Natural and Synthetic Hydroxyapatite Filled PCL: Mechanical Properties and Biocompatibility Analysis

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ABSTRACT: Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) is one of the most biocompatible ceramics because it is similar to the mineral constituents of human bone and teeth. Composites of hydroxyapatite (HA) and biodegradable polymers such as polycaprolactone (PCL) are interesting materials for medical applications, especially for bone replacement. With the aim to improve the properties of biodegradable polyester-based devices, we have prepared and characterized novel composites made of polycaprolactone and natural or synthetic hydroxyapatite. The composites were screened for cytocompatibility by a direct contact method, in view of the future application of these hydroxyapatite filled PCL polymers as scaffolds for bone engineering. Primary cultures of human bone marrow mesenchymal stem cells (MSC) were selected as the most appropriate models to study the *in vitro* performance of these materials.

The results showed that all materials have good biocompatibility and allow expression of the osteoblastic features. Scanning electron microscopy provided direct evidence of intense cell adhesion and proliferation on the tested materials.

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INTRODUCTION

The increasing number of skeleton problems caused by sport and car accidents has created a growing interest for bone substitute materials. All materials designed for biomedical applications in humans must be submitted to *in vitro* and *in vivo* tests to verify biocompatibility, response and the behavior of the interacting cells according to the rules of EN ISO 10993.

The standard EN ISO 10993–5 contains two main test methods, indirect and direct, designed to provide predictive evidence of material biocompatibility. The indirect or elution test method is applicable to the screening of all types of medical devices and analyzes the behavior of cells exposed to fluid extracts from the material under investigation. The *in vitro* approach that is suitable for bone regeneration and repair devices, that are destined to be colonized by the tissue in which they are implanted, can be accomplished by a direct contact method. In this approach, cell populations typical of the implant site are cultured directly onto the material under investigation and observed, for a period of days or weeks, in terms of morphological and functional features.

In recent studies on bone engineering, an increasing interest has been directed to bone marrow mesenchymal stem cells (MSC) that are multipotential in that they can give rise to osteoprogenitors cells as well as to precursors for cartilage and adipose tissue [1]. In addition to progenitors for connective tissues, MSC also have the potential, under appropriate microenvironment and stimuli, to differentiate into myogenic precursors [2] and also into neural cells [3].

Among the different materials proposed for bone substitution application, biodegradable polyesters have acquired a particular niche of interest. In the frame of a project aiming to improve the properties of biodegradable polyester-based devices, we have prepared and characterized novel composites made of polycaprolactone (PCL) and natural or synthetic hydroxyapatite (HA). In a previous paper, we reported that the addition of natural hydroxyapatite powder (5% by weight) has a small but detectable effect on the mechanical as well as biocompatibility properties of PCL based composites [4].

In the present paper we extend our studies to synthetic hydroxyapatite and to a larger compositional range. We have also introduced a shape factor and compare the behavior of natural powders to that of synthetic whiskers. Dense HA ceramics manufactured by using synthetic HA powders have always exhibited a low fracture toughness

($1\,\mathrm{Mpa} \times \mathrm{m}^{1/2}$) in contrast to the values observed for human bones, in the range 2–12 $\mathrm{MPa} \times \mathrm{m}^{1/2}$ [5]. Reinforcement by whiskers has been considered as a way to improve the fracture toughness of pure HA bioceramics; thus, whisker-like or needle-like crystals of HA have been synthesized in recent years, mainly by the hydrothermal method or precipitation routes [6].

Although all the individual components have recognized biocompatibility, nevertheless it is necessary to analyze their behavior in the composite, as the interfacial phenomena can play a role in the cellular response, leading to unpredictable results. Hence, the materials designed as bone substitutes were tested for biocompatibility using primary cultures of human MSC that we selected as the most appropriate model on the basis of the reported results with such cell populations in bone engineering experiments [7]. In particular we assessed parameters such as cell adhesion, material colonization as well as the expression of the early biochemical marker of osteoblastic phenotype, namely alkaline phosphatase.

MATERIALS AND METHODS

Materials

Polycaprolactone (PCL) CAPA 650 was obtained from Solvay ($T_m = 60°C$, $M_n = 50{,}000$). $Ca(NO_3)_2 \times 4H_2O$, methylcellulose, $(NH_4)_2HPO_4$ and K_2SO_4 were all Fluka reagents, used as received. Natural hydroxyapatite (Ossein PET) was treated at 450°C to remove organic materials. Tissue culture media and biochemicals were from Gibco and plastic ware was from Falcon. All chemicals (Sigma) were of the highest grade available commercially.

