J Mater Sci: Mater Med (2010) 21:343–351
DOI 10.1007/s10856-009-3839-5

# Porous scaffolds of polycaprolactone reinforced with in situ generated hydroxyapatite for bone tissue engineering

**Paola Fabbri · Federica Bondioli · Massimo Messori · Cristina Bartoli · Dinuccio Dinucci · Federica Chiellini**

Received: 29 May 2009 / Accepted: 24 July 2009 / Published online: 4 August 2009


**Abstract** Polycaprolactone/hydroxyapatite (PCL/HA) composites were prepared by in situ generation of HA in the polymer solution starting from the precursors calcium nitrate tetrahydrate and ammonium dihydrogen phosphate via sol–gel process. Highly interconnected porosity was achieved by means of the salt-leaching technique using a mixture of sodium chloride and sodium bicarbonate as porogens. Structure and morphology of the PCL/HA composites were investigated by scanning electron microscopy, and mechanical properties were determined by means of tensile and compression tests. The possibility to employ the developed composites as scaffolds for bone tissue regeneration was assessed by cytotoxicity test of the PCL/HA composites extracts and cell adhesion and proliferation in vitro studies.

P. Fabbri (✉) · F. Bondioli · M. Messori
Department of Materials and Environmental Engineering, University of Modena and Reggio Emilia, Via Vignolese 905/a, 41100 Modena, Italy
e-mail: fabbri.paola@unimore.it

C. Bartoli · D. Dinucci · F. Chiellini
Department of Chemistry and Industrial Chemistry, University of Pisa, Via Vecchia Livornese 1291, S. Piero a Grado, 56122 Pisa, Italy

P. Fabbri · F. Bondioli · M. Messori
Consorzio Interuniversitario Nazionale di Sienza e Tecnologia dei Materiali, INSTM, Via Giusti 9, 50121 Florence, Italy

C. Bartoli · D. Dinucci · F. Chiellini
BIOlab–Laboratory of Bioactive Polymeric Materials for Biomedical and Environmental Applications, INSTM Consortium Reference Center, Pisa, Italy

## 1 Introduction

Biodegradable and bioresorbable polymer scaffolds developed as substrates for cell tissue regeneration found great application and success in the last years thanks to the easier processability and formulation with respect to the traditional biocompatible metal or ceramic implants. Polycaprolactone (PCL), along with polyhydroxyalcanoates, is the most widely diffused biodegradable and non-cytotoxic polymer used as biomaterial for the production of scaffolds for tissue engineering, thanks to its rate of bioresorption which is appropriate for bone tissue regeneration, and suitable mechanical properties [1, 2]. Unfortunately, PCL has an intrinsic hydrophobic chemical nature, and its poor surface wetting and interaction with biological fluids avoid cells adhesion and proliferation. For this reason, and in order to get enhanced mechanical properties, PCL is often used as polymer matrix in composites including osteogenic and osteoinductive inorganic phases, such as hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$) (HA), which is the main mineral component of bone tissues and confers its high bioactivity to the polymer-based composite promoting bone regeneration [3–9]. Processing methodologies for the production of PCL/HA composites have been recently resumed and described in a review by Baji et al. [10], and mainly consist in the following four procedures: (1) melt blending of PCL with preformed HA micro- or nanoparticles [11–13]; (2) ring-opening polymerization of caprolactone monomer in the presence of HA particles [11, 14]; (3) HA particles dispersion in the polymer solution [15], at times using amphiphilic surfactant mediation [16]; (4) sol–gel synthesis of HA in tetrahydrofuran (THF) for 24 h followed by PCL dissolution in the resulting suspension [17]. However, when preformed HA particles are dispersed in the polymer matrix, poor interfacial adhesion is often

observed as a consequence of the different chemical nature of the components and their different surface energy, resulting in a too fast decay of the mechanical properties of the composite.

For this reason, the in situ synthesis of HA by sol–gel process starting from the precursors calcium nitrate tetrahydrate and ammonium dihydrogen phosphate directly in the presence of the polymer solution, appears to be a promising strategy for the achievement of homogeneous hybrid materials having higher degree of phase interaction between the polymer matrix and the inorganic filler. This procedure should avoid the extensive particles agglomeration typically seen in PCL/HA composites obtained by mechanical incorporation of preformed HA powders into the polymer melt or solution, causing non homogeneous materials, and it was already proved to be an effective approach for the preparation of homogeneous PCL/silica glasses composites [18, 19], poly(propylene fumarate)/HA composites [20], PCL/HA composites [17] and polyacrylic acid/calcium phosphate ceramics composites [21].

