HIGHLY POROUS HYDROXYAPATITE/POLYCAPROLACTONE COMPOSITE SCAFFOLD: PHYSICOCHEMICAL AND *IN VITRO* BIOLOGICAL PROPERTIES

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Abstract
Hydrothermal transformation of aragonitic cuttlefish bone into hydroxyapatite (HAp) was performed at 200°C retaining the natural well interconnected porous structure. The scaffold was coated with a poly(ε-caprolactone) (PCL) using vacuum impregnation technique. The prepared scaffolds were characterized by Fourier transform infra-red spectroscopy (FTIR), X-ray diffraction analysis (XRD), and scanning electron microscopy (SEM). The mechanical properties of scaffolds were assessed under compression. Biological characterization of scaffolds was performed using the preosteoblastic cell line MC3T3-E1. The attachment and spreading of preosteoblasts on scaffolds were observed by Live/Dead kit staining. DNA content on HAp/PCL scaffold was significantly higher compared to porous HAp scaffolds. The osteoblastic differentiation of the cells was evaluated by determining ALP activity. The results demonstrate the potential of the HAp/PCL scaffold to be used in bone tissue engineering.

1. Introduction

Composites of bioactive ceramics and biodegradable polymers have proved to be attractive scaffold materials for use in bone tissue engineering [1-3]. An ideal scaffold should mimic both the structure and mechanical properties of the natural bone. It should provide a highly porous matrix with interconnected pores that enables the transport of nutrients, oxygen and metabolic waste products. Its surface properties must be suitable for cell adhesion, proliferation and differentiation. Also, the scaffold should be bioresorbable with a controllable degradation rate to match the replacement by new tissue. Additionally, the scaffold should possess sufficiently high mechanical properties such as stiffness, strength and toughness. Among calcium phosphate-based ceramics hydroxyapatite (HAp), $Ca_{10}(PO_4)_6(OH)_2$, has received particular attention in recent years due to its chemical similarity to the inorganic matrix of natural bone, excellent osteoconductivity and bioactivity [4, 5]. The major drawback of the HAp scaffolds is their poor mechanical properties, especially the brittleness and low fracture toughness. Therefore, they cannot be used in load bearing application. To overcome these disadvantages HAp has been combined with polymers that provide flexibility to the brittle system. Due to its biodegradability, biocompatibility, appropriate mechanical properties, and low emission of harmful by products polycaprolactone (PCL) has been widely

used to prepare HAp/PCL composite scaffolds. In this work, PCL-coated highly porous hydroxyapatite scaffold derived from cuttlefish bone was prepared and characterized.

2. Materials and methods

2.1. Hydrothermal synthesis of porous HAp scaffold

Cuttlefish bones (*Sepia Officinalis L.*) from Adriatic Sea were used as starting material for the hydrothermal synthesis [6] of HAp by treatment with $NH_4H_2PO_4$ at 200°C for 72 hours. The pressure inside the reactor was self-generated by water vapour and reached 18 bars. After hydrothermal treatment the resulting pieces of HAp scaffold were washed with boiling demineralised water and dried at 105°C.

2.2. Preparation of the HAp/PCL composite scaffold

HAp scaffolds were impregnated with PCL solution in chloroform using the vacuum impregnation unit (CitoVac, Struers). A homogeneous 20 w/v% solution of poly(ε-caprolactone) (PCL, Mn = 45000, Sigma-Aldrich) was prepared by intensive stirring of PCL pellets in chloroform ($CHCl_3$, p.a., Kemika).

2.3. Physicochemical characterization of porous scaffolds

X-ray diffraction analysis was performed with an X-ray diffractometer (Shimadzu XRD 6000) equipped with CuK_α radiation source generated at 40 kV and 40 mA in the range of $5° < 2\theta < 70°$ at a scan speed of 0.2°/s. Identification of the phases was performed by comparing the experimental XRD patterns to standards compiled by the Joint Committee on Powder Diffraction Standards (JCPDS).

