

# Porous alginate/poly( $\epsilon$ -caprolactone) scaffolds: preparation, characterization and *in vitro* biological activity

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**Abstract.** In bone tissue engineering, scaffolds with controlled porosity are required to allow cell ingrowth, nutrient diffusion and sufficient formation of vascular networks. The physical properties of synthetic scaffolds are known to be dependent on the biomaterial type and its processing technique. In this study, we demonstrate that the separation phase technique is a useful method to process poly( $\epsilon$ -caprolactone) (PCL) into a desired shape and size. Moreover, using poly(ethylene glycol), sucrose, fructose and Ca<sup>2+</sup> alginate as porogen agents, we obtained PCL scaffolds with three-dimensional porous structures characterized by different pore size and geometry. Scanning electron microscopy and porosity analysis indicated that PCL scaffolds prepared with Ca<sup>2+</sup> alginate threads resemble the porosity and the homogeneous pore size distribution of native bone. In parallel, MicroCT analysis confirmed the presence of interconnected void spaces suitable to guarantee a biological environment for cellular growth, as demonstrated by a biocompatibility test with MC3T3-E1 murine preosteoblastic cells. In particular, scaffolds prepared with Ca<sup>2+</sup> alginate threads increased adhesion and proliferation of MC3T3-E1 cells under basal culture conditions, and upon stimulation with a specific differentiation culture medium they enhanced the early and later differentiated cell functions, including alkaline phosphatase activity and mineralized extracellular matrix production. These results suggest that PCL scaffolds, obtained by separation phase technique and prepared with alginate threads, could be considered as candidates for bone

tissue engineering applications, possessing the required physical and biological properties.

## Introduction

Regenerated bone is proposed in tissue engineering applications for the treatment of many orthopaedic diseases, such as trauma, bone cancer, metabolic diseases and congenital malformations (1-3). Several approaches have been explored, but various experimental and clinical complications have been observed, including the limited availability of bone tissue, donor-site morbidity, and post-operative pain in regards to the use of autografts (4,5), and poor functional outcomes due to the limited vascularization, low cellularity, significant risk of pathogen transmission and immune reactions in the use of allografts (6). Furthermore, some limitations in the use of synthetic grafts are the fibrous encapsulation, the lack of remodelling and the subsequent fatigue-associated graft failure (7). Since the last decade, tissue engineering has provided viable alternatives for harvested tissues, implants and prostheses. As part of the rapid development of biomaterial technologies to repair orthopaedic defects, scaffolds made by biocompatible and bioresorbable polymers and composite materials have been developed for providing temporary support of damaged bone and to sustain tissue regeneration (8,9).

Scaffolds used in tissue engineering applications must have compatible biological and physical properties to match the physiological conditions *in vitro* and *in vivo*. Their physical and structural properties, such as interconnectivity, porosity and surface morphology, play a key role in cell adhesion, behaviour and tissue development. Moreover, to be considered appropriate for bone regeneration applications the scaffolds must fulfill basic criteria, including having mechanical properties similar to those of the bone repair site, biocompatibility, biodegradability and osteoconductivity (10-13). The fundamental concepts guiding the development of bone tissue-engineered scaffolds are based on the selected biomaterial and the production method (14). To date, many synthetic polymers have been investigated as scaffolds for bone tissue engineering, including poly(glycolic acid) (PGA), the simplest linear aliphatic polyester; poly(lactic acid) (PLA),

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an  $\alpha$ -polyester used widely in medical applications; their copolymers, such as poly(lactic-co-glycolic acid) (PLGA); and poly( $\epsilon$ -caprolactone) (PCL) (13,15,16). PGA and PLA are biodegradable and biocompatible; they have a degradation rate ranging from 12 months to over 2 years for PLA, and from 4 to 12 months for PGA. Their mechanical properties are relatively weak for high porosity scaffolds, which limit their usage on the hard tissue regeneration applications (11). Moreover, the breakdown products from scaffold degradation (glycolic acid for PGA and lactic acid for PLA) have been shown to induce cytotoxicity and local inflammatory responses (17), particularly in the case of low vascularization due to slow drainage of waste products from the implant site (18,19).

PCL is a semicrystalline  $\alpha$ -polyester, approved by the Food and Drug Administration (FDA) for implantation in the human body. It degrades through hydrolytic scission and has resistance to rapid hydrolysis. Because of its biocompatibility properties, degradation rate and mechanical strength, it has been investigated as a biomaterial for medical drug delivery devices (20-25), and load-bearing bone tissue engineering applications (26,27). PCL is characterized by a resorption time slower than other polyesters (28,29), and a negligible weight loss (below 2% in 30 days) (30,31). After *in vivo* implantation, its mechanical properties appear to remain unchanged for about 5-6 months (32) and the hydrolytic degradation occurs after about 2 years (33). The slow degradation kinetics result in a reduced risk of acidosis from the rapid accumulation of acidic by-products (20).

A crucial role in bone formation is played by the pore sizes and the interconnectivity of synthetic biomaterials. Suitable pore size guarantees the migration and proliferation of osteoblasts and mesenchymal cells, as well as the diffusion of factors and vascularization (34-36), while the total porosity improves the connection of the implant biomaterial with the surrounding natural bone (37). The pore sizes which better sustain bone mineralization appear to be approximately 100-200  $\mu\text{m}$  (38) while the optimal total scaffold porosity is considered to be the one ranging between 80-90% (39).

Several different techniques have been established to obtain scaffolds with open pore structures. The most common three-dimensional biodegradable polymer scaffolding fabrication approaches include fiber bonding (40), solvent-casting/particulate leaching (41,42), gas foaming (43,44), phase separation (45-49) and electrospinning (10,50,51). Almost all techniques allow the production of porous, biodegradable scaffolds, which, however, result do not appear to be suitable for bone regeneration: fiber bonding and gas foaming techniques do not allow for a fine control of porosity, while solvent-casting/particulate leaching is used to produce only thin scaffolds (thickness  $\sim$ 3mm) which lack the mechanical property required for the load-bearing tissue. In contrast, the phase separation technique is characterized by high flexibility and could be appealing for obtaining bone tissue-engineered scaffolds: various parameters can be changed to tailor pore size and porosity and scaffolds with controlled morphology, defined shape and size (52-55).

The aim of the present study was to obtain PCL scaffolds by means of phase separation technique using different porogen agents in order to achieve three-dimensional porous matrices with defined porosity, mechanical properties and

microstructure which allow the osteoblastic adhesion, growth and differentiation.

## Materials and methods

**Reagents.** PCL (MW  $\sim$ 65 kDa, density 1.145) was purchased from the Sigma Chemical Company (St. Louis, MO). Sucrose and ethanol were obtained from Carlo Erba Reagenti (Rodano, Italy). MC3T3-E1 mouse embryo/fetus calvaria fibroblast cells were obtained from DSMZ (Braunschweig, Germany), while the  $\alpha$ -modified minimal essential medium ( $\alpha$ -MEM) and gentamicin/amphotericin were from Invitrogen (Grand Island, NY). The CellTiter 96<sup>®</sup> AQ<sub>ueous</sub> Non-Radioactive Cell Proliferation Assay was purchased from Promega (Madison, WI). All other chemicals and reagents were provided by the Sigma Chemical Company.


### *Preparation of PCL scaffolds by phase separation technique*

**PCL without porogen (PCL-WP).** PCL (17.5% w/w) was dissolved in tetrahydrofuran (THF) for 2 h at room temperature under continuous agitation. Distilled water (50% of the solution's weight) was then added to the PCL solution under stirring, and two phases (aqueous and gel) were obtained. The aqueous phase was discarded and the obtained gel was transferred into a polyethylene cylindrical mould (3 cm diameter, 4 cm high). The polyethylene moulds containing the gels were incubated at  $-30^{\circ}\text{C}$  until complete solidification (24 h). The solid cast was thrown out from the mould and the solvent THF was removed. The PCL scaffolds were incubated in ethanol three times at  $-30^{\circ}\text{C}$  for 24 h, followed by two washes with distilled water. Disk shaped slides of scaffolds were prepared (1 cm diameter, 1 mm thickness) and stored in water at  $4^{\circ}\text{C}$  until use.