Porosity is a key factor in the design of bioresorbable scaffolds for tissue engineering, since proper morphology is needed to permit and promote tissue growth and transportation of nutrients into the scaffold. The wet chemical technique proposed for the preparation of PCL/HA homogeneous composites is also particularly suited to the preparation of porous structures by the simple method of solvent casting and particulate leaching, using porogen particles (such as water-soluble inorganic salts like sodium chloride or sodium bicarbonate) that will dissolve generating a porous structure with pore size depending on the size of the porogen particles, and amount of porosity correlated to the polymer to porogen ratio [22].

In this work, PCL/HA scaffolds having a homogeneous distribution of HA and good adhesion between organic and inorganic components were prepared by promoting the generation of the inorganic phase by sol–gel process directly inside the polymer solution; highly interconnected porosity was further obtained by the salt leaching technique. Materials structure and morphology were investigated and mechanical properties were determined in tensile and compression mode. Preliminary biological evaluation of these porous hybrid materials was carried out by cytotoxicity investigations of the PCL/HA extracts by using the balb/c 3T3 Clone A31 mouse embryo fibroblast cell line. The ability of the prepared composites to sustain cell adhesion and proliferation was investigated by using the MC3T3-E1 mouse preosteoblast cell line.

## 2 Materials and methods

### 2.1 Materials

Calcium nitrate tetrahydrate ($Ca(NO_3)_2{\cdot}4H_2O$), ammonium dihydrogen phosphate ($NH_4H_2PO_4$), phosphate buffered-saline tablets (PBS), dichloromethane, methanol ($CH_3OH$), ethanol (≥99.8%) and THF were purchased from Sigma Aldrich (Milan, Italy) and used as received without further purification. PCL diol of MW = 65 000 g/mol was purchased from Sigma Aldrich (Milan, Italy) and purified by dissolution in THF and reprecipitation in cold $CH_3OH$, in order to eliminate residual polymerization catalysts. Ammonium hydroxide ($NH_4OH$) solution 28% in water was purchased from Fluka (Milan, Italy) and used to regulate pH during sol–gel synthesis. Sodium chloride (NaCl) and sodium bicarbonate ($NaHCO_3$) were food grade porogens used as purchased or after milling.

### 2.2 In situ generation of HA in PCL solution

In the sol–gel process $Ca(NO_3)_2{\cdot}4H_2O$ and $NH_4H_2PO_4$ were selected as calcium and phosphorous precursors, respectively. A typical preparation was as follows: Purified PCL (2.0 g) was dissolved in 100 ml of THF at a temperature of 50°C inside a three neck round bottom flask equipped with mechanical stirring. Ethanol was added (approx. 150 ml) until the solution was cloudy. Then the PCL solution was mixed with 0.518 M $Ca(NO_3)_2{\cdot}4H_2O$ and vigorously stirred for 15 min. A 0.156 M solution of $NH_4H_2PO_4$ in water was prepared and slowly added dropwise to the polymer solution over a period of approximately 1 h. Vigorous stirring was maintained for 3 h and pH was periodically monitored and leveled at value 9 by addition of $NH_4OH$. A white gel was obtained inside a clear solution that was eliminated by filtering. 2.0 g of a 1:1 wt/wt $NaCl/NaHCO_3$ mixture were incorporated into the gel and the blend was made homogeneous through vigorous mechanical stirring. The viscous slurry was poured into a large sterile disposable Petri dish and dried in oven at 45–50°C under vacuum for 12 h. The PCL/HA composite was obtained as a solid film, that was washed in a large excess of distilled water to leach out the porogen particles. Washing step usually took 12 h and water was refreshed every 4 h. The porous scaffold was then air-dried at room temperature until constant weight.

For the samples prepared in the absence of the polymer a similar procedure was followed, with the exceptions that no PCL was added to the $Ca(NO_3)_2{\cdot}4H_2O$ solution in THF and that different molarities were used of the starting reactants in order to get a pure HA crystalline phase.

Composites were prepared with final weight ratio 90:10 between PCL and HA phases.

The density ($\rho$) of the scaffolds was calculated using the formula $\rho = w/V$ where $w$ is the scaffold weight after salt leaching and drying, and $V$ is the volume of the scaffold. The porosity ($P$) of the scaffold was then calculated as $P = (1 - (\rho - \rho_{np})) \times 100\%$, where $\rho_{np}$ is the density of the non-porous scaffold.

### 2.3 Sterilization of the scaffolds

Prior to biological tests, scaffolds were sterilized by multiple washing in PBS solution. One PBS tablet was dissolved in 200 ml distilled water and the solution was kept at low temperature (0–5°C) for 1 h. PCL/HA films were submerged in ultrapure ethanol in two steps of 30 min each; ethanol was refreshed after each step. Then, films were submerged for five times, lasting 2 h each, in fresh PBS solution. Sterilized hybrid scaffolds were finally sealed into sterile Petri dishes until further use.