The Fourier transform infrared spectra (FTIR) of investigated materials were recorded by attenuated total reflectance (ATR) spectrometer for solids with a diamond crystal (Brucker Vertex 70) at room temperature. 64 scans were collected for each measurement over the spectral range of 400–4000 cm^{-1} with a resolution of 4 cm^{-1}.

The compression tests were carried out by a Microtest standard compression machine (Microtest S.A.) with a 15 N maximum load at a crosshead speed of 0.2 mm/min in ambient conditions. The porous scaffolds of raw cuttlefish bone, HAp and HAp/PCL composite were cut into cube blocks of 6 mm edge length. A compressive load was applied perpendicular to lamellae on each specimen. Ten to fifteen specimens for each sample group were used for the compressive testing.

2.4. Biological characterization of porous scaffolds

Each scaffold was cut into quadratic shape (7mm x 7mm x 1 mm) and sterilized by gamma radiation. Specimens were repeatedly washed in Dulbecco´s phosphate buffer saline, DPBS (Sigma-Aldrich) and preconditioned in Dulbecco's Modified Eagle's Medium, DMEM (Invitrogen), containing 1g/L glucose supplemented with 10% fetal bovine serum, FBS (Invitrogen) and 1% penicillin/streptomycin, P/S (100 unit/mL/100 mg/mL, Lonza), overnight.

Mouse pre-osteoblastic cells, MC3T3-E1 (RIKEN CELL BANK, Japan) were expanded into 75 mL culture flasks and grown in DMEM supplemented with 10% FBS, 1% P/S at 37°C and 5% CO_2. After reaching confluence, MC3T3-E1 cells were trypsinized (0.25% trypsin/EDTA solution, Sigma Chemical, USA) from the culture flask and counted with a hematocytometer. Each scaffold was transferred into the respective well of a 24 well plate. MC3T3_E1 cells were seeded onto the surface of the scaffolds in a drop wise manner at a cell density of 2×10^4 cell/scaffold. After three days of culture 1% ascorbic acid (Sigma-Aldrich) and 1% β-glycerophosphate (Sigma-Aldrich) was added to the medium to promote osteogenic differentiation for the total 21 days of culture. The culture media was renewed every three days and triplicates of each type of scaffold were used.

Cell viability was determined by staining cells using a Live/Dead® viability/cytotoxicity kit (Invitrogen). After 3 and 14 days of culture the samples were washed in DPBS and then incubated with 2 µM of calcein and 4 µM of ethidium homodimer-1 (EthD-1) for 15 minutes at 37°C in a humidified atmosphere under 5% CO_2. Live cells were stained in green due to the enzymatic conversion of the nonfluorescent, cell-permeant dye calcein AM to the intensely fluorescent calcein. Dead cells are identified by staining with EthD-1, which enters cells with damaged membranes, and upon binding to nucleic acids produces a bright red fluorescence. The samples were observed by a fluorescence microscope Nikon Eclipse 80i.

The morphology of HAp and HAp/PCL composite scaffolds before the culturing and after 21 day of culture were examined by SEM (JEOL JSM 5410) at a voltage of 15 kV.
The samples seeded with cells were carefully washed with DPBS and fixed in 2.5% glutaraldehyde solution at 4°C for 1h. The samples were dehydrated in ethanol - graded solutions (30%, 40%, 60%, 70%, 80%, 96% and 100%) for 5 min each and dried at room temperature overnight. All dried samples were sputter coated with gold.

MC3T3-E1 proliferation on the scaffolds was determined using a fluorimetric PicoGreen dsDNA quantification kit (Invitrogen) following the manufacturer protocol.

The activity of alkaline phosphatase (ALP) was evaluated by measuring the conversion of p-nitrophenylphosphate (pNPP) to p-nitrophenol as the result of the activity of a cellular extract [7].

