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Poly(ε-caprolactone) and poly(D,L-lactic acid-co-glycolic acid) scaffolds used in bone tissue engineering prepared by melt compression–particulate leaching method

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Abstract Porous bioresorbable polymers have been widely used as scaffolds in tissue engineering. Most of the bioresorbable scaffolds are aliphatic polyesters and the methods employed to prepare the porous morphology may vary. This work describes and evaluates the in vitro degradation of porous and dense scaffolds of poly(ε-caprolactone) (PCL) and poly(D,L-lactic acid-co-glycolic acid) (50/50) (PLGA50) prepared by particulate leaching-melt compression process. Biological evaluation was carried out using osteoblast cell cultures. The results showed an autocatalytic effect on the dense samples. Osteoblasts presented intermediate adhesion and the cell morphology on the surface of these materials was dispersed, which indicated a good interaction of the cells with the surface and the material.

1 Introduction

Tissue engineering has been proposed as a therapeutic approach to create new tissues and treat patients suffering from loss or failure of organs and tissues. A number of tissue engineering strategies have been developed and many involve the transplantation of cells on or within bioresorbable polymeric scaffolds [1].

The most common of these bioresorbable scaffolds are the synthetic aliphatic polyesters, such as poly(glycolic acid) (PGA), poly(lactic acid) (PLA), poly(ε-caprolactone) (PCL) and their copolymers. These polymers have been widely used in tissue engineering applications because they undergo controllable hydrolytic degradation into natural metabolites, which become incorporated in the tricarboxylic acid cycle and are subsequently excreted as water and carbon dioxide [2].

In addition to biodegradation and bioresorption processes, certain properties of the scaffolds must be considered when designing a substrate to be used in tissue engineering. Macroporous structures are desirable in many cases to facilitate cell seeding, infiltration of fluids, vascularization and tissue ingrowths. Thus, several techniques have been developed to create porous scaffolds, including solvent casting/particulate leaching, fiber bonding, gas foaming, phase inversion, solvent removal by freeze-drying, etc. The morphologies and properties of the resultant scaffolds largely depend on the fabrication process [3].

Usually, particulate leaching is a general procedure that is utilized to obtain porous scaffolds in solvent casting methods. The first step in the casting process is to dissolve the polymer in chloroform or methylene chloride, which usually includes the addition of sodium salts, with a controlled particle size. After organic solvent evaporation, the polymer/salt composite is immersed in water to remove the salt (porogen), producing porous scaffolds in the range of 100–500 μm [4]. Particulate leaching is an efficient technique to create porous scaffolds; however, residues of organic solvents used to dissolve the polymer, can remain after casting process, damaging the seed cells and

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neighboring tissue. Additionally, many biological active factors are inactivated by exposure to organic solvents [5].

Thus, from a manufacturing point of view, an alternative is the thermal processing of the polymer, mixed with leachable particles by extrusion and/or compression molding. The technique combines the advantages of particulate leaching without the use of organic solvents. The present paper investigates the melt compression process with the particulate leaching method to produce porous and dense scaffolds of PCL and PLGA, evaluated by in vitro degradation process and osteoblast culture.

2 Materials and methods

2.1 Preparation of porous and dense scaffolds

Scaffolds were prepared using poly(D,L-acid lactic-co-glycolic acid) (50/50) (PLGA50) (Purac Biochem., The Netherlands), Mw 60,000 g/mol and poly(ε-caprolactone) (PCL), Mw 100,000 g/mol (Sigma Co., USA), through the melt compression process. Dense scaffolds were fabricated by compression into a mold (4 mm × 50 mm with a central pin with 1.6 mm in diameter) using Mini Max Molder (LMM-2017, USA) at 160°C and injection pressure 230 bar. The mold was cooled at room temperature. Porous scaffolds were prepared as described above, including the addition of citrate sodium (Fluka Chemicals, Switzerland) sieved in particles of 180–250 μm in diameter, and a 2 salt/1 polymer weight ratio. The salt was added during the polymer melt compression and stirred to obtain paste-like polymer-particulate mixture. The polymer-salt composites were placed into demineralized water and stirred for 24 h to leach out the salt. The samples were dried and stored in a desiccator under vacuum until use.

2.2 Evaluation of in vitro degradation

The in vitro degradation of dense and porous scaffolds was evaluated using phosphate buffer solution (PBS), pH 7.4, maintained at 37 ± 0.5°C. PLGA50 samples were removed from the solution after 1, 2 and 3 weeks, and PCL after 8, 20, 32 and 52 weeks. The samples were washed with distilled water and ethanol before vacuum drying. The in vitro evaluation experiments were done in triplicate.