Methods

Preparation of HA Powders

A methyl cellulose solution (3 mL of 0.1 g/L) was mixed with 1440 mL of distilled water. Then 0.152 mol (35.89 g) of $Ca(NO_3)_2 \times 4H_2O$ was dissolved in the above solution along with 0.09 mol (11.89 g) of $(NH_4)_2HPO_4$. After complete dissolution, 115 mL of 24 vol% NH_4OH was added and the solution was heated and stirred at 60–70°C for 3 h. The precipitates formed were recovered from the supernatant by filtering/centrifugation and washed five times with distilled water. The filtrates were dried at 100°C overnight. Finally the HA powder was calcined in air atmosphere at 1000°C for 6 h.

Synthesis of HA Whiskers

Calcined HA powders were ground by hand and dry-mixed with potassium sulfate ($T_m = 1069°C$) at a K_2SO_4 to HA weight ratio of 1.6. The HA weight after calcination was 12.63 g and 20.20 g of potassium sulfate were added. The mixture as a compact powder was heated to a peak temperature of 1190°C for 3.5 h. The sample was allowed to cool to room temperature within the shut off furnace. The whiskers were separated from the solidified mass by washing the mass several times with hot (~90°C) distilled water. Washing was repeated ten times; the washed whiskers were dried in an oven at 100°C overnight.

Preparation of PCL/Hydroxyapatite Composites

In a single screw extruder, specially designed for mixing, the two components were extruded at $T = 100°C$ at a screw speed of 30 rpm.

Sheets for testing of mechanical properties and for biocompatibility tests were prepared by compression molding at $T = 100°C$, $p = 4.5$ tons, time under pressure was 2.5 min.

Listed in Table 1 are the samples prepared and identified by abbreviations that, for simplicity, will be used from now on.

The mechanical properties of the composites were evaluated by tensile tests on an Instron Model 4301 machine. The size of the specimen (five samples for each composition) were 5 cm × 4 mm and 1 mm thick. Tests were performed at room conditions at a rate of 10 mm/min.

Scanning electron micrographs were taken on a Au/Pd coated film surface by using a Philips XL 20 SEM of the HA powder, HA whiskers and tensile specimen fracture surfaces.

Cytocompatibility Tests

Each polymer was obtained in four different preparations and each tested in triplicate. The samples as disks, with 4 cm^2 surface, were attached with biological silicone (NuSil Silicone Technology, CA, USA) onto the bottom of twelve multiwell plates and sterilized overnight with PBS containing penicillin (1000 units/mL), streptomycin (1000 μg/mL) and fungizone (12.5 μg/mL) at 37°C in a 5% CO_2 humidi-

Table 1. List of samples tested.

Composite	Code
PCL/HA Natural Powder 90/10	NP10
PCL/HA Natural Powder 80/20	NP20
PCL/HA Synthetic Whiskers 90/10	SW10
PCL/HA Synthetic Whiskers 80/20	SW20

fied atmosphere. The materials were then placed in complete culture medium and Opti-MEM was added with antibiotics and fungizone (at concentrations ten-fold lower than those indicated above), 10% FCS and sodium ascorbate (50 μg/mL) and kept in the incubator for an additional 36 h before plating the cells.

Preparation of Mesenchymal Stem Cells

The preparation of MSCs was performed employing heparinized human bone marrow and following the method of Friedenstein et al. with some modifications [8] (informed consent was provided according to the Declaration of Helsinki). Briefly, 2 mL-sample was diluted 1:5 with Opti-MEM, containing penicillin 100 units/mL and streptomycin 100 μg/mL, placed in 100 mm polystyrene dish and incubated at 37°C in a 5% CO_2 humidified atmosphere. After 24 h, the medium was collected and centrifuged at $800 \times g$. Half of the supernatant (conditioned medium, CM) was aspirated, mixed with an equal volume of fresh medium and placed in the first plate. The medium remaining in the tube was used to re-suspend the cellular pellet; after the addition of an equivalent volume of fresh culture medium, the entire cellular suspension was plated in a second dish. After one day, the medium of the second plate was centrifuged; the pellet, containing all nonadherent cellular elements, was discarded and the corresponding CM was added again to the dish. In 3–4 days, a few small foci of adherent fibroblast-like cells started to appear and develop until the whole dish surface was colonized in 2–3 weeks. During this period, fresh medium was added every 3 days, never removing at all the conditioned medium. After the reaching confluence, cells were trypsinized and plated at a density of $5 \times 10^3\,cm^{-2}$, always diluting the CM. Cultures between the third and sixth passages were used in our experiments.