3. Results and discussion

3.1. X-ray analysis

The powder X-ray diffraction pattern of cuttlefish bone after hydrothermal transformation process (HAp in Figure1) showed well-resolved peaks indicating a high degree of crystallinity of the inorganic material. Comparing the experimental XRD pattern to JCPDS standards the crystalline phase was identified as HAp mineral (JCPDS; 09-0432). Besides the diffraction peaks belonging to HAp, the diffraction pattern of HAp/PCL composite showed two additional strong diffraction peaks at Bragg angles $2\theta = 21.2°$ and 23.6° attributed to the (110) and (200) crystallographic planes of semi-crystalline PCL [8].

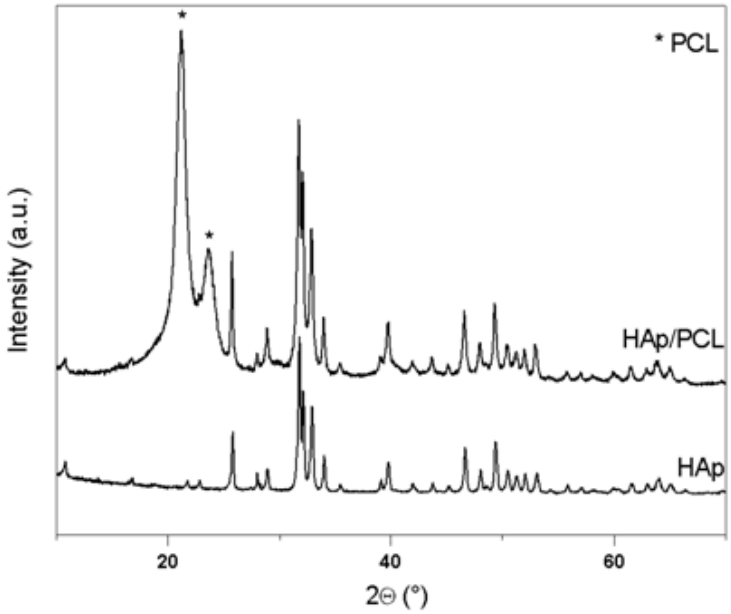

Figure 1. XRD patterns of HAp and HAp/PCL composite.

3.2. FTIR studies

FTIR spectra of HAp, PCL and composite HAp/PCL samples are shown in Figure 2. FTIR spectrum of prepared HAp/PCL composite appears as a superposition of the spectra of HAp and PCL. No other bands or band shifts were observed in the spectrum indicating that no chemical reactions occurred between HAp and PCL.

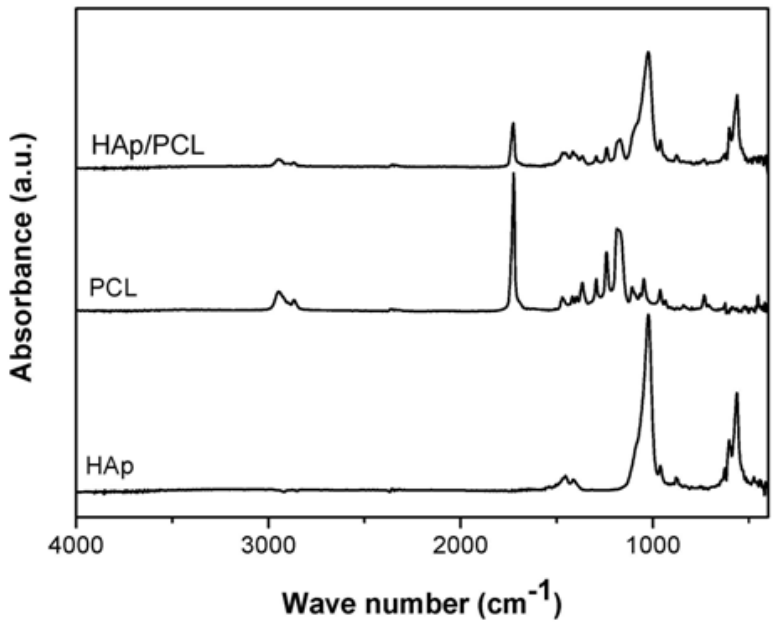

Figure 2. Characteristic infrared spectrum of HAp, PCL and HAp/PCL scaffolds.Spectra are vertically shifted for the sake of clarity.