**Porous PCL scaffolds.** Poly(ethylene glycol) (PEG), sucrose, fructose, or calcium alginate were used as porogen agents for the preparation of PCL scaffolds.

**PCL-PEG.** PEG 1000 was dissolved in THF together with PCL in order to obtain the following three PEG/PCL weight ratios: 15/100, 30/100 and 100/100. Distilled water (50% of the solution's weight) was added to the PCL-PEG solution under stirring, and two phases (an aqueous and a gel phase) were obtained. The gel phase was transferred into a polyethylene cylindrical mould. The samples were incubated at  $-30^{\circ}\text{C}$  until complete solidification (24 h). Subsequently, the solvent and PEG were removed by cold ethanol ( $-30^{\circ}\text{C}$ ) and water washings.

**PCL-sucrose (PCL-S).** Distilled water (50% of the solution's weight) was added to the initial PCL solution under stirring, and the aqueous and gel phases were collected. Sucrose particles were milled, sieved and three particle sizes (50-140  $\mu\text{m}$ , 140-180  $\mu\text{m}$  and 180-600  $\mu\text{m}$ ) were selected and added to the PCL gel phase at various weight ratios. The finest sucrose particles (50-140  $\mu\text{m}$  and 140-180  $\mu\text{m}$ ) were added to PCL in a 15/100 ratio (PCL-S<sub>1</sub>), while the largest ones (180-600  $\mu\text{m}$ ) were added in 15/100, 30/100 and 100/100 ratios (PCL-S<sub>2</sub>). The gel phase with porogen was transferred into a polyethylene cylindrical mould and incubated at  $-30^{\circ}\text{C}$  until complete solidification (24 h). The solvent and sucrose were then removed as previously described.

 SPANDIDOS PUBLICATIONS *fructose spheres (PCL-F)*. Fructose spheres were

by an emulsion technique (56). Fructose (20 g) was melted for 90 min at 120°C until complete liquefaction. An emulsion was obtained by adding mineral oil (20 ml) and Span 80 (0.52 ml) under constant stirring. The resulting mixture was cooled in an ice bath to allow the formation of fructose spheres. After removing the mineral oil, the obtained spheres were washed five times with hexane, heated for 30 min at 37°C to compact the spheres and eliminate the hexane, were dried under vacuum and finally sieved selecting the sizes from 150-400 μm. The PCL gel was loaded with the fructose spheres using the following fructose/PCL weight ratios: 25/100, 50/100 and 100/100. The gel was then placed into a mould and incubated at -30°C until complete solidification (24 h). The solvent and fructose were removed as previously described.

*PCL-calcium alginate spheres (PCL-AS) and PCL-calcium alginate threads (PCL-AT)*. Sodium alginate (0.9% w/v) was dissolved in water by constant agitation overnight at room temperature (57). Calcium alginate threads or beads were obtained by dripping the solution of sodium alginate into a CaCl<sub>2</sub> solution (50 mM) using a syringe needle of 800 μm bore constantly or not immersed into the solution. Ca<sup>2+</sup> alginate beads (with alginate/PCL weight ratios of 50/100 and 100/100) or Ca<sup>2+</sup> alginate threads [with alginate/PCL 100/100 weight ratio (PCL-AT<sub>1</sub>) or with alginate/PCL 200/100 weight ratio (PCL-AT<sub>2</sub>)] were added to the gel phase. The gel was then transferred into the mould and incubated at -30°C until complete solidification (24 h). The THF solvent was removed by incubating the PCL scaffolds in ethanol for three times at -30°C for 24 h. Ca<sup>2+</sup> alginate spheres/threads were dissolved and removed with a sodium phosphate solution (0.1 M, pH 7.0) and then with water.

*Scaffold characterization*. The absence of THF residual solvent within the scaffolds was verified by analyzing the wash solutions by gas chromatography-mass spectrometry (GC-MS) (Varian 3800 GC and Varian Saturn 2000 ion trap MS). Dioxane was used as an internal standard.

Morphological and pore size analysis was performed using a scanning electron microscope (SEM) (58-60). The samples were lyophilized, frozen in liquid nitrogen, fractured, coated with gold and observed using the Stereoscan-205 S SEM (Cambridge Instruments, Cambridge, MA).

Porosity is conventionally considered the measure of void spaces over the total volume (61). Density and micro-computed tomography (MicroCT) (2,62) methods were used to determine the total porosity of the scaffolds. By the first method, total porosity was calculated applying the following equation:

$$\Phi = V_0/V_1$$

where  $\Phi$  is the total porosity,  $V_1$  is the geometrically measured volume, and  $V_0$  is the difference between  $V_1$  and the volume calculated by dividing the scaffold weight by the PCL bulk density.

MicroCT analysis was performed only for the PCL-WP and PCL-AT scaffolds. To preserve the scaffold's morphology all the specimens were cut (height, 1 cm; side, 0.5 cm) with a

lancet preparing regular surfaces. The upper side of each sample was marked to keep the same orientation during the MicroCT acquisition and compression test. Samples were scanned following standard processing procedure using the SkyScan 1172 high resolution MicroCT (SkyScan, Aartselaar, Belgium). The MicroCT settings used were as follows: voltage 57 kV; current 167 μA; exposition time 242 msec; field of view (FOV) 1280 x 1024 pixels; isotropic voxel size 8 μm. All the samples underwent a 360° rotation, 0.4° rotation step and a frame averaging 1. The raw data acquired were reconstructed using the NRecon software (SkyScan). Axial subsequent images (saved as bitmap format) were reconstructed using a back projection algorithm. The bitmap images were analyzed by the SkyScan MicroCTAnalyzer software: the selected volume of interest (VOI) (3x1.59 mm) was focused in the centre of each PCL scaffold to avoid artifacts from cutting. All the samples were binarized using the same parameters. Porosity and pore quantitative analysis were obtained based on the structural indices measured for bone samples as reported by Hildebrand *et al* (63). Sample porosity was calculated as:

$$\Phi = 1 - BV/TV$$

where  $\Phi$  is the total porosity, BV the bone volume and TV the total volume.

Trabecular thickness (Tb.Th) (or pore wall) and trabecular separation (Tb.Sp) (or pore diameter) were computed by direct measurements. Three-dimensional images were reconstructed by the CTVol software (SkyScan) and by the open source software, Osirix.

PCL-AT scaffolds were subjected to compression testing by the SkyScan Material Testing Stage and were compressed in a continuous mode. The maximum load was 22 kg with real-time visualization of the displacement curve.

*Modelling PCL replicas of a human finger phalanx*. A silicon mould of a human finger phalanx was prepared at the Paleontologic Laboratory-Ca' Foscari University of Venice, Italy. To obtain a replica of the phalanx bone, PCL-WP gel was injected into the pre-cooled mould (-20°C) and then incubated at -30°C until complete solidification (24 h). Subsequently, the solvent was removed by cold ethanol (-30°C) and water washings.

*MC3T3-E1 cell culture*. MC3T3-E1 murine pre-osteoblastic cells were cultured in T75 culture flasks (BD Biosciences, San Jose, CA) with  $\alpha$ -MEM and 10% foetal bovine serum (FBS) without antibiotic and were incubated at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. Cells from passage 8-12 were used for the experiments. PCL-AT scaffolds were sterilized in 95% ethanol for 2 h, incubated in PBS solution containing 2% gentamicin/amphotericin (Invitrogen) for 2 h, and then washed three times of 30 min each in  $\alpha$ -MEM. Scaffolds were placed in Millicel culture plate inserts (Millipore, USA), seeded with MC3T3-E1 cells (3x10<sup>4</sup> cells/cm<sup>2</sup>) and incubated at 37°C in a 5% CO<sub>2</sub> humidified atmosphere.

*Cell morphology*. At different time points (24 h, 7 and 14 days), samples were removed from the culture medium, rinsed

Table I. Different PCL scaffolds obtained using phase inversion technique.