MTT Assay

The MTT assay is a very simple and useful tool for evaluating cell vitality and proliferation [9]. The key component is 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Mitochondrial dehydrogenases of living cells reduced the tetrazolium ring, yielding a blue formazan product, which was measured spectrophotometrically. The amount of formazan produced is proportional to the number of viable cells present. MTT (5 mg/mL in DMEM without phenol red) was added to the wells in an amount equivalent to 10% of the culture medium. After an incubation of 4 h at 37°C, the liquid was aspirated and the insoluble formazan produced was dissolved in isopropanol. The optical densities were measured at 570 nm, subtracting background absorbance determined at 690 nm.

Cell Extracts Preparation

After the removal of the medium, the wells were rinsed with 20 mM TRIS.HCl−0.5 M NaCl, pH 7.4 (TBS) and the cells were solubilized with lysis buffer that is TBS containing DTT (0.5 mM), PMSF (0.5 mM) and that is Triton X–100 (0.25%) by carrying out three freezing-thawing treatments. The cell lysates were then centrifuged in microfuge at 12,000 × g for 5 min and the supernatants assayed for alkaline phosphatase activity. The protein concentration was determined using the protein assay kit (Bio-Rad Laboratories, CA, USA), according to the method of Bradford [10]. This protein assay was based on the complexing proteins with Coomassie blue. The protein sample was mixed with the reagent and then read at 595 nm after a short incubation at room temperature.

Alkaline Phosphatase Assay

Alkaline phosphatase (AP) activity was determined by measuring the release of p-nitrophenol from disodium p-nitrophenyl phosphate (PNPP) [11]. The reaction mixture contained 0.1 M diethanolamine buffer, pH 10.5, 0.5 mM $MgCl_2$ and 12 mM PNPP in a final volume of 0.5 mL. The reaction was initiated by the addition of the cell extract and the mixture was incubated at 37°C for 30 min. The reaction was stopped by adding 0.5 mL of 0.5 M NaOH and the absorbance was determined at 405 nm. Results are expressed as units/mg protein. One unit was defined as the amount of enzyme that hydrolyzes 1 nmol of PNPP/min under the specified conditions.

Scanning Electron Microscopy

After the removal of the medium, the specimens were fixed with 0.5% glutaraldehyde in 0.01 M-phosphate buffer, pH 7.4, for 20 min at room temperature. After being rinsed several times with the same buffer, they were dehydrated in an ethanol–water series of 30, 50, 70, 95% ethanol. The samples were submitted to critical-point drying and then coated with a thin layer of an alloy of gold and palladium. Successively, the samples were examined by using a Philips XL 20 electron microscope.

RESULTS AND DISCUSSION

Molten salt synthesis (MSS) is one technique for preparing ceramic powder bodies with whisker-like, needle-like or plate-like morphology [12]. The MSS technique is based on the use of low melting solvents, such as alkali chlorides, sulfates, carbonates or hydroxides, as the medium of reaction for the ceramics. We synthesized submicrometer-sized HA powders as one starting material for the MSS method. We then prepared HA whiskers by the MSS technique using K_2SO_4 salt as solvent. For the natural HA powder, we performed a simple solid state calcination process at 1100°C.