### 2.4 Instrumental characterization

Structural characterization on HA and PCL/HA composites was performed by means of X-rays diffraction (XRD) using a X'Pert PRO diffractometer (PANalytical), operating at $2\theta$ between 5°–80° with step-size 0.02 and step-time 1 s. Thermogravimetric (TG) and differential thermal analysis (DTA) were performed in inert atmosphere in the range 20–1400°C using a heating rate of 20°C/min, with a STA 449C Jupiter simultaneous analyzer (Netzsch) equipped with a PU1.851.01 unit power. Morphology and porosity of the scaffolds were investigated by scanning electron microscopy (SEM) using a XL 30 instrument (Philips) over gold-coated samples; transmission electron microscopy (TEM) was performed using a JEM 2010 (Jeol).

Mechanical testing was performed using an electromechanical Instron 4502 dynamometer (Instron, MA) equipped with a 10 kN load cell, operating either in tensile or compression mode; the measurement cell was held at 30°C. UNI EN ISO 604 standard was followed for the determination of compression properties, and tests were done in different modalities for the determination of compression modulus and yielding properties. Elastic modulus was determined as a secant modulus using the stress values corresponding to strain values of 0.05 and 0.25%, using the formula (Eq. 1):

$$E = \frac{\sigma_{0.25\%} - \sigma_{0.05\%}}{\varepsilon_{0.25\%} - \varepsilon_{0.05\%}} \quad (1)$$

yielding stress ($\sigma_y$) was determined as the maximum stress value in the stress–strain curves. Results were averaged over five specimens for each kind of sample.

### 2.5 Biological tests

#### 2.5.1 Materials

Cell lines BALB/3T3 Clone A31 mouse embryo fibroblasts (CCL163) MC-3T3-E1 (CRL 2594) were obtained from American Type Culture Collection (ATCC) and propagated as indicated by the supplier. Dulbecco's Modified Eagles Medium (DMEM), 0.01 M pH 7.4 phosphate buffer saline without $Ca^{2+}$ and $Mg^{2+}$ ($PBS_1$), Fetal Bovine Serum (FBS), Calf Serum (CS), trypsine/EDTA, glutamine, and antibiotics (penicillin/streptomycin) were purchased from GIBCO Brl. Cell proliferation reagent WST-1 was purchased from Roche Diagnostic. PhalloidinAlexa488® and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Invitrogen (NewYork, NY, USA). Cell Proliferation Assay WST-1 was purchased from Roche Diagnostic. Tissue culture grade disposable plastics were obtained from Corning Costar.

#### 2.5.2 Cytotoxicity tests on PCL/HA Extracts

To assess the cytotoxicity of possible substances that could leach from the prepared PCL/HA composites, 3 $cm^2$ specimens were cut and immersed in 1 ml of DMEM supplemented with 10% calf serum, 4 mM L-Glutamine and 100 U/ml:100 μg/ml penicillin:streptomycin for 48 h at 37°C in an enriched 5% $CO_2$ atmosphere. The medium containing the extracts was tested undiluted and diluted at a volume ratio of 1:1 using the complete culture medium. Balb/c 3T3 Clone A31 cells were seeded at a density of $1 \times 10^3$/well in a 96 wells plate and allowed to proliferate for 24 h. Then the culture media was changed with the DMEM containing the extracts and cells were allowed to proliferate for further 24 h at 37°C in an 5% $CO_2$ enriched atmosphere. Cells incubated with complete DMEM and wells containing only complete DMEM were used as controls. At the end of the exposure time, cell viability was measured using WST-1 tetrazolium salt. Absorbance was measured at 450 nm and values relative to control were reported. Experiments were performed in triplicates.

#### 2.5.3 Cell adhesion and proliferation onto PCL/HA scaffold

To investigate the ability of the prepared PCL/HA scaffolds to support cell adhesion and proliferation, a film sample was cut into 100 mm diameter disc and placed into a Cell Crown24 sample holder (Scaffdex, Finland) in 24 well culture plates. MC3T3-E1 mouse preosteoblast cells were seeded onto the film at a concentration of $1 \times 10^4$/well and allowed to proliferate for 5 days at 37°C in an 5% $CO_2$ enriched atmosphere. DMEM supplemented with 10%

FBS, 2 mM L-glutamine, 1 mM sodium pyruvate and 100 U/ml:100 μg/ml penicillin:streptomycin was used as complete culture media and renewed every 48 h. At the end of the incubation time samples were analyzed for cell proliferation by means of WST-1 tetrazolium salts.

