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Characterization of calcium phosphate layers grown on polycaprolactone for tissue engineering purposes

M. Lebourg [a,b,c,*], J. Suay Antón [a,b,c], J.L. Gomez Ribelles [a,b,c]

[a] CIBER en Bioingeniería, Biomateriales y Nanomedicina, Campus Río Ebro, Edificio I+D Bloque 5, 1a planta C./Poeta Mariano Esquillor s/n, 50018 Zaragoza, Spain
[b] Centro de Biomateriales e Ingeniería Tisular, Universidad Politécnica de Valencia, 46022 Valencia, Spain
[c] Regenerative Medicine Unit, Centro de Investigación Príncipe Felipe, Autopista del Saler 16, 46013 Valencia, Spain

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ABSTRACT

Composites fabricated by biomimetic mineral precipitation on polymeric substrates are of interest for tissue engineering. As biological properties of such mineral layers vary with slight changes in composition, a good physical characterization is necessary in order to study their biological activity. In this work polycaprolactone sheets were subjected to air plasma treatment followed by nucleation of calcium phosphate seeds to activate the growth of an apatite-like coating when immersing in simulated body fluid. Two compositions of the SBF were prepared, one of them highly carbonated and the other with no carbonate or magnesium ions. Immersion of PCL in the high carbonate composition produced a low-crystallinity apatite-like layer while the absence of carbonate and magnesium ions yielded a high crystallinity apatite with low Ca/P ratio that is likely partially hydrolyzed octacalciumphosphate (OCP). The morphology, crystal structure and composition of both types of coatings were characterized; osteoblast-like cell adhesion behaviour on different surfaces was observed by fluorescence and electron microscopy.

1. Introduction

Polymer–ceramic composites are of interest in the field of bone tissue engineering [1]: traditional polyesters used in biomedical field, as polylactide or polycaprolactone, are easily reabsorbed, show ductile properties, and can be easily processed to porous bodies as those used in tissue engineering, but they lack intrinsic bioactivity, thus not favouring any repair response from the body. On the other hand, ceramics from the calcium phosphate family, for example tricalcium phosphate and hydroxyapatites, have shown to induce a good response from bony cells. In fact they are currently in use in orthopaedics, though always with the drawback of fragility, scarce remodelling [2], and a difficulty in cutting to fill the defect shape. A combination of both material types thus reduces their drawbacks while benefiting from their respective advantages [3–5].

In recent years, much attention has been paid to the in vitro mineralisation of materials in protophysiological solutions (called simulated body fluid, hereafter mentioned as SBF) as an important step for demonstrating corresponding in vivo bone bonding.

The deposited calcium phosphate layer is described in the literature mostly as "biomimetic apatite" for its composition is similar to that of bone mineral, that is to say a carbonated, calcium deficient and poorly crystalline apatite. Such nanostructured calcium phosphates, due to their high specific surface and soft preparation methods, are thought to be useful in tissue engineering as in vitro cell culture substrates for the study of cell–material interactions, or as carriers for proteins [6] or drugs (like biphosphonates or strontium containing drugs) while being easily degraded in the body if implanted. Based on the assumption that such a "bone-like mineral" would show good bioactive properties for the culture of cells, many studies have been carried out to prepare composites by precipitating mineral on different substrates to study the response of cells from bone lineage (osteoblasts, SAOS cells, mesenchymal cells, etc.) to such mineral, with variable results [7–13]. Nevertheless, many papers do not characterize properly the mineral layer deposited, and as has been described among others by Chou et al. [14], the composition, Ca/P ratio, present phases, crystallinity [15] and biosolubility of the mineral layer is of outermost importance with respect to the cell response, since cells show exquisite sensitivity to the smallest changes in roughness, chemistry, surface tension, crystal structure or culture medium composition [16]. In this paper, we describe how we obtained two differentiated calcium phosphate layers and their characterization: we also present initial results of osteoblast-like cell culture on them.

* Corresponding author at: Centro de Biomateriales e Ingeniería Tisular, Universidad Politécnica de Valencia, Edificio 8E, Bloque F-Nivel 1, Camino de Vera s/n, 46022 Valencia, Spain. Tel.: +34 963877007x88938; fax: +34 963877276.
E-mail address: myle1@upvnet.upv.es (M. Lebourg).