2.3 Scaffold characterization

2.3.1 Scanning electron microscopy (SEM)

The samples were fractured in liquid nitrogen and coated with gold using a sputter coater SCD 050 Cool Sputter System (Bal-Tec, Balzers, Switzerland). The fractured surfaces of the samples were observed with a JXA 840 scanning electron microscope (Jeol, Peabody, USA) at a tension of 10 kV. The porosity of the samples was determined using an image software analyzer, Scion Image®.

2.3.2 Thermogravimetric analysis (TGA)

TGA analysis was carried out in a STA 409C Netzsch (Gerätebau GmbH Thermal, Selb, Germany). The samples ($n = 3$) were heated from 25°C up to 400°C at a heating rate of 10°C/min under helium atmosphere.

2.3.3 Differential scanning calorimetry (DSC)

The DSC analysis was carried out in a STA 409C Netzsch (Gerätebau GmbH Thermal, Selb, Germany), and the samples ($n = 3$) were submitted to heat from 25°C up to 200°C at a rate of 10°C/min. After cooling at the same rate, each sample was re-heated. The changes in the glass transition temperature (Tg), crystallization temperature (Tc) and melting temperature (Tm) were evaluated during the degradation process. The degree of crystallinity of the PCL samples were calculated from the enthalpy change (ΔH) using the equation below, where $\Delta H_{100\%} = 139.5$ J/g [6] (melting enthalpy for 100% crystalline polymer).

$$\text{Degree of crystallinity}\,(\%) = 100 \times (\Delta H_{melt} - \Delta H_{crystallization})/\Delta H_{100\%.}$$

2.3.4 Mechanical test

Samples of of dense and porous scaffolds, prepared as described above in Sect. 2.1, were submitted to the tests of three-point bending. Cylindrical segments ($n = 8$) were carried out at room temperature using a MTS (TestStar II) according to the ASTM D790-95a. Young's modulus was determined by the angular coefficient of the linear curve of stress×strain.

2.3.5 Gel permeation chromatography

The average molar mass in weight (Mw), in number (Mn) and the rate of polydispersity was determined by gel permeation chromatography (GPC) coupled to a refraction index detector. Samples of 200 ml were dissolved in 10 ml THF (Merck) and injected with chloroform used as an eluent at a rate of 1 ml/min. Molar mass and rate of polydispersivity were calculated using polystyrene as a standard.

2.4 Cell culture

Cells hFOB 1.19, obtained from the American Type Culture Collection (Manassas, VA, USA), were cultured in Ham-F12 medium (Sigma, St. Louis, USA) supplemented

with 15% fetal calf serum (FCS) (Nutricell Nutrientes Celulares, Campinas, SP, Brazil) at 37°C.

2.4.1 Cell adhesion assay

Cell adhesion was studied using the method described in Murakami et al. [7] with some modifications. Briefly, the scaffolds ($n = 6$) were added to 96 well plates (Corning/Costar Corporation, Cambridge, MA, USA) in Ham-F12 for 24 h at 37°C. After this incubation time, 200 µl of a cell suspension (1.0×10^5 cells/ml) in Ham-F12 medium containing 15% FCS were added to the wells. The cells were cultured for 2 h at 37°C and then washed with 0.1 mmol/l PBS in pH 7.4 at 37°C, before fixing in 10% formalin for 15 min. After washing in PBS, the cells were stained with 100 µl of fresh medium and 50 µl of yellow tetrazolium MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) (5 mg/ml) for more 4 h. After this time, 100 µl of isopropanol acid were added in each well plate. The wells were read in a Multiskan Biochromatic microplate reader (Labsystems, Helsinki, Finland) at 540 nm. As a positive control, an empty polypropylene culture plate was read. Teflon membranes were used as a negative control. Absorbance of all wells was also determined without cells as a control for dye staining. One-way ANOVA was used to evaluate the results. Statistical differences among groups were detected by Newman-Keuls test. All experiments were done in triplicate.

2.4.2 Scanning electron microscopy

For morphological analysis, 1.0×10^5 cell/ml were incubated with polymers in Ham-F12 medium supplemented with 15% FCS. Cells cultured on glass cover slips under the same conditions were used as control. After 24 h, the samples were fixed with Karnovisky solution (4% of paraformaldehyde in 0.1 mol/l PBS), pH 7.2, for 45 min at 4°C, and postfixed with 1% OsO_4 for 2 h at 4°C. The samples were then dehydrated in an ethanol series, critical point dried (Balzers CDT 030) and coated with gold in a sputter coater (Balzers CDT 050). The coated specimens were viewed and photographed with a JEOL JSM–5800 Low Vacuum scanning electron microscope. Energy dispersive X-ray spectroscopy (EDS) (JEOL JXA-840A Electron Probe Microanalyses) was used to characterize granules observed during microscopy. All experiments were done in triplicate.