3.3. Mechanical properties

Compression tests were performed on raw cuttlefish bone, HAp and HAp/PCL composite specimens. The compressive stress-strain curves shown in Figure 3 are characterized by three different regions typical for porous structures [9]. The initial increase in stress at low strain (linear-elastic region) is followed by a long stress plateau region. The fluctuations observed in this region could be attributed to the layer by layer collapse of the microstructure under compression. Further increase in load did not result in a catastrophic failure but in a densification region accompanied by a steep increase in stress where the specimens crumbled into powder.

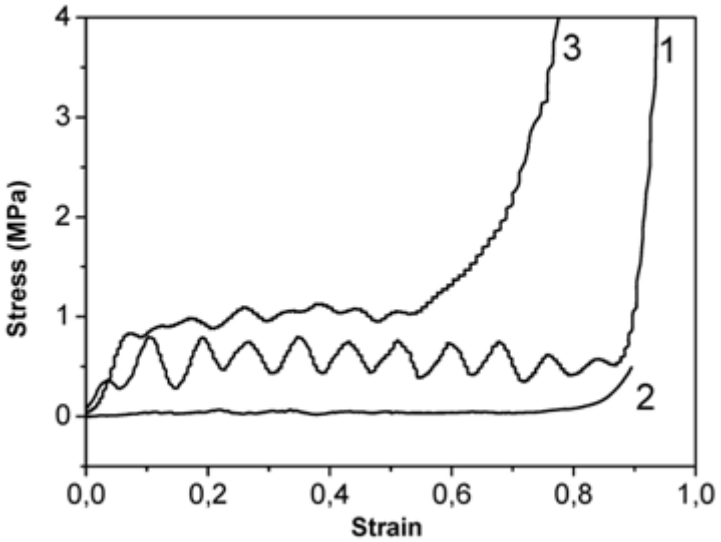

Figure 3. Stress-strain curves of the raw cuttlefish bone (1); HAp (2) and HAp/PCL composite scaffold (3).

The compressive strength and elastic modulus of the porous scaffolds were quantified from the maximum stress and the initial slope of the stress-strain curve, respectively. The average values of ten to fifteen measurements are summarized in Table 1.

Scaffold	Elastic modulus [MPa]	Compressive strength [MPa]
Raw cuttlefish bone	6.2 ± 1.8	0.46 ± 0.06
HAp	0.7 ± 0.3	0.15 ± 0.09
HAp/PCL	15.5 ± 1.2	0.88 ± 0.11

Table 1. Elastic modulus and compressive strength of porous scaffolds.

The results given in Figure 3 and Table 1 indicate that the removal of the organic component from the cuttlefish bone and the HT transformation of aragonitic cuttlefish bone into HAp had a negative effect on the mechanical properties of the scaffold and that PCL coating on HAp was very effective in increasing the mechanical properties of the scaffold. The HAp/PCL composite scaffold displayed the highest compressive strength and the elastic modulus compared to the raw cuttlefish and the HAp scaffold. The applied vacuum infiltration of PCL probably facilitates the filling of the crack-like defects inhibiting crack propagation on HAp scaffolds' surface. The mechanical properties of the prepared scaffolds are within the lower range of properties reported for human trabecular bones.