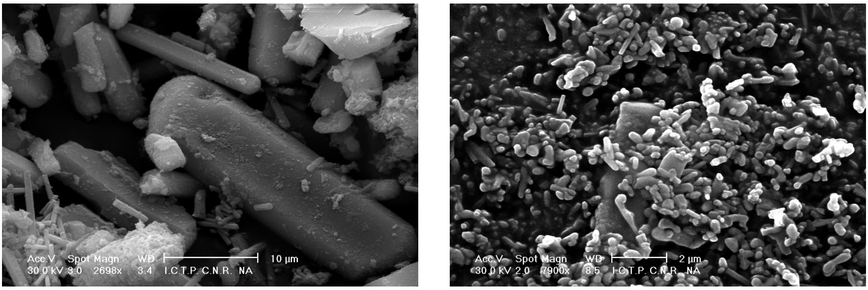
An FTIR (Fourier Transform InfraRed) spectrophotometer was used to measure the infrared transmission spectra of the whiskers, while Wide Angle X-Ray Spectroscopy (WAXS) was employed to determine their structural characteristics. The FTIR spectrum and WAXS diffraction pattern (not reported) of the synthesized whiskers are characteristic for hydroxyapatite formation and agree with reported data [12]. Typical IR bands originating from phosphate (1190 to 980 cm^{-1}) and hydroxyl groups (3570 and 635 cm^{-1}) are clearly visible, while most intense diffraction peaks are observed at 2θ of 31.7870 ($I/Io = 26$, $d = 2.8128$, $hkl = 211$) and at 2θ of 32.9093 ($I/Io = 100$, $d = 2.7194$, $hkl = 300$).

The morphology of the HA powder before and after calcination and of the HA whiskers was evaluated by scanning electron microscopy. The synthesized powder consisted of small (sub-micrometer sized) agglomerated particles. After the MSS treatment, a mixture of whiskers and larger crystals were obtained from both natural and synthesized powders. Most of the whiskers were 10–20 μm long and ~2 μm in diameter, so that the aspect ratio of whiskers was between 5 and 10.

Figure 1(a) refers to the SEM micrograph of synthetic whiskers, while the material in Figure 1(b) shows the appearance of natural hydroxyapatite after calcination; the cylinder shape of synthetic HA is evident.

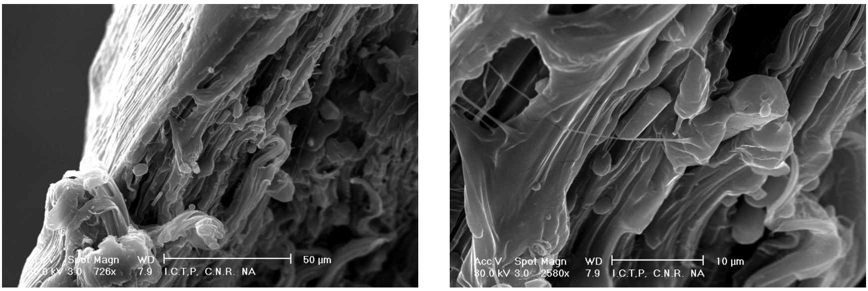
PCL is characterized by a low melting temperature and a very low glass transition temperature. To improve the mechanical response of the PCL-matrix, we prepared composites at two different percentage of filler, 10 and 20%. In Table 2, the tensile strength data for the prepared samples are listed.

For the composites with 10% of filler, some increase in toughness may be due to a nucleating effect of HA with either powder or whiskers. By increasing the filler content, the plasticity was decreased while rigidity increased. The shape of filler influenced mechanical response. In fact, whiskers are able to orient in the direction of flow, hence the composites were elongated.



(a) (b)

Figure 1. Scanning electron micrographs of (a) synthetic hydroxyapatite whiskers (2700×), (b) natural hydroxyapatite powder (2000×).



(a) (b)

Figure 2. Scanning electron micrographs of fracture surfaces of (a) SW20 (750×) and (b) SW20 (2500×).

Table 2. Mechanical properties of PCL and PCL-HA composites.

Sample	Stress at Peak (MPa) (+/−2)	Strain at Break (%) (+/−20%)	Young Modulus (MPa) (+/−10)
PCL	15	1200	328
NP10	35	1254	339
SW10	33	1297	288
NP20	16	637	479
SW20	24	999	337

Following the mechanical analyses, the scanning electron microscope was used to observe the broken surfaces on the composites. Figures 2(a) and 2(b) are SEM micrographs that show the broken surface at two different magnifications for sample SW20. Whiskers are clearly visible on the broken surface and most of them were oriented in a longitudinal direction. The whiskers showed poor adhesion to the PCL matrix as seen in the magnification (see Figure 2(b)).

BIOLOGICAL RESPONSE

All the samples and the polystyrene bottoms used as controls were subjected to the same treatment outlined in the Materials and Methods section, and then seeded with 10^4 cells/cm^2. Since the materials were opaque to microscopic observation, due to their semi-crystalline nature, the visual observation of the cells adherent to the samples was not possible and, consequently, it was not possible to evaluate cell adhesion. Thus, after 24 h, the medium was removed from the wells and the unattached cells were counted; all the composites allowed good cell adhesion, even better than the controls.