Cell morphology was investigated by means of confocal laser scanning microscopy (CLSM) and SEM. For CLSM analysis cells grown onto PCL/HA films were fixed with 3.8% paraformaldehyde in PBS 0.01 M pH 7.4, permeabilized with a $PBS_1$ 0.01 M/Triton X-100 solution (0.2.%) for 5 min and incubated with a PBS 0.01 M solution of DAPI and phalloidin-Alexa488 for 45 min at room temperature. After dyeing incubation samples were washed with $PBS_1$ before mounting on a glass slide and sealing with resin for microscopic observation.

A Nikon Eclipse TE2000 inverted microscope equipped with a EZ-C1 confocal laser (Nikon, Japan) and Differential Interference Contrast (DIC) apparatus and a 60× oil-immersion objective were used to analyze the samples. A 405 nm laser diode (405 nm emission) and Argon ion laser (488 nm emission) were used to excite, respectively, DAPI and FITC fluorophores. Images were captured with Nikon EZ-C1 software with identical settings for each sample. Images were further processed with GIMP (GNU Free Software Foundation) Image Manipulation Software and merged with Nikon ACT-2U Software.

For SEM analysis samples were fixed with solution of 2.5% glutaraldehyde in $PBS_1$ 0.01 M for 1 h at room temperature and dehydrated by the use of a series of ethanol solutions (25, 50, 70 and 100% v/v). Afterward constructs were air dried overnight at room temperature and sputter coated with gold and analyzed under a JEOL LSM5600LV scanning electron microscope.

#### 2.5.4 *Statistical analysis*

For every test, the data are expressed as means plus or minus the standard deviation. The statistical analysis was performed with a Students'*t*-test at a 0.05 level.

## 3 Results and discussion

### 3.1 Sol–gel synthesis of HA in water/THF mixture

Several sol–gel approaches starting from different calcium and phosphorous precursors have been proposed for the preparation of HA powders, and between them aqueous routes have demonstrated to be the most effective and easy to handle, mainly for the control of Ca/P stoichiometry [23]. The first step of the present work was the optimization of the sol–gel synthesis of a pure HA crystalline phase starting from the precursors $Ca(NO_3)_2{\cdot}4H_2O$ and $NH_4H_2PO_4$, in the absence of the polymer. Figure 1 reports the XRD pattern of the HA crystalline phase obtained starting from 0.156 M solution of $NH_4H_2PO_4$ in water and 0.256 M solution of $Ca(NO_3)_2{\cdot}4H_2O$ in THF, with Ca/P in 1.66 stoichiometric ratio.

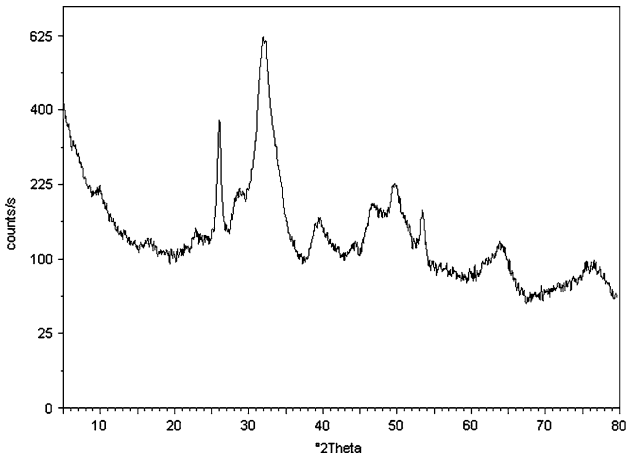


**Fig. 1** XRD pattern of HA synthesised from Ca/P ratio of 1.66

During the formation of HA and during the ageing of the suspension, pH was maintained constant at value 9. The final Ca/P ratio in the synthesised HA remained at the value of 1.66, such as that of HA present in natural bone tissues. The peaks in the XRD pattern were those characteristic of pure HA and closely matched with the JCPDS 09-432 of stoichiometric calcium hydroxyapatite, and indicated the absence of different calcium phosphate phases.

TEM micrographs of the synthesised HA are reported in Fig. 2, showing crystallites of acicular shape that tend to form roughly spherical agglomerates of about 100 nm.

TG-DTA analysis performed on the same sample confirmed the absence of secondary calcium phosphate phases; the curves revealed the endothermic loss of water molecules at 100°C and the exothermic crystallization of the amorphous HA phase at 280°C.

### 3.2 In situ generation of HA in the presence of PCL

The same procedure above reported for the synthesis of pure HA was repeated in the presence of the polymer that was dissolved in THF along with the calcium precursor and then reacted with $NH_4H_2PO_4$. Preparations were done in order to have a PCL/HA ratio in the final composite equal to 90/10 by weight. XRD pattern reported in Fig. 3, registered on the PCL/HA composite prepared by starting with an initial Ca/P stoichiometric ratio of 1.66 between the reactants, revealed a strong calcium deficiency in the inorganic phase generated in situ inside the polymer solution, that was mainly composed by a calcium phosphate having a Ca/P ratio of 1.0, namely brushite ($CaHPO_4{\cdot}2H_2O$, JCPDS # 09-0077).