3.4. Cell viability

The Live/Dead assay, a two-part dye, staining live cells green and dead cells red, has been used to study the cell viability on both the HAp and HAp/PCL scaffolds for 3 and 14 days. From the fluorescence microscopy images (not shown) it was observed that MC3T3-E1 cells proliferated and remained viable after 14 days on both substrates. At 14 days, the cell density on both scaffolds was much higher than that at 3 days. Dead cells were present in low numbers as detected by the low bright red fluorescence. The cells grown on the HAp/PCL composite scaffold exhibited greater spreading than cells grown on the HAp scaffold.

3.5. Scanning electron microscopy

Figure 4 shows SEM images of the macroporous HAp and HAp/PCL scaffolds before the cell culturing. As observed, the interconnected structure of the cuttlefish bone is maintained after the hydrothermal conversion into HAp (Figure 4 a)) as well as after polymer impregnation (Figure 4 b)).

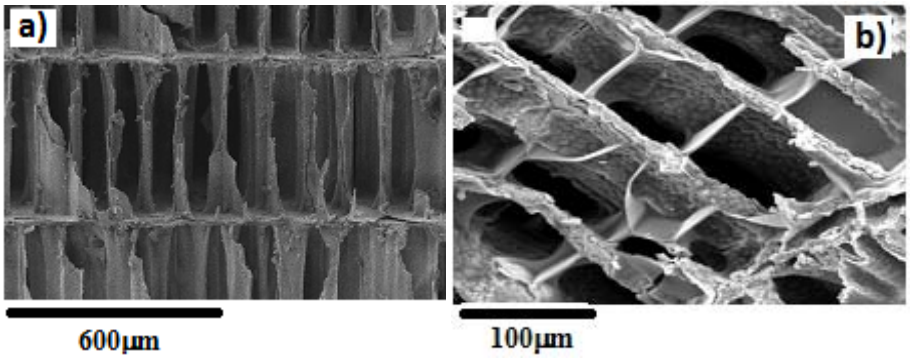

Figure 4. SEM images of transverse cross-section of the macroporous HAp (a) and HAp/PCL (b) scaffolds before the cell culturing.

In Figure 5, SEM micrographs of the surface of the HAp and the HAp/PCL scaffolds seeded with MC3T3-E1 cells after 21 days of culturing are given. As seen, the surface of both scaffolds was almost entirely covered by the cells and the extra-cellular matrix (ECM) they secreted. Most of the pores at the surface of the HAp/PCL scaffold are covered by a layer of cells and matrix (Figure 5b)).

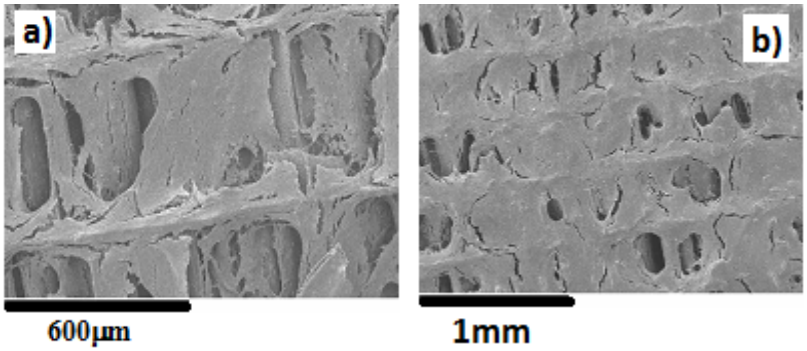

Figure 5. SEM micrographs of the surface of the HAp (a,) and the HAp/PCL scaffolds (c,) seeded with MC3T3-E1 cells after 21 days of culturing.

3.6. Cell proliferation by DNA quantification

Cell proliferation studies were performed at different time points over a period of 21 days. As seen from Figure 6 the DNA content increased on HAp scaffold at each time point until day 14 and on the HAp/PCL scaffold until day 21. Following day 14, DNA content remained statistically unchanged on the HAp scaffold resulting in typical sigmoidal curve. Despite the lower number of cells attached at the earliest time point the DNA content is significantly higher on HAp/PCL scaffold compared to porous HAp scaffold at every time point with the exception of day 14.