Scaffold	Porogen	Porogen:PCL (w/w)
PCL-WP	None	0
PCL-P	PEG-1000	15:100; 30:100; 100:100
PCL-S <sub>1</sub>	Sucrose (range, 50-140 and 140-180 $\mu\text{m}$ )	15:100
PCL-S <sub>2</sub>	Sucrose (range, 180-600 $\mu\text{m}$ )	15:100; 30:100; 100:100
PCL-F	Fructose spheres (diameter, 150-400 $\mu\text{m}$ )	25:100; 50:100; 100:100
PCL-AS	Calcium alginate spheres (diameter, 1500 $\mu\text{m}$ )	50:100; 100:100
PCL-AT <sub>1</sub>	Calcium alginate threads (diameter, 1000-1500 $\mu\text{m}$ )	100:100
PCL-AT <sub>2</sub>	Calcium alginate threads (diameter, 1000-1500 $\mu\text{m}$ )	200:100

with PBS and fixed using glutaraldehyde (3%) in sodium cacodylate buffer (0.1 M, pH 7.2) for 24 h at 4°C. The scaffolds were then washed three times with sodium cacodylate buffer, dehydrated through a graded series of ethanol and air dried. The samples were coated with gold and observed by SEM.

**Cell viability.** Cell viability was monitored after 24 h, 72 h, 7 days and 14 days by the colorimetric MTS assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium). Metabolically active cells react with the tetrazolium salt in the MTS reagent to produce a soluble formazan dye that can be detected at 490 nm. At each time point, the cellular constructs were rinsed with PBS, in order to wash out unattached cells, and transferred into another well for a 3 h incubation with 20% MTS reagent prepared in culture medium. Thereafter, aliquots were pipetted into 96-well plates and the samples were read at 490 nm with an ELx808 Ultra microplate reader (BioTek Instruments). A calibration curve (cell number/cm<sup>2</sup> = 2.5x10<sup>5</sup>A<sup>490</sup>-8.6x10<sup>4</sup>, R<sup>2</sup>=0.99) was prepared by carrying out the assay with MC3T3-E1 cells cultured for 4 h.

**In vitro biological behaviour of MC3T3E1 cells on porous PCL-AT scaffolds.** The cells were seeded (3x10<sup>5</sup> cells/cm<sup>2</sup>) on PCL-AT<sub>1</sub> and PCL-AT<sub>2</sub> scaffolds placed in uncoated 48-well plates (BD Biosciences) containing 600  $\mu\text{l}$  culture medium. After 48 h of seeding, the cells were exposed for 7-21 days to a medium containing  $\alpha$ -MEM, 10% FBS, 1% APS, 0.1  $\mu\text{M}$  dexamethasone, 10 nM  $\beta$ -glycerophosphate and 0.05 mM ascorbate. In parallel, samples were maintained in proliferation medium as a negative control. The differentiation progression of MC3T3-E1 cultured on PCL scaffolds was analyzed as the alkaline phosphatase (ALP) activity, and the extracellular matrix calcium deposition at different time-points (7, 14, 21 days).

ALP activity was analyzed using p-nitrophenyl phosphate (p-NPP) as a substrate. The cell lysate was centrifuged at 12,000 x g for 10 min and the supernatant was incubated with p-NPP at 37°C for 30 min. The reaction was terminated by adding 3N NaOH and the absorbance was measured at 405 nm. All results were expressed as mean value  $\pm$  SD of three separate experiments consisting of triplicates.

Mineralized matrix deposition was detected conducting an *in vitro* mineralization assay using the Osteogenesis Assay

kit (ECM810, Millipore). The samples previously fixed with 10% formalin solution for 15 min were washed twice with distilled water and then incubated with Alizarin Red S for 20 min at room temperature. After washings with distilled water, and incubation for 30 min with 10% acetic acid under agitation, cellular monolayers were transferred to microcentrifuge tubes, heated to 85°C for 10 min, placed on ice for 5 min and centrifuged at 20,000 rcf for 15 min. After adding ammonium hydroxide, the amount of extracted Alizarin Red dye was measured at 562 nm and quantified against an Alizarin Red standard curve. All results were expressed as mean concentration ( $\mu\text{M}$ )  $\pm$  SD of three separate experiments performed in triplicate on differentiated and undifferentiated samples.

**Statistical analysis.** Data are expressed as mean value  $\pm$  SD of at least three different samples. Significant differences were estimated by analysis of variance (ANOVA) followed by the Student-Newman-Keuls t-test as a post-hoc test. P-values <0.05 were considered significant.

## Results

**Scaffold characterization.** Table I lists all PCL scaffolds and corresponding porogen agents used. As THF is cytotoxic, its residuals were completely removed from matrices before cell seeding, as demonstrated by the GC-MS profile of the ethanol solution used for the third washing of the samples (Fig. 1).

PCL scaffold characterization and pore size measurements were based on SEM observations while the overall porosity evaluation was performed using the density method and MicroCT.

### SEM analysis

**PCL-WP.** The PCL-WP scaffolds were characterized by a smooth external surface, while SEM analysis of their internal structure showed globular and hallow formations (20-30  $\mu\text{m}$  diameter) with macropores (~10  $\mu\text{m}$  diameter) (Fig. 2). The estimated total porosity was 70% and interconnected interstitial voids were detected.

**PCL-P.** SEM micrographs showed that PCL-PEG scaffolds, prepared with a PEG/PCL weight ratio of 15/100, demonstrated pore sizes ranging from 10 to 50  $\mu\text{m}$  (Fig. 3A),

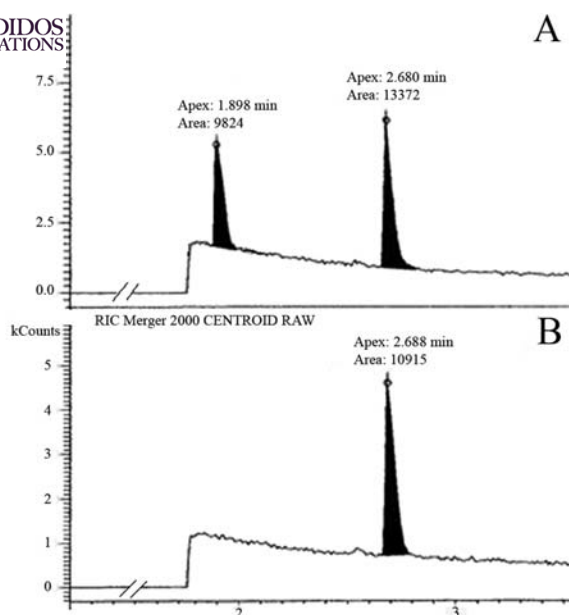


Figure 1. Detection of THF residual solvent within PCL scaffolds by gas chromatography-mass spectrometry (Varian 3800 gas chromatograph and Varian Saturn 2000 ion trap mass detector). (A) Gas chromatogram of a reference THF solution (0.01 M). (B) Gas chromatogram of the third ethanol wash solution used for solvent extraction.

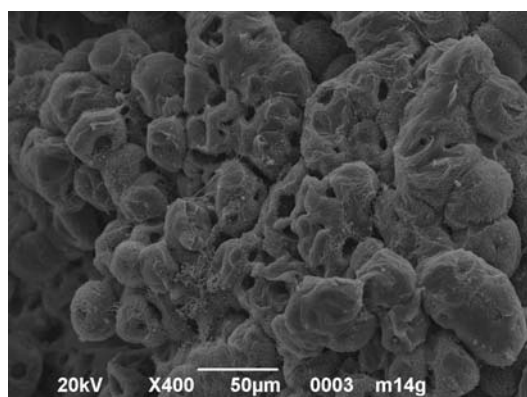


Figure 2. SEM micrograph of PCL scaffolds prepared without a porogen agent. Magnification x400.

while the internal structure of the one obtained using the 30/100 weight ratio demonstrated a higher microporosity grade (Fig. 3B). The total porosity measured was 72%. A more compact structure was observed when the 100/100 weight ratio was used (Fig. 3C).

**PCL-S<sub>1</sub> and PCL-S<sub>2</sub>.** When unm micronized sucrose was used as porogen, macropores were detected in the polymeric matrices with a mean dimension nearly the size of the used sucrose particles and appeared to be regularly distributed (Fig. 4A, B, C). Only interconnected trabecular micropores of <5 µm diameter were observed in PCL-S scaffolds obtained with micronized sucrose (Fig. 4D, E, F). A small increase in total porosity (from 75-85%) was detected in the scaffolds which was dependent on the increasing sucrose/PCL ratio.