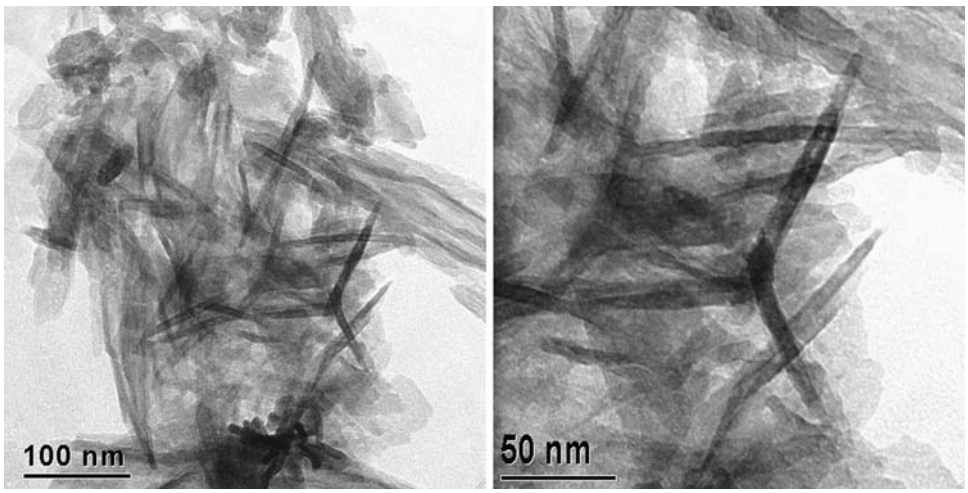


**Fig. 2** TEM micrographs of pure HA synthesised in the absence of PCL

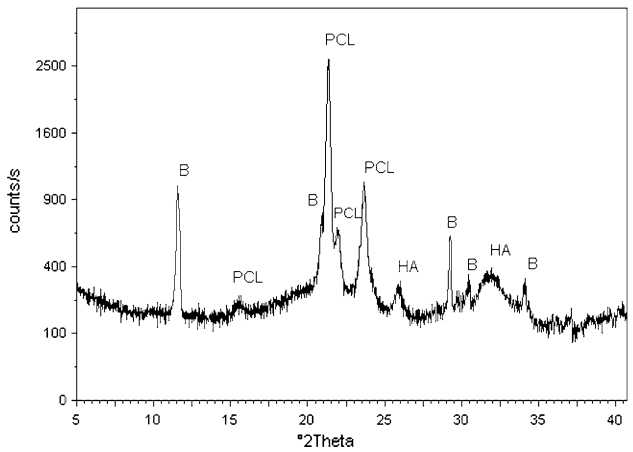


**Fig. 3** XRD pattern of HA synthesised in the presence of PCL from Ca/P ratio of 1.66

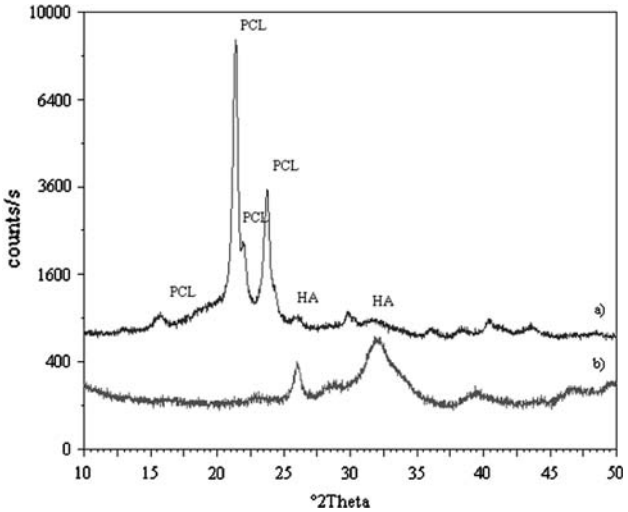


**Fig. 4** XRD patterns of the in situ PCL/HA 90/10 composite obtained by synthesising HA from Ca/P ratio of 3.32 (a) compared to pure HA (b)

Calcium deficiency in the inorganic phase in the in situ PCL/HA composites can be explained by the chelating action of the ester groups $COO^-$ belonging to the polymer chain on the $Ca^{2+}$ ions present in solution. The negatively charged functional groups can initiate heterogeneous nucleation of the inorganic phase by forming a complex with positively charged $Ca^{2+}$ ions, but the overall effect is the decrease of the calcium ions available for the formation of stoichiometric HA, and the subsequent formation of phosphates with lower Ca/P ratio [24, 25].