3 Results

3.1 Scaffolds morphological analysis

The results of scanning electronic microscopy of PCL and PLGA50 samples prepared without the addition of salt showed dense similar morphologic aspect. The analysis of the dense undegraded samples (Figs. 1a, 2a) shows tubular geometry with flat and regular surfaces with no pores due to the contact with the mold surface during the process. The morphology of the external surfaces of porous scaffolds (Figs. 1b, 2b) was similar to those of the dense samples. The internal morphology was irregular with concavities generated by the encapsulation of salt particles during the melting process and pores caused by the penetration of water due to salt leaching. During the degradation process, the comparison between PCL and PLGA50 scaffolds indicates a clear difference between degradation rates. While samples of PLGA50 lose their geometry in 3 weeks (Fig. 2c, d), samples of PCL are stable morphologically for about 1 year (52 weeks) (Fig. 1c, d).

Both dense scaffolds showed a marked morphologic change compared with their porous equivalent. In PCL scaffolds, the material maintains the initial tubular geometry, but presents superficial erosion (Fig. 1c). PLGA50 dense samples lose their tubular geometry, becoming a viscous material when manipulated and appear as a plane film in SEM analysis (Fig. 2c).

Using an image analyzer software, the average pore size (130–200 µm) of the undegraded samples were shown to be smaller than the size of the particles of salt used (180–250 µm). The pores sizes are homogeneous in relation to their distribution, and porosity was estimated to be 80% for both materials. During the degradation process, decrease in the pores sizes of PLGA50 samples was directly proportional to degradation time.

3.2 Thermal analysis

The DSC thermograms obtained for dense and porous PCL samples showed glass transition temperature (Tg) at about −63°C obtained in the second heating, and melting temperature (Tm) at about 67°C in the first heating. Crystallization peaks (Tc) of about 20°C present only in PCL samples suggested that the rate of cooling employed (10°C/min) was rapid enough to promote slow nucleation and crystal growth. The degree of crystallinity was calculated from the melting enthalpy and showed variations between 55% and 57%. During the 52 weeks of in vitro degradation, the PCL samples showed stable thermal properties with no significant changes of the values above (Fig. 3).

The PLGA50 samples Tg was about 42°C. There was no significant effect on Tg values when the leaching process was carried out before degradation. After 3 weeks, dense samples presented a crystalline peak at about 182°C. During degradation, dense and porous samples of PLGA50 presented a decrease in Tg and by regression of the values, it was possible to compare the angular coefficient, higher in

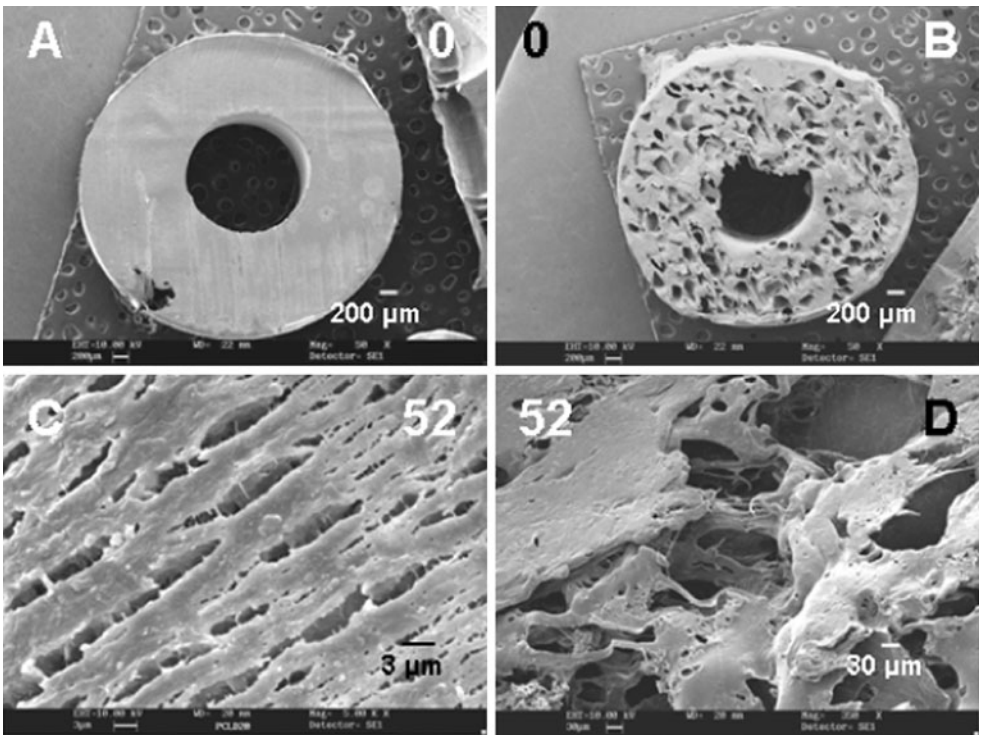

Fig. 1 Scanning electron micrographs of cross section of PCL samples. **a**, **c** Dense scaffolds. **b**, **d** Porous scaffolds. The *numbers* reported on the top of each image indicate the degradation time in weeks