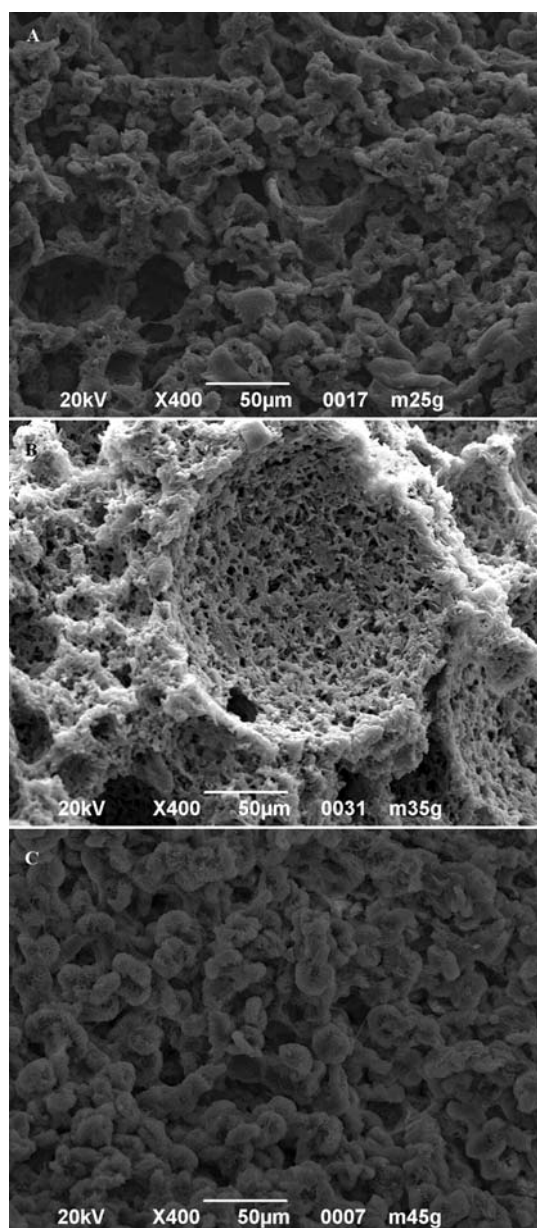


Figure 3. SEM micrographs of PCL scaffolds obtained using polyethylene glycol 1000 (PEG) as a porogen agent. (A) PCL-15% PEG, (B) PCL-30% PEG, (C) PCL-100% PEG. Magnification x400.

**PCL-F.** PCL-F scaffolds were loaded with fructose spheres (150-400 µm) as a porogen. PCL-F with a fructose/PCL ratio of 25/100 (Fig. 5A), 50/100 (Fig. 5B), and 100/100 (Fig. 5C) were characterized by the presence of evenly distributed spherical cavities, ranging in size from ~50 to 200 µm, within a continuous and microporous PCL matrix. Global porosity was dependent on the fructose loading and was <80% in all samples.

**PCL-AS and PCL-AT.** The PCL-AS (Fig. 6A and B) and PCL-AT (Fig. 6C and D) scaffolds had similar internal structure and morphology. Both PCL scaffolds showed bimodal porosity with large (50-250 µm) and small (5-10 µm) cavities and total porosity varying between 72-79%. Using calcium alginate as a porogen agent, either in spheres or in threads, we obtained scaffolds with a good porosity and a

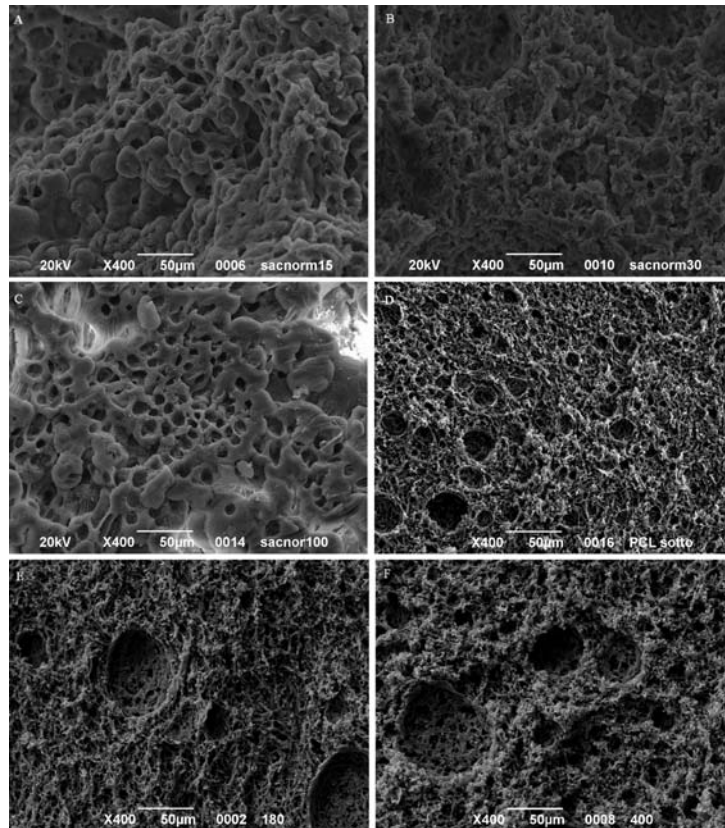


Figure 4. SEM micrographs of PCL scaffolds obtained using unmicronized (A-C) and micronized sieved (D-F) sucrose as a porogen agent. (A) PCL-15% sucrose, (B) PCL-30% sucrose, (C) PCL-100% sucrose, (D) PCL-15% sucrose (diameter 140  $\mu\text{m}$ ), (E) PCL-15% sucrose (diameter 180  $\mu\text{m}$ ), (F) PCL-100% sucrose (diameter 400  $\mu\text{m}$ ). Magnification x400.

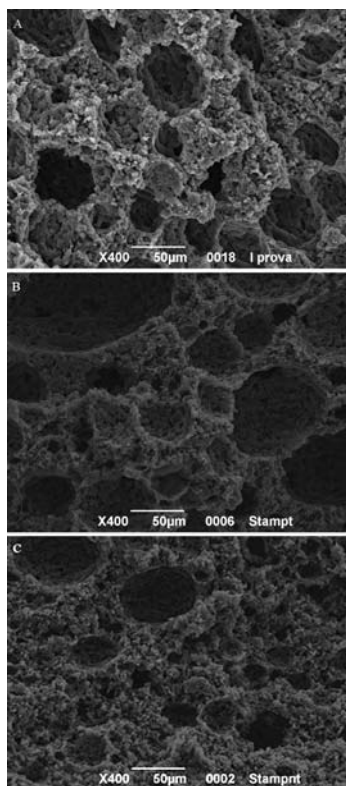


Figure 5. SEM micrographs of PCL scaffolds obtained using fructose spheres (size range, 150-400  $\mu\text{m}$ ) as a porogen agent. (A) PCL-25% fructose spheres, (B) PCL-50% fructose spheres, (C) PCL-100% fructose spheres. Magnification x400.

wide distribution of pore size, which are the two required properties for a bone substitute. Moreover, the scaffolds obtained with  $\text{Ca}^{2+}$  alginate threads showed appropriate macropore interconnectivity (Fig. 7B) in comparison to the PCL-AS scaffolds (Fig. 7A). On the basis of this evidence, scaffold characterization by MicroCT analysis and cell viability and biological activity studies were performed only on PCL-AT and PCL-WP scaffolds.

*MicroCT.* As reported in Table II, porosity and pore diameter (Tb.Sp) values increased proportionally to the increase in the amount of the porogen  $\text{Ca}^{2+}$  alginate threads, in contrast to BS/BV and trabecular thickness (Tb.Th) which remained nearly constant. Only the PCL-AT<sub>2</sub> scaffold was characterized by parameters which were all in accordance with those of natural bone and especially in regards to the distribution of pore diameter (Fig. 8) (63). The three-dimensional scaffold reconstruction confirmed the higher number of interconnected pores in PCL-AT<sub>2</sub> (Fig. 9), suggesting a trabecular bone-like framework.