For this reason the stoichiometry of the sol–gel reaction was varied by increasing the amount of calcium precursor up to the value of Ca/P ratio of 3.32. In this way a clean crystalline phase of HA having the correct final Ca/P ratio of 1.66 was generated in situ inside the PCL solution. Figure 4 reports the XRD pattern of the PCL/HA 90/10 composite with in situ generated pure HA, compared with the pattern of the pure HA phase having Ca/P ratio of 1.66, generated by sol–gel process in the absence of the polymer.

### 3.3 Morphology of in situ generated PCL/HA composite

The molecular-level mixing of the calcium and phosphorous precursors along with the polymer chains offered by the sol–gel process, resulted in composites having enhanced dispersion and good interaction between the inorganic phase and the polymer matrix. The resulting morphology is clearly shown in the SEM micrograph reported in Fig. 5. Problems of preformed particles agglomeration and phase separation, usually described for composites prepared by melt or solution blending, were widely overcome by this wet method.

Porosity was generated by blending porogen salt particles with the PCL/HA wet composite, complete solidification of the composites through thermal heating, followed

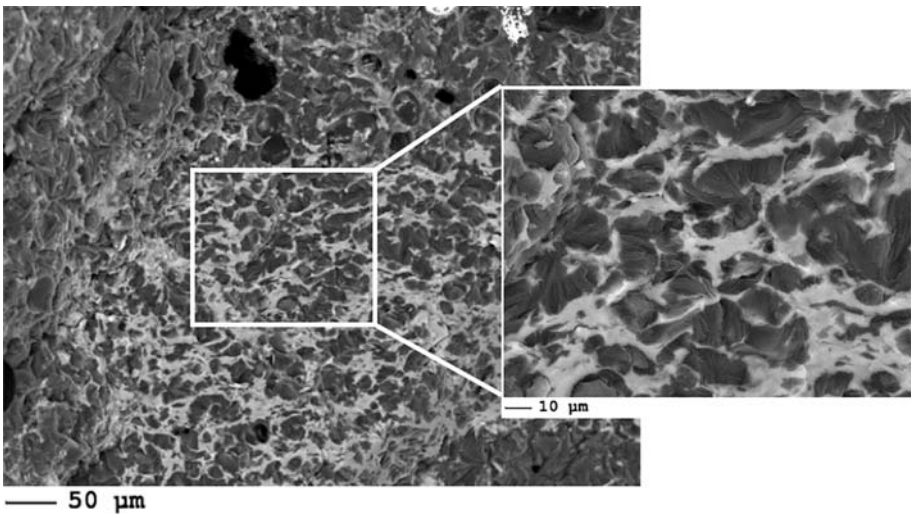


**Fig. 5** SEM micrograph of in situ generated PCL/HA 90/10 composite

by a leaching technique. This represents the easiest way of pore generation, and porosity degree and pores dimension can be tailored by opportune choice of particle size and concentration of the porogens.

The porous morphologies resulting by salt-leaching on the in situ PCL/HA composite is demonstrated by SEM micrographs reported in Fig. 6. The use of porogen particles having different particle size (NaCl and $NaHCO_3$ at different degrees of milling) clearly resulted in a proper combination of macro- and micro-porosities, that is particularly suited to the biomedical application in bone tissue engineering. Porosities of 70–80% were usually obtained for the composites.

### 3.4 Mechanical properties of the scaffolds

Among all the bioresorbable polymeric materials suitable for application in bone tissue engineering, PCL exhibits higher and more prolonged mechanical strength, and degrades at a rate compatible with the bone regeneration. With respect to pure PCL scaffolds, PCL/HA composite scaffolds have the advantage of a more hydrophilic character, promoting a better interaction and improved adhesion with the biological environment. Furthermore, the presence of an inorganic phase dispersed in the polymer matrix also improves its mechanical performance, and this aspect results to be decisive in bone tissue engineering, mainly for mass bone defects repair. For instance, HA lowers the reduction in mechanical strength and distortion and cracking of PCL as degradation processes proceed during implantation [26].