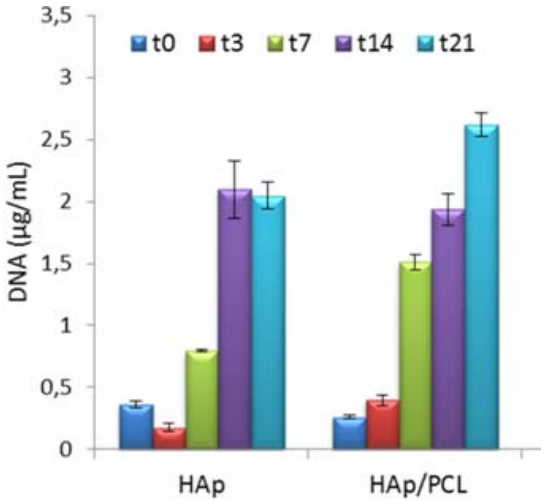

Figure 6. DNA content of MC3T3-E1 pre-osteoblasts cultured on HAp and HAp/PCL scaffolds after 0, 3, 7, 14 and 21 days in culture. The values are represented as the mean ± standard deviation of three replicates.

3.7. Alkaline phosphatase activity

The activity of intracellular alkaline phosphatase (ALP) of MC3T3-E1 cells cultured on HAp and HAp/PCL was monitored at 3, 7, 14, and 21 day as shown in Figure 7, as an indication of osteoblastic differentiation. ALP activity of the cells grown on HAp and HAp/PCL scaffolds increased with the increase of culture time between day 3 and 14 reaching a maximum on day 14. On day 21 a decrease of ALP activity on the scaffolds was observed.

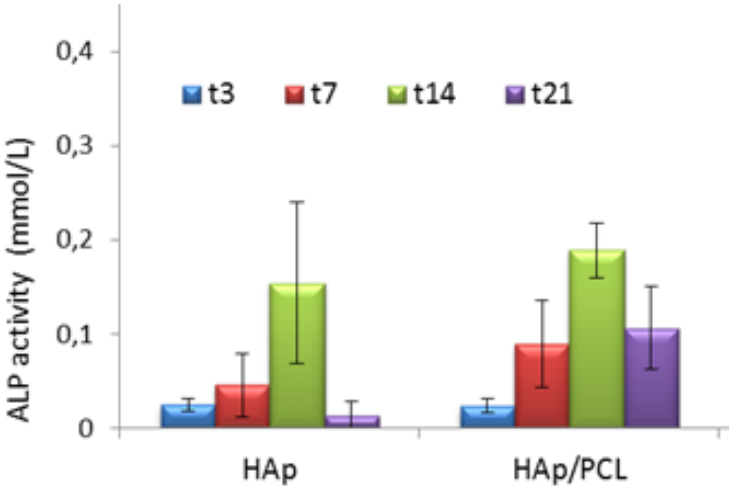

Figure 7. ALP activity of MC3T3-E1 cells cultured on HAp and HAp/PCL at different time points. The values are represented as the mean ± standard deviation of three replicates.

4. Conclusions

Natural aragonite from cuttlefish bone was hydrothermally transformed into hydroxyapatite (HAp) at 200°C preserving the natural well interconnected porous structure. The obtained HAp scaffold was coated with a poly(ε-caprolactone) (PCL) using vacuum impregnation technique. *In vitro* cell culture studies showed that the scaffold is nontoxic and provide an adequate 3D support for the attachment, proliferation and differentiation of MC3T3-E1. Moreover, the proliferation and differentiation was more favourable on PCL coated HAp scaffold with respect to HAp scaffold itself. The results demonstrate the potential of the HAp/PCL scaffolds to be used in bone tissue engineering.

Acknowledgements

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