Load-displacement tests (Fig. 10) showed that PCL-AT<sub>2</sub> scaffolds underwent less displacement (3 mm vs. 4.6 mm for the PCL-AT<sub>1</sub> and 4.5 mm for the PCL-WP scaffolds) and showed a lower load of shift/deformation at start (1.794 g for the PCL-AT<sub>2</sub> vs. 3.447 g and 3.200 g for the PCL-AT<sub>1</sub> and PCL-WP scaffolds, respectively). On the other hand, the elastic recovery of PCL-AT<sub>2</sub> (~2 mm) was higher than that of the PCL-AT<sub>1</sub> (1.3 mm) and PCL-WP (0.6 mm) scaffolds.

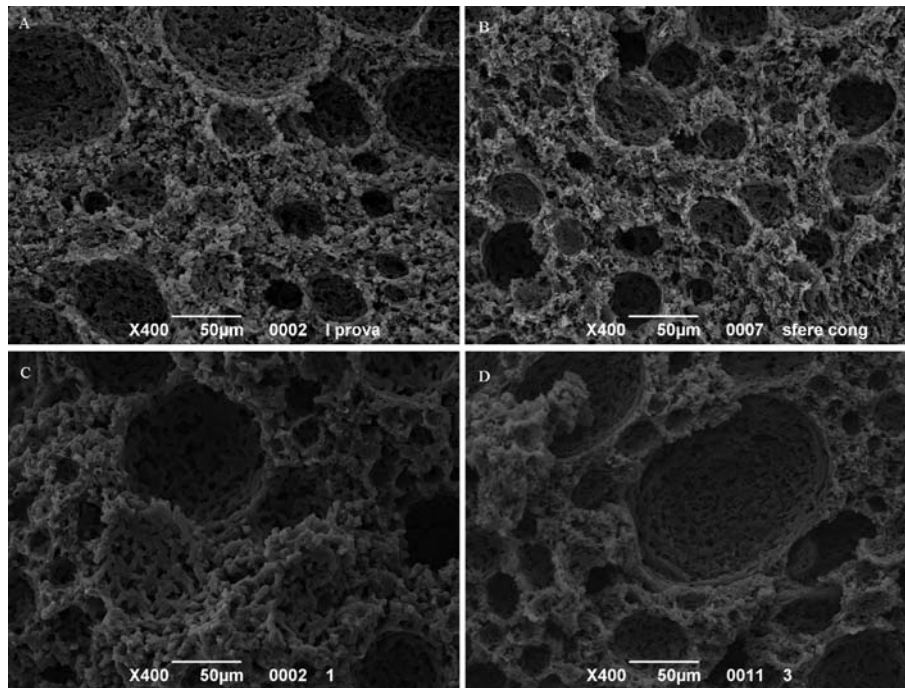


Figure 6. SEM micrographs of PCL scaffolds obtained using  $\text{Ca}^{2+}$  alginate spheres (diameter  $800\ \mu\text{m}$ ) (A, B) and  $\text{Ca}^{2+}$  alginate threads (C, D) as porogen agents. (A) PCL- $\text{Ca}^{2+}$  alginate spheres (200/100 ratio), (B) PCL- $\text{Ca}^{2+}$  alginate spheres (100/100 ratio), (C) PCL- $\text{Ca}^{2+}$  alginate threads (200/100 ratio), (D) PCL- $\text{Ca}^{2+}$  alginate threads (100/100 ratio). Magnification  $\times 400$ .

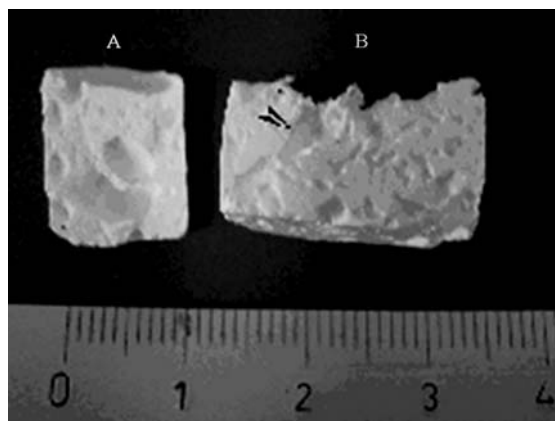


Figure 7. Transversal section of porous PCL scaffolds prepared with  $\text{Ca}^{2+}$  alginate spheres (A) and threads (B). More interconnected pores were observed within the PCL  $\text{Ca}^{2+}$  alginate thread scaffolds.

**Modelling PCL replicas of finger phalanx.** Using a silicon mould (Fig. 11A) prepared on a model of a human finger phalanx bone, we obtained PCL-WP matrix replicas shaped in a pre-determined form (Fig. 11B). The PCL gel technique approach used herein allowed the construction of PCL scaffolds presenting a volume contraction of about 8% in respect to the original bone. This effect could be overcome by making a mould slightly larger than the original bone target using a computer-assisted three dimensional modelling and printing. Thus, the replica could be obtained with the exact required dimension. All the external typical characteristics of phalanx bone, as epiphysis and periosteum, were successfully reproduced using the PCL gel technique (Fig. 11C).

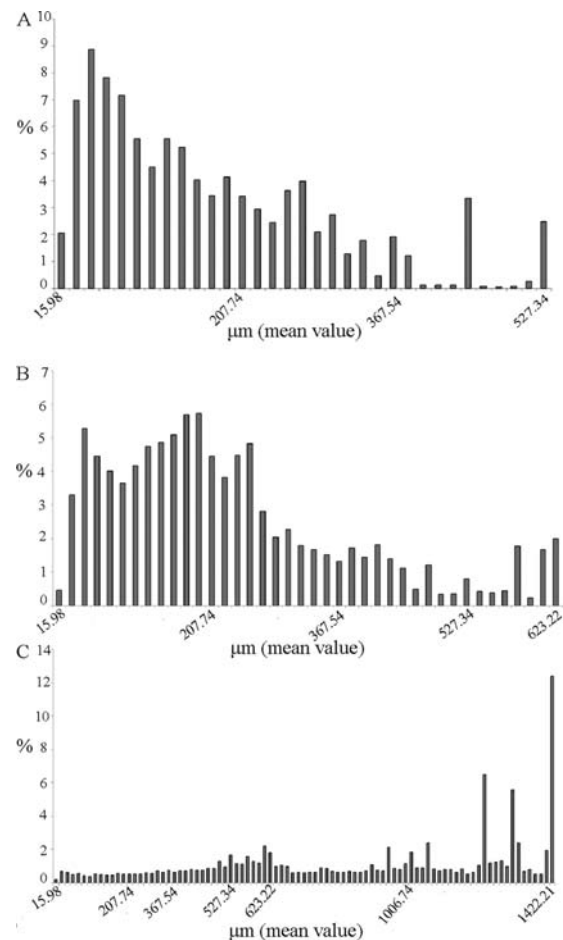


Figure 8. Trabecular separation (Tb.Sp) values measured by MicroCT in PCL scaffolds. (A) PCL-WP; (B) PCL- $\text{Ca}^{2+}$  alginate threads (100/100 ratio) (PCL-AT<sub>1</sub>), (C) PCL-  $\text{Ca}^{2+}$  alginate threads (100/200 ratio) (PCL-AT<sub>2</sub>).

Table II. MicroCT characterization of PCL-WP and PCL/Ca<sup>2+</sup> alginate threads scaffolds in comparison to native trabecular bone.

Sample	$\Phi$ (%)	BS/BV (1/ $\mu\text{m}$ )	Tb.Sp mean ( $\mu\text{m}$ )	Tb.Th mean ( $\mu\text{m}$ )
PCL-WP	21 $\pm$ 1.52	0.017 $\pm$ 0.0035	172 $\pm$ 6.65	101 $\pm$ 7.75
PCL-AT <sub>1</sub>	52 $\pm$ 2.1	0.024 $\pm$ 0.0037	231 $\pm$ 6.03	91 $\pm$ 3.51
PCL-AT <sub>2</sub>	73 $\pm$ 4.04	0.018 $\pm$ 0.0036	913 $\pm$ 8.88	103 $\pm$ 4.58
Trabecular Bone <sup>a</sup>	52-96	0.007-0.034	450-1031	80-280

$\Phi$ , total porosity; BS, bone surface; BV, bone volume; TV, total volume; BV/TV, percent of bone volume; Tb.Sp, trabecular separation (pore diameter); Tb.Th, trabecular thickness (pore wall). <sup>a</sup>Values as reported by Hildebrand *et al.* (63).