In this work, the mechanical properties of the in situ generated PCL/HA 90/10 composite were tested in tensile and compression modes, and compared with the properties of unfilled PCL. Tensile modulus increased from the value of 405 (±13) MPa of pure PCL up to 996 (± 28) MPa due to the presence of HA dispersed in the polymer matrix, confirming the good dispersion and interface adhesion between organic and inorganic phases as observed by SEM. From tensile stress–strain curves (reported in Fig. 7),

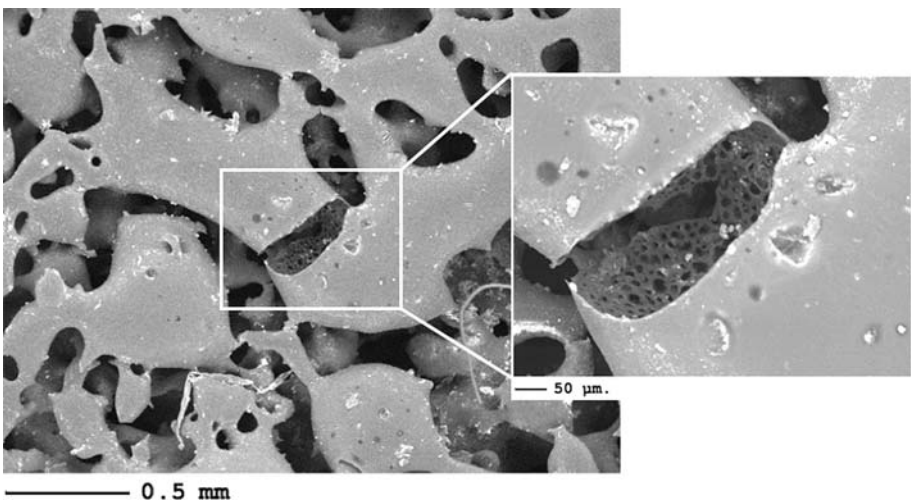


**Fig. 6** SEM micrographs of in situ generated PCL/HA 90/10 composite after salt leaching

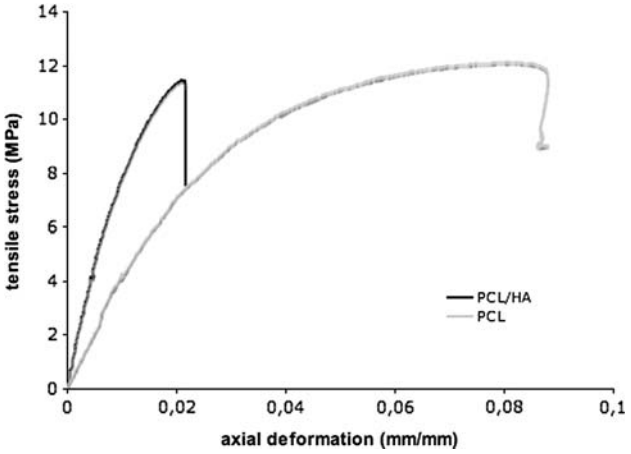


**Fig. 7** Tensile stress–strain curves for PCL and PCL/HA 90/10 composite

the maximum stress resulted almost unvaried at the value of approximately 12 MPa.

The evaluation of compression mechanical properties was performed since for a scaffold to be functional to bone tissue regeneration implies bearing compressive loads. Compression modulus of the in situ generated PCL/HA 90/10 composite resulted 632 (±30) MPa compared to 285 (±17) MPa of unfilled PCL, corresponding to a 120% increase in compression rigidity. Compression yielding stress was also slightly increased from 13.5 MPa of pure PCL to 15.6 MPa of PCL/HA composite.

### 3.5 Biological evaluation

#### *3.5.1 Cytotoxicity*

Materials intended to be use for tissue engineering applications should not release any agent that may be cytotoxic. To know whether the prepared PCL/HA composite extracts might be harmful to cells, the fibroblast cell line balb/c 3T3 Clone A31 was cultured in the presence of the extractables of this film over 24 h at 37°C. WST-1 assay was carried out to evaluate the potential cytotoxicity of the PCL/HA extracts. Aqueous extracts of the investigated samples, both undiluted and diluted 1:1 were used. The result of this study revealed that no release of cytotoxic compound occurs from the prepared composite and that cells not only remained viable but also proliferated similar to the control also in the case of the undiluted extract.

#### *3.5.2 Cell adhesion and proliferation onto PCL/HA 90/10 scaffold*

A preliminary biological evaluation of the prepared PCL/HA composite scaffolds to sustain cell adhesion and proliferation in the view of a potential application of such scaffolds for bone tissue engineering, was carried out by using the mouse calvaria-derived pre-osteoblastic cell line MC-3T3-E1. Quantitative evaluation of cell proliferation onto the PCL/HA 90/10 sample was carried out after 5 days of static culture and evaluated by means of WST-1 tetrazolium salt. Results highlighted a cell proliferation onto the investigated sample of approximately 30% with respect to the cells grown onto tissue culture polystyrene (TCPS), used as control.

