to sample center) may have provoked the highly anisotropic pore structure. When the nHA particles were introduced into the polymer solution, the crystallization of the solvent was perturbed and it changed the crystal growth, forming more irregular crystals in the solvent. Both polymer and nHA particles were expelled from the crystallization front forming a rich phase and a continuous PLLA/nHA skeleton, and the spaces formerly occupied by the solvent crystals became the pores of the scaffold. As a result of irregular solvent crystal growth, the pores and the structure became irregular (more isotropic) [18]. This behavior was observed for the composite scaffolds with PLLA/nHA molar ratios higher than 70:30 (see figure 6.b); the pore structure became so irregular that no regular channels or ladder-like structure were observed. Moreover, the introduction of nHA particles, even in small amounts, decreased pore font size from 300  $\mu\text{m}$  to 50  $\mu\text{m}$ . A good, homogeneous adhesion between the polymer and nHA particles also was observed and most of the nHA particles were found on the surfaces of the pore walls [20]. The scaffolds with compositions higher than the PLLA/ NHA molar ratio of 70:30 were observed to have highly porous pore walls (see figure 6.b). The perturbation due to the nHA particles was small at the highest concentration, due to their nanometric scale and uniform distribution. As a result PLLA/nHA composite scaffold maintained the main characteristic pore architecture of solid-liquid phase separation, which is anisotropic and regular. In this case, the effect of a low content of nHA on solvent crystallization was not significant enough to alter the pore structure.

As indicated earlier, the selected samples were subjected to a process of *in vitro* degradation, over 8 weeks. No appreciable changes were observed in the micrographs of these samples, nor did any degradation-related products appear (see Figure 6 c,d,e and

f). On the contrary, a progressive increase in the porosity of the pore walls was observed over the course of various weeks [21]. It may therefore be concluded that a process of degradation over 8 weeks, even if it was not enough to appreciate morphological changes under SEM, was enough to appreciate significant changes in other aspects of the study.

### **3.5 pH**

The change in pH of the aqueous media with degradation time was determined to check for the release of acid residues from the PLLA samples: the greater the lactic acid formation, the larger the pH decrease. This determination also provided information on total acid production [1,11]. Figure 7 shows the pH changes of PLLA and PLLA/nHA composite scaffolds as a function of *in vitro* degradation time. All the samples in the study followed a similar behavior until the fourth week of degradation; the pH fell very little and returned to the original value at around the fourth week, staying constant thereafter, except for the two composites that have a larger content of nHA, the pH of which continued to rise slightly throughout the period of degradation under study [16]. This increase in the pH may be due to the high quantity of nHA particles in those scaffolds, which, in the dissolution, counteracted the effect of the acids produced in the degradation process: this increase of pH is believed to be from the release of OH<sup>-</sup> from the degradation of nHA particles in composite scaffolds.

### **3.6 Water absorption**

Water absorption is the first event that occurs when a scaffold is in contact with an aqueous solution. So, it is the cause of and it reflects hydrolytic degradation [11]. Figure 8 shows the % water absorption as a function of degradation time. We can see through the first week of degradation is a very important water absorption for all the scaffolds studied. Between the first and fourth week, it appeared that the samples suffered a stabilization process in which the absorption of water was very small and as from the fourth until the eighth week they began to absorb a large amount of PBS. In other words, the greater the nHA content of the scaffolds, the less likely they were to absorb water and to degrade; a behaviour that has also been noted by other authors [1,16]. This

would appear logical with regard to the morphology of the scaffolds, as the greater the nHA content, the smaller the pore size (as may be seen in the SEM) and therefore the lower the absorption of water. The water absorption process is a balance between the dissolution of oligomers in solution and the material consumption PBS residue. An increase in water uptake reflects the degradation rate in the initial state. The accumulation of hydrophilic degradation products inside 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. These products introduce nHA particles which slow down the rate of degradation of the PLLA scaffolds, because they are an alkaline solution which acts as a physical barrier that causes a decrease in the rate of degradation.

### **3.7 Weight loss**

According to Figure 9, the increase in degradation time, for all samples, led to an increase in the weight loss values. However, weight loss in PLLA did not begin until the fourth week, while the composite scaffolds began to degrade after the first week. At the end of the eighth week, all the scaffolds had undergone a similar weight loss of about 22%. These results may indicate that nHA particles cause weight loss from the start of the degradation process, but the degradation rate is slower compared to PLLA scaffolds [22]. The degradation mechanism in poly-lactides, such as PLLA, is bulk degradation and when the water up take is increased, the degradation rate quickens, although the incorporation of nHA decreases the degradation rate [11-12].

### **3.8 Conclusions**

The effect of nHA content has been studied on the mechanical properties and degradation of a set of PLLA/nHA composite scaffolds. Good, homogeneous adhesion between the polymer and nHA particles was observed. The incorporation of nHA particles, even in small amounts, decreased pore font size from 300  $\mu\text{m}$  to 50  $\mu\text{m}$ . The presence of nanoparticles was not sufficient to affect the morphology of the samples. The percentage of crystallinity increased during the degradation time and there was a

considerable increase in the glassy transition temperature. The nHA particles slow down the rate of degradation of the PLLA scaffolds, because they are an alkaline solution which acts as a physical barrier that causes a decrease in the rate of degradation. As from the fourth week, the mechanical properties decreased continuously throughout the degradation time, in such a way that, after 8 weeks, they had reached very low values for the samples with more nHA wt%.

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