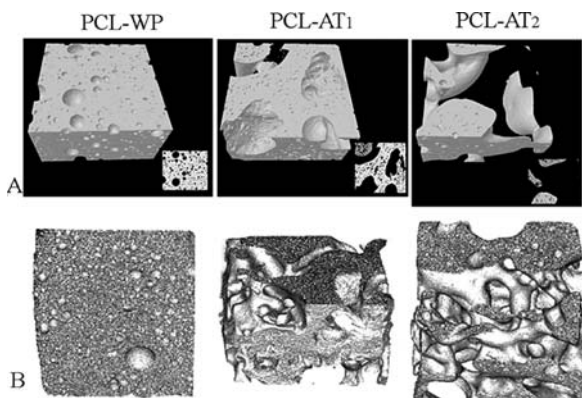


Figure 9. (A) 3D MicroCT isosurface image of PCL scaffolds with various porogen ratios. The insert represents a 2D MicroCT image. (B) 3D image obtained by the Osirix software.

#### Scaffold biocompatibility

**Cell viability.** PCL biocompatibility was evaluated by the MTS assay. Cell viability (expressed as cell number) at 24 h, 72 h, 7 and 14 days after seeding on PCL-WP, PCL-AT<sub>1</sub> and PCL-AT<sub>2</sub> (Fig. 12). Cell number generally increased until 72 h and subsequently decreased in a scaffold-type dependent mode. While no significant difference in cell number was detected between samples at 24 h and 72 h, a strong decrease in cell viability was observed on the PCL-WP scaffolds at 7 and 14 days. In contrast, PCL-AT<sub>1</sub> maintained a good cell viability until 14 days while PCL-AT<sub>2</sub> showed a statistically significant decrease ( $p < 0.05$ ) in cell viability at 14 days compared to 7 days.

**Cell morphology.** SEM micrographs revealed that, after 24 h incubation time, MC3T3-E1 cells adhered to the polymeric scaffolds (Fig. 13A, B and C) and appeared flattened showing an irregular and elongated form. All polymeric surfaces were completely covered by cells after 7 (Fig. 13D, E and F) and 14 days (Fig. 13G, H and I). Moreover, in the case of PCL scaffolds loaded with alginate threads, cells seemed to grow with a multilayer arrangement.

**ALP activity.** Low ALP enzyme activity of MC3T3 E1 cells was found in all constructs at 7 days independent of the culture conditions. In contrast, at 14 days the enzyme activity

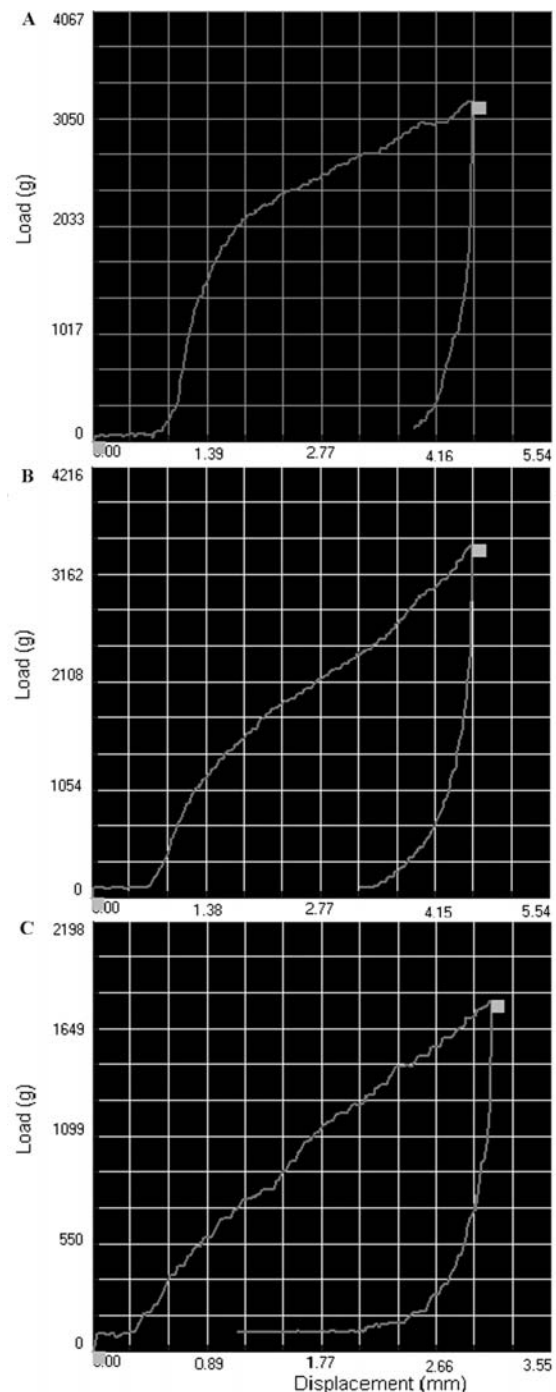


Figure 10. Load-displacement test by MicroCT. (A) PCL-WP, (B) PCL-AT<sub>1</sub>, (C) PCL-AT<sub>2</sub>.

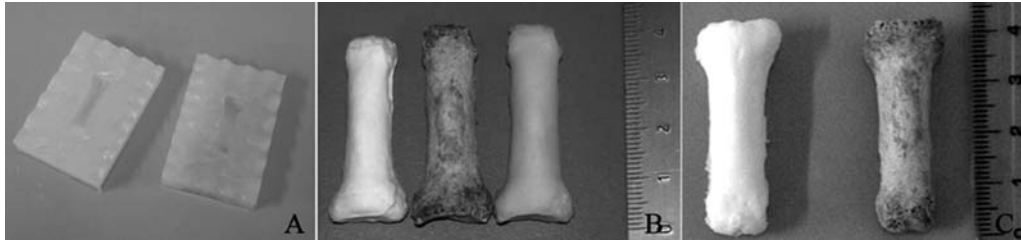


Figure 11. A silicon mould reproducing a human finger phalanx (A) was used to obtain a pre-determined form scaffold. Comparison of human phalanx bone (center) with replicas obtained using PCL (left), or resin (right) (B). Using a mould 8% larger than the original structure, the size of the PCL phalanx was comparable to the one of native bone.

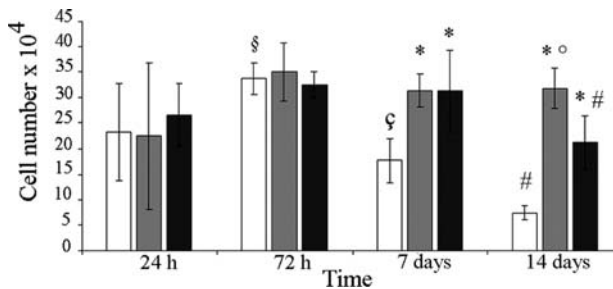


Figure 12. Cell number/cm<sup>2</sup> of MC3T3-E1 cells on PCL-WP (white), PCL-AT<sub>1</sub> (grey) and PCL-AT<sub>2</sub> (black) evaluated by the MTS viability assay. The values were obtained from absorbance data of the proliferation assay using a straight calibration line. Data are expressed as the mean  $\pm$  SD of three similar experiments carried out in triplicate mode. Statistical significance: \* $p < 0.05$  from PCL-WP;  $\circ p < 0.05$  from PCL-AT<sub>2</sub>; # $p < 0.05$  from same scaffold at 24 h,  $\S p < 0.05$  from same scaffold at 72 h,  $\S p < 0.05$  from same scaffold at 72 h.

in osteogenic stimulated and unstimulated PCL-AT<sub>1</sub> and PCL-AT<sub>2</sub> constructs consistently increased in comparison to PCL-WP ( $p \leq 0.05$ ). Although the ALP activity was significantly decreased in all samples at 21 days of culturing, PCL-AT<sub>2</sub> constructs under osteogenic stimulation conditions showed a significant difference in ALP expression ( $p \leq 0.05$ ) in comparison to PCL-WP and PCL-AT<sub>1</sub> (Fig. 14).