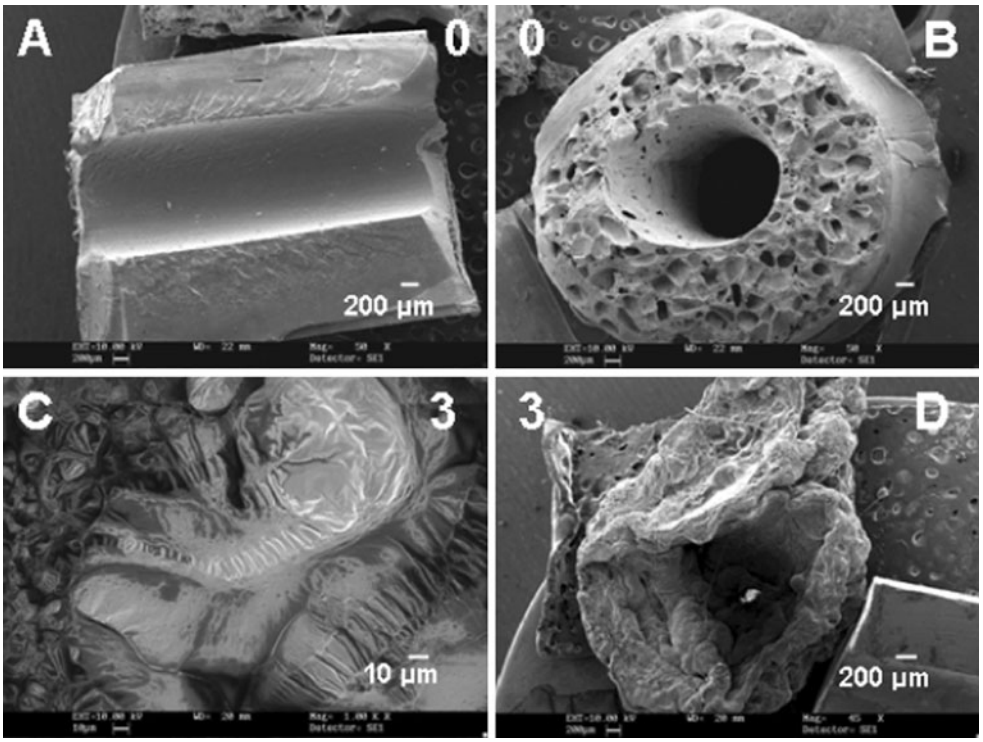

Fig. 2 Scanning electron micrographs of cross section of PLGA50 samples. **a**, **c** Dense scaffolds. **b**, **d** Porous scaffolds. The *numbers* reported on the top of each image indicate the degradation time in weeks

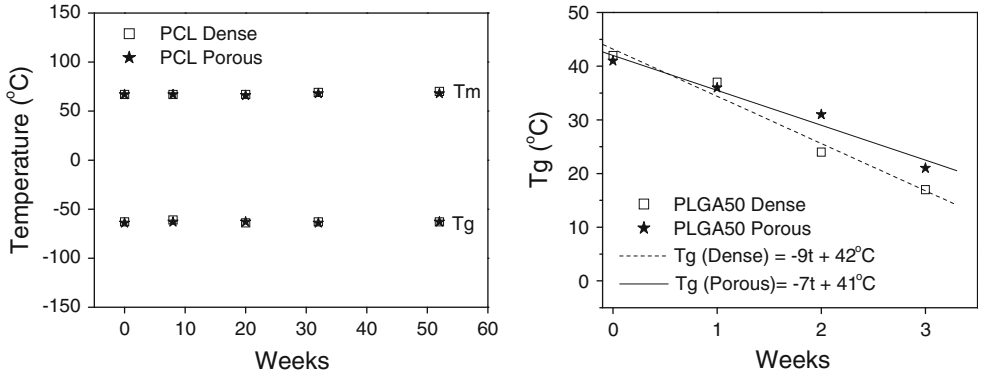

Fig. 3 Differential scanning calorimetric data for PLGA50 and PCL samples. *Tg* glass transition and *Tm* melting temperature

Table 1 Thermogravimetric analysis of samples of porous and dense PCL and PLGA50 as a function of in vitro degradation time (weeks). Tonset is the initial temperature of loss mass

	PCL (Tonset) (°C)				
	Without processing	0 week	20 weeks	32 weeks	52 weeks
Dense	390	389	392	390	388
Porous		392	391	389	387
	PLGA50 (Tonset) (°C)				
	Without processing	0 weeks	1 week	2 weeks	3 weeks
Dense	330	333	323	312	296
Porous		330	321	308	298

dense samples (9°C/week) when compared to porous samples (7°C/week).