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2. Materials and methods

2.1. Sample preparation

PCL (Polysciences, Mw = 43,000–50,000 Da) and 1–4 dioxane (Scharlab 98% pure) were used without further purification. PCL bulk samples were prepared by solvent casting from a PCL solution in dioxane (15% by weight) in Petri dishes. PCL with hydroxyapatite nanoparticles used in cell culture (Hap particles, $d < 100$ nm, Sigma–Aldrich) were prepared by solvent casting of PCL solution (15% by weight in dioxane) with 20% of Hap (dispersed with ultrasound); these samples are referred thereafter as PCL-HAp-P samples. After solvent evaporation, films were rinsed in ethanol and vacuum dried to constant weight. Then 12 mm-diameter disks were stamped out of the films and treated for apatite nucleation as described in [17]. Samples were first treated with air plasma treatment, 300 W power, 90 s on each side in an Electronic Piccolo plasma chamber (Plasma Electronic GmbH, Germany). PCL and PCL-HAp-P samples were submitted to plasma treatment just before cell culture as effect of plasma on surface tension decreases with time. Then, samples were immersed for ten seconds in a $CaCl_2$ alcoholic solution (0.1 mM, water/alcohol, w/a, 50/50), washed for 1 s in w/a 50/50 mixture, then immersed for 10 s in a K_2HPO_4 alcoholic solution (0.1 mM, w/a = 50/50), and washed in water–alcohol mixture. This process was repeated 5 times. Subsequently, samples were freely suspended in modified SBFs, prepared following the methodology of Müller et al. [18] out of concentrated solutions of KCl, NaCl, $NaHCO_3$, $MgSO_4$–$7H_2O$, $CaCl_2$, Tris–HCl buffer, NaN_3, KH_2PO_4. The final electrolyte concentrations in SBFs are presented in Table 1. The main difference with Müller protocol was the concentration of sodium azide used as an antibacterial agent (when not prepared in totally aseptic conditions, SBF is likely to be colonised by phosphate eater bacteria [19]), this concentration was lowered from 1 g/l to 10 mg/l as sodium azide is very toxic and 10 mg/l is sufficient for antibacterial activity: as a result, the sodium content in the prepared SBF was slightly lower than in traditional SBF, but it is hypothesized that since sodium is not a constituent of hydroxyapatite it does not modify its solubility product and thus does not influence its precipitation from the solution. Previous experiments (results not shown) showed that these SBFs are able to produce an apatite layer as do original SBF.

The SBF-A, (for amorphous), is a carbonate rich SBF prepared following the method by Müller and Müller and contains 15 mM HCO_3^{2-} per litre. It was buffered at pH = 7.4 at 37 °C. It is expected to produce small-crystallite, low crystalline apatite deposit.

The SBF-C, for crystalline, is mainly a SBFx2 (with concentration of electrolytes doubled) containing neither magnesium nor carbonate, since these ions are known to act as crystal growth inhibitors, and buffered at pH = 7, (so as to lower the probability of spontaneous nucleation in the solution). It is expected to produce large-crystallite, highly crystalline apatite deposit.

SBF was changed every 4 days and SBF immersion lasted 14 days. After SBF treatment, the samples were washed with distilled water and dried (except samples for cell culture). During washing, leaching of some mineral particles was observed.

Table 1
Electrolyte concentration of simulated body fluids employed.

Electrolyte concentration	SBF-A (mM/l)	SBF-C (mM/l)
Na	130.0	287.5
K	3.7	7.4
Ca	2.5	5.0
Mg	1.0	0.0
Cl	122.7	302.8
HCO_3	15.0	0.0
SO_4	1.0	0.0
HPO_4	1.0	2.0

2.2. SEM observation

Samples were cut and stuck to a metallic support using graphite adhesive tape, so that a piece of each face of the sample could be observed. Samples were gold sputtered and observed in a Hitachi S3000 electron microscope with acceleration tension of 20 kV and work distance of 15 cm. EDX analysis was performed and Ca/P values were calculated from at least three large field analysis measurements.

2.3. FTIR characterization

The surface of the samples was scraped and the resulting powder was mixed with KBr in relation 1:1000 and compacted to a disk. FTIR analysis was performed in a FT-IR Bruker IFS60v analyzer provided with MCT detector in transmission mode from 550 to 4000 cm^{-1} with a sensitivity of 4 cm^{-1}. For evaluation of peaks, data was analyzed using the review on HAp characterization by Koutsopoulos [20].

2.4. X-ray diffraction

XRD analysis was carried out using a Siemens D-5000 diffractometer with $\theta/2\theta$ geometry equipped with parallel beam device for grazing incidence measurements and secondary monochromator. Measurements were performed in grazing incidence mode using monochromatic Cu (Kα) radiation, with incidence angle 1° in order to observe mainly the mineral deposit, and scanning 2θ between 10° and 100° with increment steps of 0.04. For evaluation of peaks, data were compared with data from respective ICDD card files 026-1056 (OCP) and 009–0432 (HAp).

2.5. Cell culture

Materials were sterilized with cold ethanol during two hours before cell culture. Before seeding, samples of pure PCL, PCL Hap-P, PCL-HAp-A and PCL-HAp-C (and glass coverslips as a control) were washed with PBS and coated with fibronectin solution in PBS (fibronectin from human plasma, Sigma, 20 μg/ml) overnight. MT3C3 osteoblastic cells (Riken Cell Bank, Japan) at passage 20 were seeded at a density of $\sim 9 \times 10^3$ cells/cm^2 in 400 μl standard growth medium (DMEM-LG supplemented with 1% Penicillin and Streptomycin, 1% glutamine), and allowed to adhere for 3 h. At the end of the culture time, samples were washed twice with PBS, blocked with 3.7% paraformaldehyde (Sigma–Aldrich) during 60 min at 4 °C and then stored in PBS at 4 °C until biological characterization was performed.

2.6. Biological characterization

2.6.1. Fluorescence microscopy

The actin cytoskeleton was visualized using the green-fluorescent Bodipy FL Phalloidin (Invitrogen). After staining according to standard protocols, samples were glued on microscope slides with mounting medium containing DAPI (Vectashield, ATOM) for visualization of nuclei and stored in the dark at 4 °C until fluorescence microscopy was performed. Samples were observed in an inverted vertical microscope Axiovert200 (Zeiss) coupled to a CCD monochrome and color camera.

2.6.2. SEM

Samples were dehydrated in graded alcoholic solutions and air dried, then stuck to a metallic support using graphite adhesive