**In vitro mineralization assay.** As shown in Fig. 15, calcium deposits of cells cultured under differentiation conditions were significantly higher in PCL-AT<sub>1</sub> and PCL-AT<sub>2</sub> constructs compared to PCL-WP scaffolds at 14 days, and progressively increased at 21 days of culture. Under basal conditions, cells produced mineralized extracellular matrix at 14 days of culturing in PCL-AT<sub>2</sub> ( $p \leq 0.05$ ) and at 21 days in PCL-AT<sub>1</sub> compared to PCL-WP ( $p \leq 0.05$ ).

## Discussion

Scaffolds with physical and mechanical properties represent a keystone in tissue engineering to guarantee a structural support for cell adhesion and growth (64). The porosity of bone structures was thoroughly investigated in order to identify a synthetic substitute structure suitable for bone tissue engineering approaches as the appropriate pore size and its distribution facilitate a complete tissue regeneration (65-67). Hildebrand *et al* (63) reported that the average

trabecular thickness of cancellous bone is 100-150  $\mu\text{m}$ , while Jowsey (65) calculated the cross-section of Haversian systems to be about 50-250  $\mu\text{m}$  in a human cortical bone. According to Hulbert *et al* (38), the estimated minimum recommended pore size for a bone scaffold to be 100  $\mu\text{m}$ . In contrast, further studies have shown that larger pores (diameter,  $>300 \mu\text{m}$ ) (68) enhance direct osteogenesis, while smaller ones sustain osteochondral ossification. Although scaffolds with a porosity not less than 70% and with a pore size between 100-900  $\mu\text{m}$  are commonly proposed for bone tissue engineering applications, the presence of pores with 5-15  $\mu\text{m}$  diameter could be useful to promote neovascularization (69), while pore sizes ranging between 15-50  $\mu\text{m}$  permit a proper nutrient diffusion (38,70).

Scaffold mechanical properties for bone regeneration applications are dependent on pore size and other factors including the biomaterial composition and its degradation rate. Scaffolds fabricated from biomaterials with a high degradation rate should not have high porosities (90%), since its rapid depletion will compromise the mechanical and structural integrity before substitution by the newly formed bone. In contrast, scaffolds produced with biomaterials with low degradation rates and robust mechanical properties can be highly porous, because the higher pore surface area interacting with the host tissue can accelerate degradation due to macrophages via oxidation and/or hydrolysis. To ensure a proper mechanical support as sufficient strength and stiffness in bone regeneration, the scaffold must have an *in vivo* degradation time of at least 6 months (11).

In this work, the material used, PCL, is a wide-spread biocompatible and biodegradable aliphatic polyester, with a low melting point and good solubility in most solvents (28,29,33). PCL has been approved by the FDA for preparing biomedical devices as urethral catheters, drug delivery systems and resorbable sutures (71), and it has been proposed as a material for tissue engineering of bone and cartilage (11,20-25). Due to its hydrophobic characteristics and semi-crystalline solid phase structure, the entry of biological fluids within PCL scaffolds is hindered and therefore, subsequent chemical degradation is delayed thus, avoiding the local accumulation of toxic substances and the onset of inflammatory processes (20). The long degradation time (12-24 months) of PCL in *in vivo* settings is a property desirable particularly, for bone substitute prosthesis (28-30).

The purpose of this study was to investigate the phase separation technique in order to obtain a three-dimensional PCL porous scaffold with a structure, porosity and mechanical

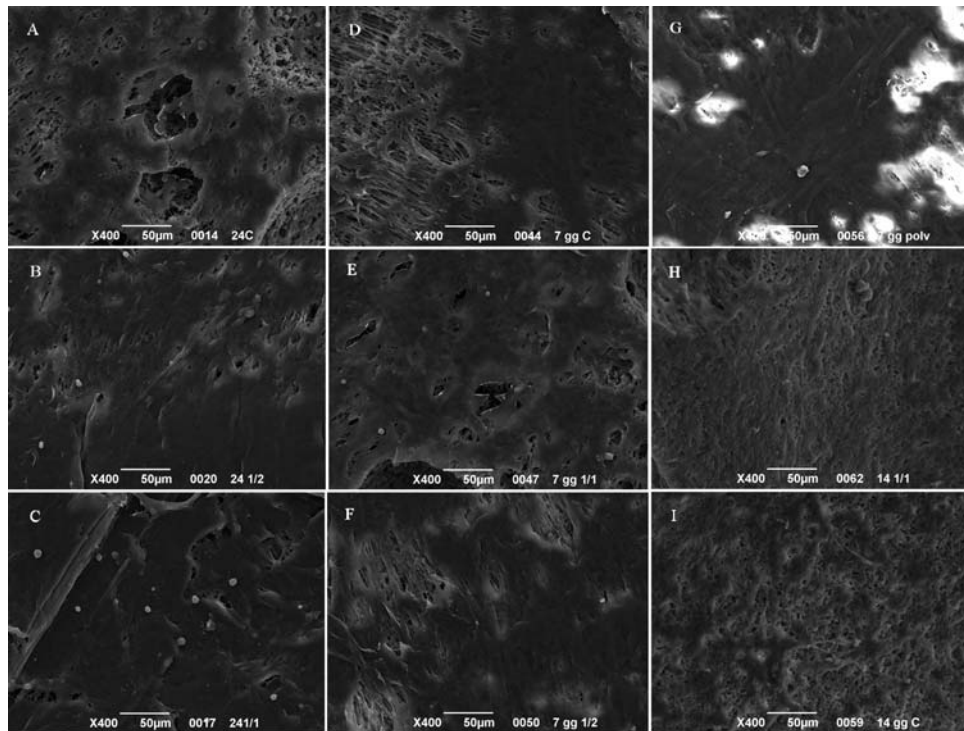


Figure 13. SEM micrographs of MC3T3-E1 osteoblasts on PCL-WP (A, D, G), PCL-AT<sub>1</sub> (B, E, H) and PCL-AT<sub>2</sub> (C, F, I) at incubation time of 24 h (A, B, C), 7 days (D, E, F) and 14 days (G, H, I). Magnification x400.

properties suitable for adhesion, growth, proliferation and differentiation of bone cells. The phase separation technique investigated herein has been proven to be very flexible and useful to prepare scaffolds for bone tissue engineering applications. The polymeric gel obtained can be moulded in any shape and size (52-55). Microporosity of the final object is dependent on the ratio between THF (good solvent for PCL) and water (bad solvent); instead macroporosity is obtained by adding an insoluble material (porogen) with a defined size to the gel phase. The porogen is subsequently removed with an appropriate washing procedure.

The organic solvent THF employed for initial solubilization of PCL is known to be cytotoxic (52) and requires to be removed from scaffolds before cell seeding. Using GC-MS analysis we have demonstrated that after three rinsings with cold ethanol any THF residual was completely removed from the mould.

PCL scaffolds obtained without adding any porogen agent (sample PCL-WP) showed a 70% porosity when evaluated by density measurement but only a 21% porosity when using the MicroCT analysis. These conflicting results could be explained by the fact that the SEM analysis of the PCL-WP revealed the presence of micropores with mean dimensions between 5-10  $\mu\text{m}$  a range that is clearly below the threshold of the MicroCT instrument. Therefore, the MicroCT analysis in the PCL-WP sample accounted for pores bigger than approximately 10  $\mu\text{m}$  which account only for 21% of the sample volume, while the porosity due to the micropores, accounting for nearly 50% of the sample volume, was not accounted for by this technique. Using four different porogen agents (PEG 1000, sucrose, fructose and calcium alginate) we have tried to introduce a macroporous structure in PCL

scaffolds making them more suitable for application in bone regeneration.

Adding the porogen PEG 1000 we have obtained PCL scaffolds presenting regular pores with size ranging between 10-50  $\mu\text{m}$ , as observed with SEM. This even pore distribution is, probably, a consequence of the co-solubility of PCL and PEG 1000 in THF.