After 6 days of incubation, cell proliferation was monitored. Some samples were subjected to trypsin treatment to proceed to cell counting and cell extract preparation, while others were analyzed by the MTT test. For both polystyrene control and unfilled PCL, trypsinization was completed in a few minutes. However, for all the composites only a small percentage of cells were detached by trypsin, the majority of them remaining strongly linked to the material. Accordingly, the formazan crystals produced in the cells by the MTT assay also remained trapped inside the composites, and it was necessary to overnight treat with isopropanol to completely solubilize the formazan and correctly evaluate the real absorbance (Figure 3). The MTT test exhibited a strong composite-cell adhesion and also gave a clear indication of a good colonization of these novel materials by cultured cells that was better than control and neat PCL (Figure 4). The composite most homogeneously colonized appeared to be SW10, as small areas devoid of cells were visible onto the other materials.

Successively, we analyzed the expression of the early biochemical marker of osteoblastic phenotype, namely alkaline phosphatase. The results confirmed that the cells cultured on the composites were able to maintain their phenotype (Figure 5).

In addition, in order to directly analyze the cell colonization on the composites, scanning electron microscopy was carried out. The SEM images confirmed the previous results and evidenced that MSC attached

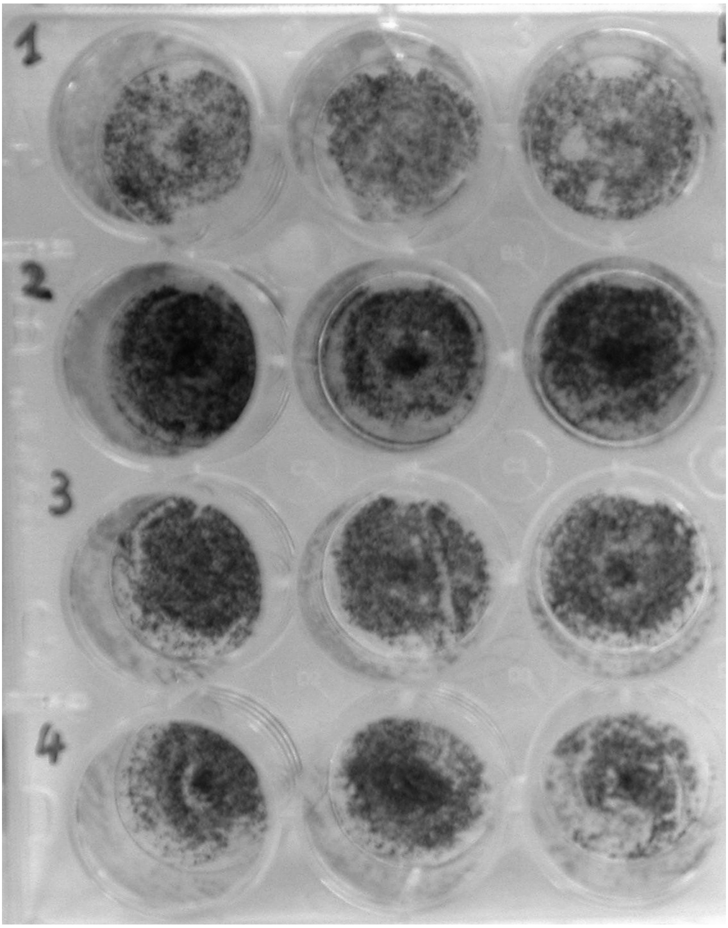


Figure 3. MTT assay carried out on MSC cultured for 6 days (1 = NP10; 2 = SW10; 3 = NP20; 4 = SW20).

and colonized the samples forming a network with ruffles and filapodia tightly anchoring the cells to the biomaterials, as can be clearly seen in Figures 6(a) and 6(b) that show the cells grown for 48 h on SW 10%.