Tonset supplied by TGA analysis, showed differences in temperature values (PCL at about 390°C and PLGA50 at about 330°C) before and after degradation. During the 1-year degradation process, PCL was thermally stable, whereas the PLGA50 Tonset decreased after few weeks of degradation. Table 1.

3.3 Mechanical bending tests and gel permeation chromatography (GPC)

Samples of dense and porous PCL showed average molar mass in weight (Mw) values of 100,000 g/mol. During the degradation process, the variation of Mw is smaller (approximately 5%), confirming the scaffolds stability observed by SEM and DSC, TGA and mechanical property data. Similar behavior was also observed in the analysis of average molar mass in number (Mn) and rate of polydispersity (data not shown). It was not observed significant variations in GPC analysis when PCL morphologies were compared. On the other hand, it was observed an increase in the tendency of degradation speed of PLGA50. Figure 4 shows the rate of decrease of the molar mass of PLGA50.

Dense and porous scaffolds were submitted to the three-point mechanical bending tests that showed a fragile behavior of PLGA dense samples, while PCL dense and porous samples and PLGA50 presented a ductile behavior. Table 2 shows the variation in the Young's modulus values and maximum stress as a function of time of degradation. Before and during the degradation period, samples of PLGA50 presented superior Young's modulus when compared to their PCL equivalent. The porosity reduces the modulus considerably. During the 52-week in vitro degradation, the Young's modulus of dense and porous PCL samples presented a decreasing tendency. Similar behavior was observed for the maximum stress. With regards to PLGA50 mechanical property, the Young's modulus decrease during 3-week degradation period. GPC results for PLGA50 can be related to its thermal and mechanical properties (Fig. 5).

3.4 Osteoblast culture

The adhesion test on the studied scaffolds demonstrated an intermediate behavior ($F = 37.83858$ and $P = 0.000001779$). Newman-Keuls indicated that the positive control (polypropylene plate) and negative (Teflon®) were significantly

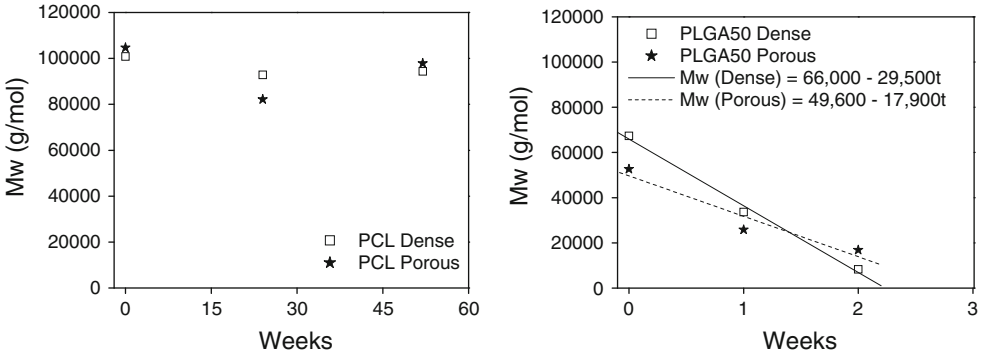

Fig. 4 Variation of average molar mass in weight (*Mw*) as function of degradation time (*weeks*) to PCL and PLGA50 samples

Table 2 Young's modulus (E) e maxim stress (σ_{max}) in the three-point mechanical bending tests of dense and porous scaffolds of PCL and PLGA50 as function of degradation time (weeks)

Mechanical bending tests									
PCL					PLGA50				
	Dense		Porous			Dense		Porous	
Weeks	E ± SD (MPa)	σmax ± SD (MPa)	E ± SD (MPa)	σmax ± SD (MPa)	Weeks	E ± SD (MPa)	σmax ± SD (MPa)	E ± SD (MPa)	σmax ± SD (MPa)
0	461 ± 42	44 ± 3.8	115 ± 18	4 ± 1.4	0	4120 ± 860	124 ± 11	529 ± 120	14 ± 3.0
24	422 ± 66	41 ± 5.2	98 ± 13	5 ± 1.1	1	6300 ± 1403	133 ± 9	502 ± 170	11 ± 2.5
52	345 ± 53	39 ± 5.6	84 ± 17	4 ± 0.7	2	2039 ± 408	78 ± 13	232 ± 89	8 ± 2.0