The relative low value of cells proliferation onto the PCL/HA samples could be related to the lower number of cells that were able to attach on the rough and hydrophobic surface of the scaffolds in comparison to the smoother and more hydrophilic surface of TCPS. Indeed, as reported by the literature, MC3T3-E1 cell line shows a better adhesion on hydrophilic surfaces [27]. The morphological analysis carried out by CLSM and SEM confirmed the quantitative data in terms of cell proliferation. As shown in Fig. 8a and b, the number of cells present in the control sample was higher than the one detected onto the PCL/HA scaffold, but interestingly the cells grown onto the scaffold showed a morphology similar to the control with expansion of the cytoplasm on the surface of the scaffold, surrounding the pores (Fig. 8b–e).

These preliminary data, obtained from the biological evaluation, suggest a possible role of the prepared composites for bone tissue engineering. Further investigations will be devoted to the assessment of the ability of the prepared PCL/HA scaffolds to support a full osteogenic differentiation of MC3T3-E1.

## 4 Conclusions

In this paper, an in situ processing methodology for the production of polycaprolactone/hydroxyapatite composites having enhanced properties has been presented. The method consists in the sol–gel synthesis of hydroxyapatite starting from the precursors calcium nitrate tetrahydrate and ammonium dihydrogen phosphate directly inside a polycaprolactone solution in tetrahydrofuran. Resulting morphology showed a homogeneous dispersion and good degree of phase interaction between the polymer matrix and the inorganic filler. Porous structures were obtained by the salt leaching technique. Mechanical testing demonstrated an effective reinforcing action of hydroxyapatite on polycaprolactone. Biological evaluation evidenced complete absence of cytotoxicity in the composites, and cells proliferation assays confirmed that preosteoblastic cells are able to adhere and to grow on the hybrid porous scaffolds prepared. Results suggest that the procedure described by this work can represent an easy method for the preparation of polycaprolactone/hydroxyapatite composite materials

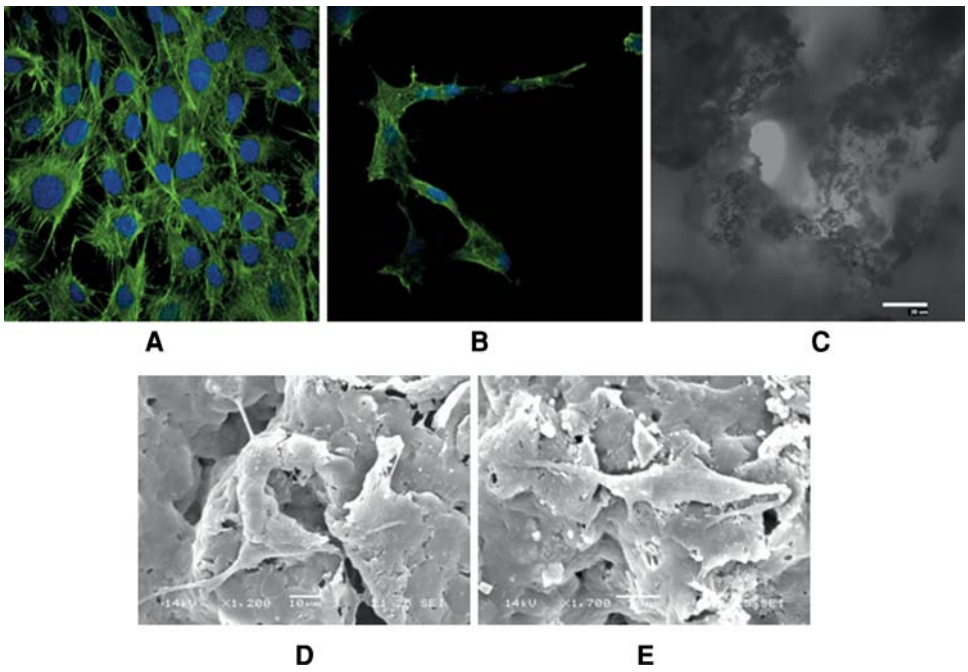


**Fig. 8** Morphology of MC3T3-E1 and Scaffold; **a** Cells grown onto TCPS; **b** Cells grown onto PCL/HA 90/10 scaffold; **c** DIC micrograph of PCL/HA 90/10 scaffold; **d** and **e** SEM micrographs of cells grown onto PCL/HA 90/10 scaffold

useful for the preparation of porous scaffolds for bone tissue engineering.

**Acknowledgments** Mr. Piero Narducci of the University of Pisa is gratefully acknowledged for the recording of the SEM images of the cells grown onto PCL/HA scaffolds. Dr. Ing. Andrea Dorigato of the Engineering Faculty at the University of Trento is also gratefully acknowledged for support in measuring the mechanical properties of the scaffolds in compression mode. Ms Giulia Bartalucci and Ms Sara Menozzi are acknowledged for support in experimental work.

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