CONCLUSIONS

Composites of natural and synthetic HA, in the shape of powder and whiskers, respectively, in variable contents of 10 and 20% in PCL, have

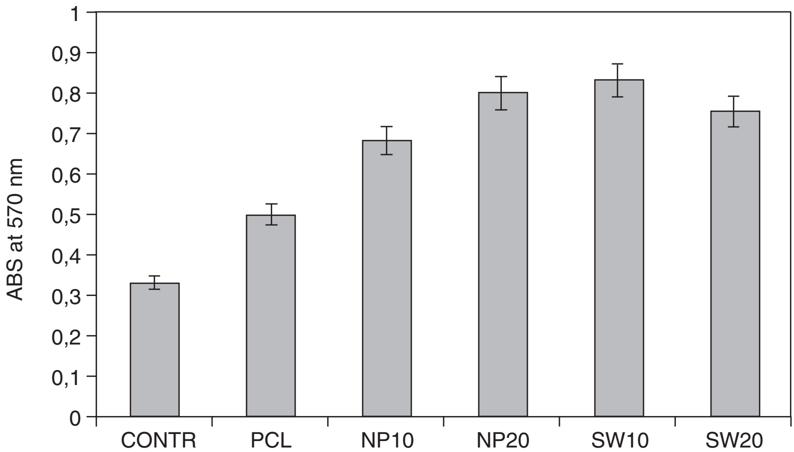


Figure 4. MTT assay on cells grown on tested materials for 6 days.

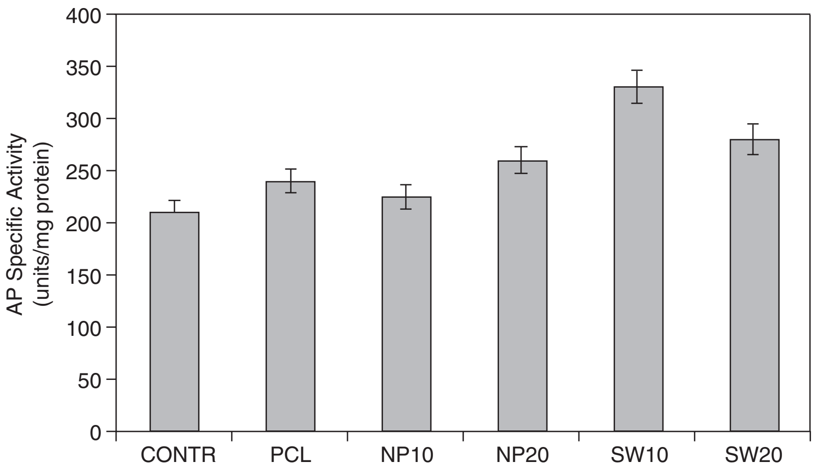
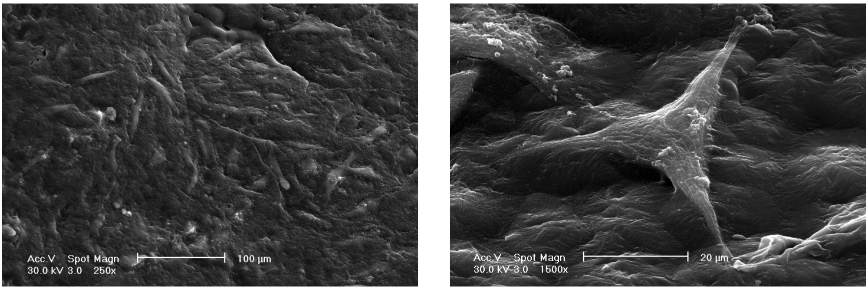


Figure 5 Alkaline phosphatase assay on MSC cultured on materials for 12 days.

been prepared and analyzed in terms of mechanical properties. In addition, by means of cytocompatibility tests effected in the direct contact mode, using MSC, we have investigated the biological response of the obtained composites.

In terms of mechanical properties, it seems that a whisker-like shape helps PCL to retain better its viscoelastic behavior, while



(a) (b)

Figure 6 SEM micrographs of cells grown on SW10 at two different magnifications: (a) (250×) and (b) (1500×).

powders have a larger influence on the elastic response that provided a higher moduli. In the future, we will concentrate on the improvement of interfacial adhesion of whiskers, as SEM analysis seems to indicate a weak interface, originated by the absence of any specific interaction between the fillers and the matrix polymer. In terms of biological response, it has been found that the presence of fillers provide a beneficial response in terms of adhesion and proliferation, with respect to the neat PCL. Both MTT test and AP activity assay indicated that the most promising composite appeared to be SW10. While for this preliminary biocompatibility analysis, the composites have been tested as two-dimensional disks, the future step will be the preparation of three-dimensional scaffolds of the most promising of all materials tested.

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