SD standard deviations

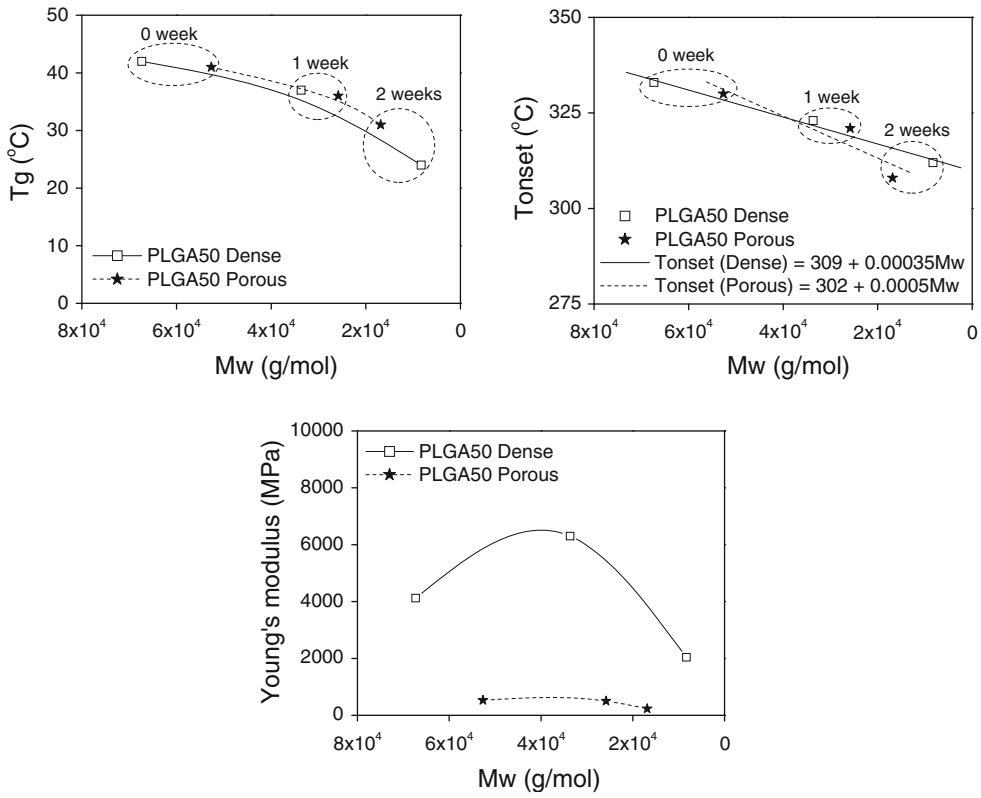

Fig. 5 Glass transition temperature (*Tg*), initial temperature of mass loss (*Tonset*) and Young's modulus of dense and porous samples of PLGA50 as function of the average molar mass in weight (*Mw*) and of degradation time (*weeks*)

different ($P < 0.01$) among themselves and in relation to the analyzed samples (Fig. 6). Cells cultivated on PCL and PLGA50 scaffolds showed a dispersed morphology with long and thin intercellular connections, known as filopodia (Fig. 7b to PCL and 7c to PLGA50). A similar morphology was observed in control cell (Fig. 7c). In samples of PCL and

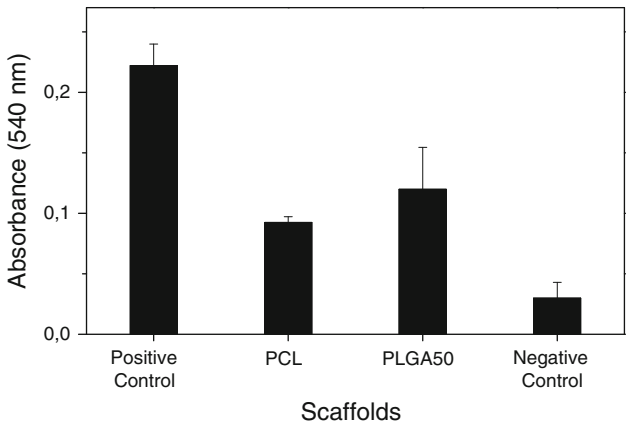

Fig. 6 Human osteoblasts adhesion of PCL and PLGA50 scaffolds. ($P < 0.01$)

PLGA50, the cells were not as densely packed as in the glass control surface; however, they showed a semi-confluent monolayer disposition. During the electronic microscopy analysis, it was observed crystalline granules on cells, suggesting deposition of organic material. EDS was used to characterize the samples. Figure 8 presents the results of EDS analysis of granules and shows different chemical elements.

4 Discussion

A first analysis of morphology of the scaffolds showed that the difference of degradation rate is attributed to the type of material and their chemical composition. The hydrolysis rate, and consequent mass loss, is directly proportional to the number of ester bonds of the polymeric chain (greater in the chains of PLGA50 when compared to the chains of PCL). Several factors influence the degradation rate of the bioresorbable scaffolds, such as chemical composition, crystallinity, geometry and morphology of the scaffold, surface energy and others parameters [8]. These factors are interrelated and to treat them separately represents an oversimplification of the degradation process.