The removal of porogens like sucrose, fructose and calcium alginate from the gel phase by washings ensures the formation of macropores with a mean dimensional distribution related to the porogen particles size used embedded in a continuous interconnected PCL micropore structure. Regarding sucrose and fructose, the macropores size distribution observed in the final scaffolds always resulted in lower than the initial dimension of porogen added to the gel phase. For example, addition of fructose spheres of 150-400  $\mu\text{m}$  resulted in macropores with a dimension ranging of 50-200  $\mu\text{m}$ . This effect could be explained considering the partial solubility of sucrose and fructose in the gel phase containing water.

In this work, the total porosity of all scaffolds were found to be dependent on the PCL:porogen ratio and varied from 72-85%. A biomaterial requirement to guarantee the cell ingrowth is that the interconnected macropores are of appropriate size and geometry. Our MicroCT data showed that only PCL scaffolds obtained using Ca<sup>2+</sup> alginate threads fulfill this requirement. The MicroCT analysis revealed that the PCL-AT<sub>2</sub> scaffold is characterized by morphometric indices (porosity, interconnectivity and distribution of pores values) very similar to those of natural trabecular bone (63). Moreover, as a major surface area stimulates cell attachment and growth (72), PCL-AT were supposed to improve the cellularity in comparison to PCL-WP. The scaffold PCL-AT<sub>2</sub> was shown

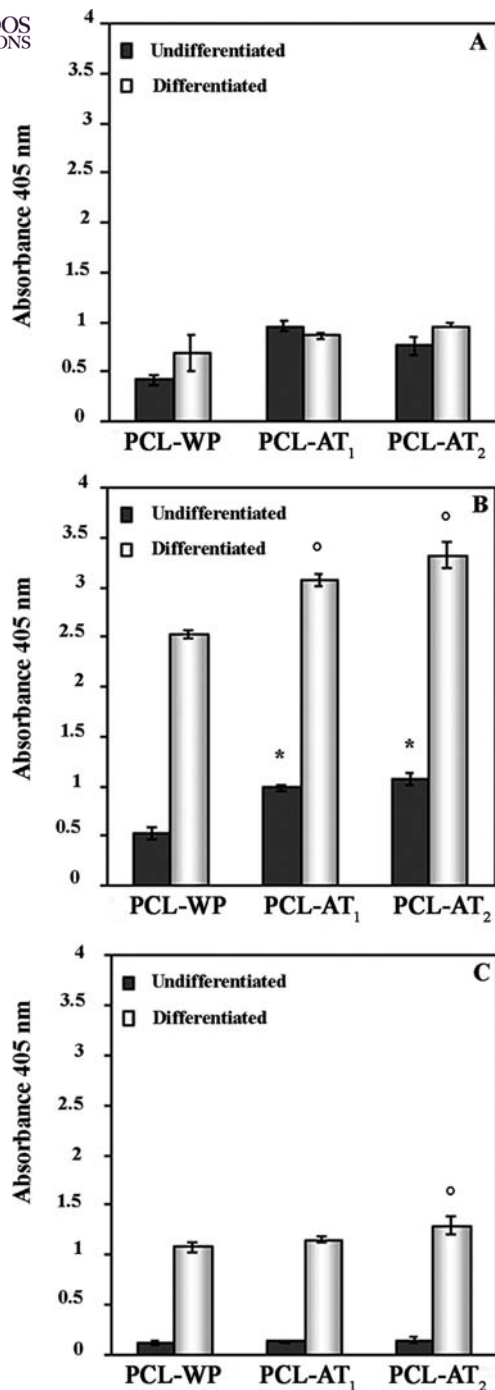


Figure 14. Effects of porosity grade induced by  $\text{Ca}^{2+}$  alginate threads on alkaline phosphatase activity in MC3T3-E1 cells at 7 (A), 14 (B) and 21 days (C). Absorbance detection at 405 nm. \* $p < 0.05$  vs. control.

to possess high elastic recovery, but a low shift/deformation and load-displacement unsuitable for bone substitute applications (36,73). The addition of a mineral component, like hydroxyapatite, to PCL-AT<sub>2</sub> could improve tensile strength and mechanical properties (74).

Biocompatibility analyses (MTS assay and SEM) demonstrated that PCL-AT<sub>2</sub> scaffolds present an environment more suitable for preosteoblastic cell cultures compared to PCL-AT<sub>1</sub> and PCL-WP. At each evaluation time, the MTS analysis revealed a significant increase in cell growth on PCL-AT<sub>2</sub> compared to PCL-AT<sub>1</sub> and PCL-WP. The interconnected

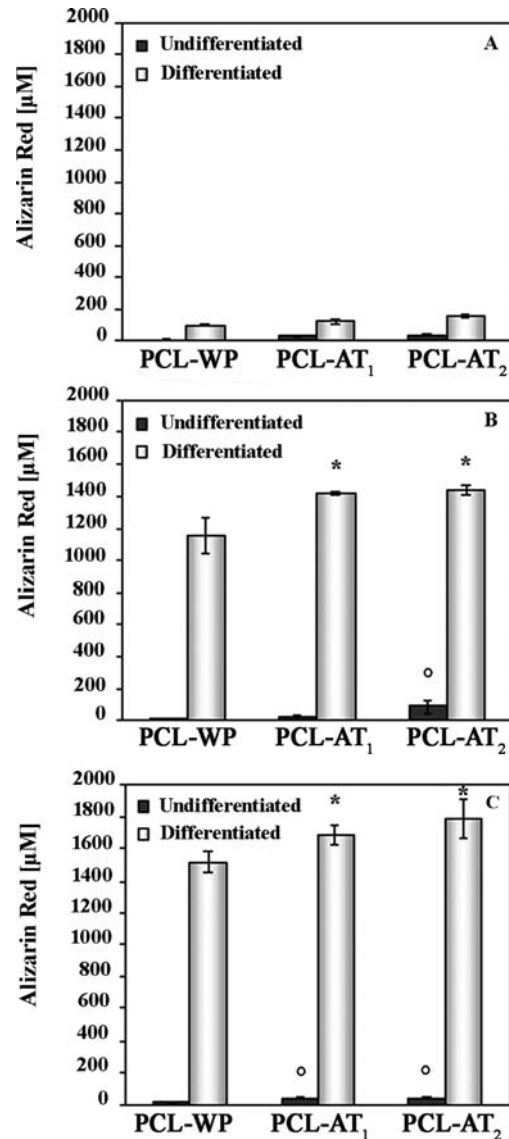


Figure 15. Quantification of calcium deposition by Alizarin Red staining by spectrophotometrical measurement at 562 nm at 7 (A), 14 (B) and 21 days (C). Values are reported as the mean concentration of Alazarin Red solution extracted from samples of three independent experiments consisting of triplicates. \* $p < 0.05$  vs. control.

pores within the scaffold ensures a major exchange of nutrients and oxygen favoring cell growth and removal of waste products (69-71). The decreased cell number observed after 7 days of culture in PCL scaffolds could be attributed to bone cell cycle properties. Indeed, it has been demonstrated that MC3T3-E1 cells at maximal confluence, reduce the proliferation rate and then start the differentiation process (75).

In this work, using  $\text{Ca}^{2+}$  alginate threads as a proagen, we obtained scaffolds of PCL with a porosity grade which met the criterion commonly used to obtain an excellent bone cell ingrowth and differentiation. PCL-AT<sub>1</sub> and PCL-AT<sub>2</sub> were shown to guarantee a better cellular microenvironment and the properly sized interconnected pores seemed to improve the biological properties of PCL scaffolds as demonstrated by the higher ingrowth and responsiveness of MC3T3E1 under basal culture conditions. Furthermore, the total porosity and the pore size in PCL-AT<sub>1</sub> and PCL-AT<sub>2</sub> scaffolds were

demonstrated to be suitable for sustaining osteogenic differentiation as demonstrated by the increased ALP activity and mineralized extracellular matrix deposition. These results are in accordance with Wang *et al.* (76) that reported that pores with a diameter  $>150 \mu\text{m}$  facilitate internal mineralized bone formation.


In conclusion, our results demonstrate that scaffolds prepared with PCL and  $\text{Ca}^{2+}$  alginate threads might be considered for bone prosthesis applications as they guarantee a microstructure suitable for bone cell growth and differentiation and ideal porosity grade.

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