Differences between dense and porous morphologies exemplify the autocatalytic effect of the bioresorbable polymers, previously presented and discussed [4]. The smaller the area of diffusion of the degradation products, the larger the effect of the acidic products will be [9].

The control of porosity of the polymeric supports can be done by varying the amount of salt and the size of salt particles. Widmer et al. [10] obtained samples of PLGA (75/15) and porous PLLA using the extrusion process. The authors evaluated the variation of the size of salt particles and salt concentration (between 70 and 90%). The study concluded that salt concentration is the most significant

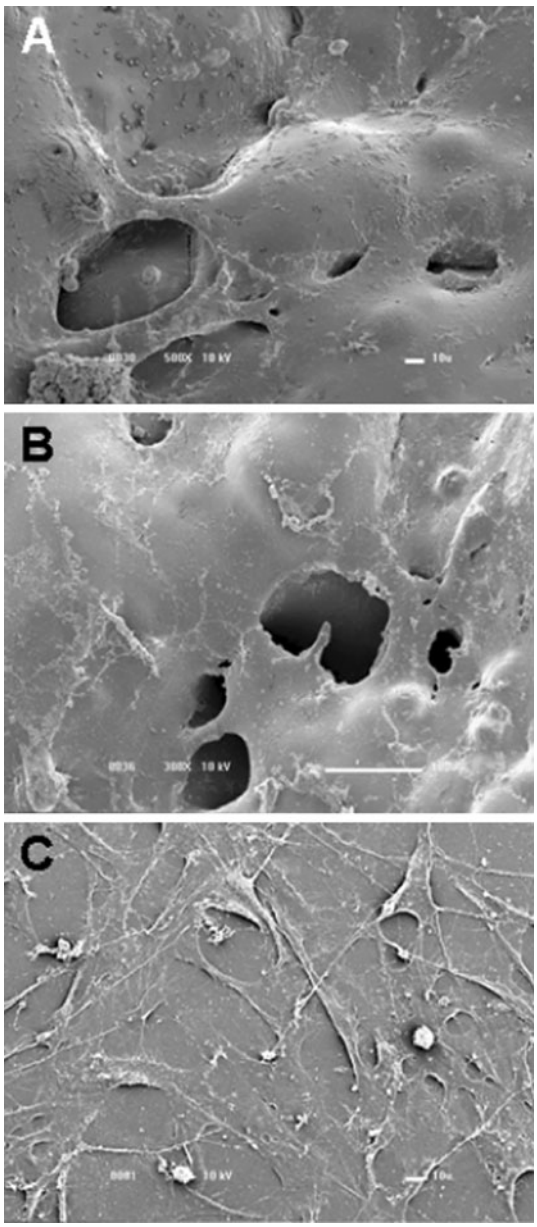

Fig. 7 Scanning electron micrographs of osteoblasts on porous PCL and PLGA50 scaffolds. **a** Cells on PCL. **b** Cells on PLGA50. **c** Glass control

parameter for the formation of scaffold porosity. Hence, higher salt concentrations result in higher porosity. However, significant increases in the porosity reduce mechanical resistance. When applied to tissue engineering, the porosity allows a flow of nutrients and metabolic products through the scaffolds, aiding in the process of local vascularization, indispensable for tissue growth [11]. Tubular samples similar to the ones obtained in our experiment with regards to geometry and morphology have been developed for applications in guided tissue regeneration, such as peripheral nerves [12].

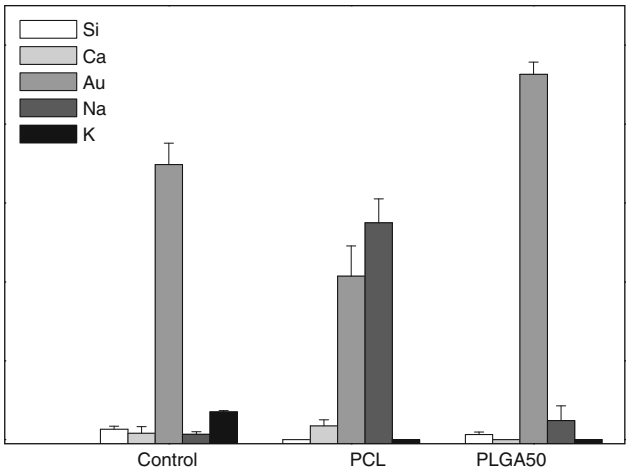

Fig. 8 Energy dispersive X-ray spectroscopy (EDS) on PCL and PLGA50 scaffolds

Concomitantly the degree of crystallinity plays a critical role in the degradation process and biocompatibility. Given the preferential attack in amorphous areas due to their greater susceptibility to water penetration during degradation, tissue ingrowth occurs much faster in amorphous materials than in the semicrystalline ones. In the melt process associated to particulate leaching, the addition of salt particles does not show differences in the degree of crystallinity. Similar results were observed with PLLA and PLGA (50/50) prepared by casting particulate leaching and submitted to degradation in cell culture medium [4]. During degradation, the polymeric chains undergo reorganization to produce crystals. The crystalline peak observed in amorphous materials, as PLGA, is related to the rearrangement of the small polymeric chains generated during the degradation process [13].

From TGA analysis, the thermal stability is an important factor in the methods employed to produce bioresorbable scaffolds. During heat processing of the material, such as extrusion, fusion or FDM process, thermal degradation can generate smaller molecules, as well as degradation byproducts that can interfere with the chemical composition of the material and alter its cytotoxicity and the scaffolds biocompatibility [14]. Comparing the TGA curves obtained for porous and dense samples and the polymer without processing, it was observed that the melting process did not modify the Tonset. Our results show that there was no significant influence of the melt process/particulate leaching used to prepare the scaffolds on the values of Tonset. Thermal degradation of bioresorbable polymers has been described as a process involving several stages and mechanisms, and dependent of ester groups [15]. Thus, the differences of the materials were considered as the result of their composition and crystallinity: the greater the content of long carbonic chains, and the proximity of polymeric chains, the greater thermal stability of the material.

Given the increase in the chains flexibility, the smaller the molar mass is, the lower Tg and Tonset will be. Also, Tg decrease has been related to the presence of water molecules absorbed during the degradation process, which acts as a plasticizing agent as described previously. With regard to PLGA50 mechanical property, the scaffolds decrease the Young's modulus during degradation period. The increase observed after one week of degradation can be due to reorganization of the polymeric chains by increasing intermolecular connections and producing additional resistance [13]. The rates of decrease of the molar mass of PLGA50 reaffirm the differences observed in the thermal analyses of DSC and TGA. The declining rate of Mw values is higher in dense samples (29,500 g/mol/week) when compared to the porous ones (17,900 g/mol/week), confirming the autocatalytic effect [4, 9].

The mechanical property was evaluated by the Young's modulus and presented a decreasing tendency of PCL and PLGA50 samples. Comparing the thermal and mechanical properties, our results are in accordance with those proposed by Pietrzak et al. [16] in which the decrease of the mechanical property precedes the loss of mass or changes in thermal properties.

For bone fixation devices, the porous morphology of the material has been described as a limitation parameter when the scaffold needs mechanical supports. The porosity allows larger diffusion of the culture medium, cellular invasion and formation of new tissue; however, it decreases the mechanical properties considerably, as observed for PLGA50 samples. In order to improve the scaffold mechanical properties, PCL and PLGA blend represents an alternative to the use of single materials [17]. Another technique employed to obtain improved scaffolds is the formation of composites with hydroxyapatite [18].

Furthermore, the interaction between cells and their substrates depends fundamentally on the surface characteristics of the material, like the hidrofilicity. The topography, chemical properties and surface energy determine whether biological molecules will be adsorbed and the subsequent stages of spreading, proliferation and cellular differentiation [4]. Thus, with different cell-substrate interactions, the cells respond with different growth patterns [19]. Calcium ions were not present in significant amounts by EDS analysis. The deposition of calcium salts on synthetic scaffolds is usually detected after about 20 days of culture [20].

Poor cellular adhesion does not necessarily imply low proliferation. Adhesion of osteoblasts to the PCL and PLGA50 samples was slow; however, a good interaction of the cells with the substrate was observed by SEM. The slow adhesion of cells to the bioresorbable materials is a

phenomenon that has already been described [19, 21], and the present results suggest that this phenomenon happens on porous PLGA50 and PCL samples. In protocols that allowed longer time for adhesion evaluation, the cells begin multiplication on the substrate [22]. In this sense, systems based on bioresorbable polymers to which growth factors are incorporated are thoroughly studied [23].

5 Conclusion

Different types of bioresorbable polymers were used in the study. The degradation of the PLGA50 samples was rapid when compared to PCL because PLGA50 is an amorphous material and has a higher number of ester bonds. The autocatalytic effect of poly(α-hydroxy acids) was seen in dense PLGA50 samples as compared to the porous samples. The samples of PCL were stable morphologically for 1 year in phosphate buffer solution. Cultured osteoblasts showed a favorable proliferation and differentiation on the samples. These results show that scaffolds of PCL can be used when a substrate with a prolonged degradation is required to serve as a physical support for cell cultures, before and after implantation. Scaffolds of PLGA degrade quickly and may be useful for the formation of mature tissue prior to implantation.

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