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Formation of carbonated hydroxyapatite films on metallic surfaces using dihexadecyl phosphate–LB film as template

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ABSTRACT

Hydroxyapatite serves as a bioactive material for biomedical purposes, because it shares similarities with the inorganic part of the bone. However, how this material deposits on metallic surfaces using biomimetic matrices remains unclear. In this study, we deposited dihexadecyl phosphate, a phospholipid that bears a simple chemical structure, on stainless steel and titanium surfaces using the Langmuir–Blodgett (LB) technique; we employed the resulting matrix to grow carbonated hydroxyapatite. We obtained the calcium phosphate coating via a two-step process: we immersed the surfaces modified with the LB films into phosphate buffer, and then, we exposed the metal to a solution that simulated the concentration of ions in the human plasma. The latter step generated carbonated hydroxyapatite, the same mineral existing in the bone. The free energy related to the surface roughness and composition increased after we modified the supports. We investigated the film morphology by scanning electron and atomic force microscopies and determined surface composition by infrared spectroscopy and energy dispersive X-ray. We also studied the role of the surface roughness and the surface chemistry on cell viability. The surface-modified Ti significantly increased osteoblastic cells proliferation, supporting the potential use of these surfaces as osteogenic materials.

1. Introduction

Since the early 1970s, different studies have demonstrated that the titanium bioactivity alone is not high enough to induce direct bone tissue growth [1,2]. This has driven researchers to search for various techniques to grow bioactive ceramics [3], specially apatites, and deposit them on titanium surfaces [4,5], aiming to increase the osteointegration related to the surface chemistry and topography and thus control tissue–implant interactions and diminish the fixation time [2,6]. Among the various coating procedures, the plasma spraying technique is the most often employed for biomedical applications regarding bone substitution implants [7,8]. However, this process poses several difficulties: (1) it requires extremely high temperatures and expensive equipment; (2) it does not elicit well-organized growth, so the mineral arises in non-specific sites; (3) it cannot cover porous surfaces or incorporate biologically active agents [9]; and (4) the poor coating-substrate binding frequently culminates in serious clinical problems [10].

In this sense, the assembly of biomimetic apatite composites guided by naturally occurring biological molecules like phospholipids and proteins represents an interesting strategy [11,12]. The spreading of insoluble lipids on air/water interface affords Langmuir monolayers, which constitute a well-known matrix for calcium phosphate and carbonate growth [13,14]. Nevertheless, few studies have reported on the transfer of Langmuir monolayers to solid supports, the so-called Langmuir–Blodgett (LB) films, to grow minerals, especially calcium phosphates (Ca–P) [15,16]. Organic LB matrices enable nucleation of the first mineral seeds, guiding the subsequent growth process at the nanoscale level [17,18]. Zhang et al. [15] observed that calcium phosphates nanodots, nanowires, and nanoislands originate on the matrix during the early nucleation stages, depending on the stearic acid LB films orientation. A further advantage of these matrices is that they are biocompatible and consist of lipids, and proteins, mimicking the cell wall structure. Also, the lipids bind strongly to the solid surfaces [19] improving attachment of the biomineral grown as guided by the LB matrix. Kokubo et al. [20] studied another important biomimetic approach consisting of a method that is currently used to grow hydroxyapatite (HAP) through surface exposure to simulated body fluid (SBF) [21]. This supersaturated solution

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mimics the ionic composition of human blood plasma and helps to evaluate the bioactivity of different materials [22]. The overall HAP growth process in SBF solution takes at least seven days [7], which limits the development of the biomimetic coatings using this procedure. Moreover, in this approach the biominerals do not specifically adhere to the surfaces. In the present study, we take the advantages of both the moleculary organized LB templates and the biomimetic approach using SBF to grow HAP in an organized and controlled manner. We modified the metallic supports by transferring Langmuir monolayers consisting of dihexadecyl phosphate spread on aqueous calcium chloride solution. This strategy also transferred the calcium ions bound to the phosphate groups to the solid supports during deposition of the molecularly organized LB film. By exposing the modified metallic surfaces to SBF solution, we achieved continuous carbonated-HAP films nucleation and growth after a few hours.

2. Materials and methods

Dihexadecyl phosphate (DHP); 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT); β-glycerophosphate; and dexamethasone were purchased from Sigma-Aldrich Co., St. Louis, MO, USA. Ascorbic acid, α-MEM, fetal bovine serum, gentamicin, fungizone, and trypsin were supplied by Gibco–Invitrogen Technologies, USA. The reactants used to prepare the SBF (calcium chloride, sodium choride, sodium bicarbonate, potassium chloride, magnesium chloride, sodium phosphate dibasic, sodium sulphate, and tris-hydroxymethilaminemethane) were analytical grade; the concentrations of the reactants are described elsewhere [23]. All the aqueous solutions were prepared using ultrapure dust-free Milli-Q® water.

2.1. Support

Before use, the stainless steel and titanium supports were treated in KH_2PO_4/NaOH buffer solution (pH 7.5) containing the non-ionic surfactant Span 20 (4.0×10^{-5} mol L^{-1}) for 5 min, at 65 °C, under ultrasound agitation. The supports were then exhaustively washed with Milli-Q® water.

2.2. Langmuir-monolayers

Surface pressure–surface area (π–A) isotherms were constructed at 25.0 ± 0.5 °C in a 216-cm^2 Langmuir trough (Insight-Brazil) by spreading a 1.0 mmol L^{-1} dihexadecyl phosphate solution dissolved in chloroform/methanol (3:1, v/v), HPLC grade (J.T. Baker). The phospholipid was spread on subphases containing either 0.1 mmol L^{-1} $CaCl_2$ aqueous solution or pure Milli-Q®. Calcium ions were included in the subphase solution to promote adhesion between the DHP phospholipidic layers in the LB films and to serve as a primary calcium ion source for mineral growth.

2.3. Langmuir–Blodgett (LB) films

The monolayers were transferred to the metallic supports by the LB technique. Y-type LB films were prepared by initially immersing the solid support in the subphase containing 0.1 mmol L^{-1} $CaCl_2$ solution before spreading the phospholipid solution. The DHP monolayers were transferred to the metallic supports using the following sequence: withdrawal (1 layer) and then immersion (2 layers); this procedure was repeated until the desired number of deposited layers was achieved. The immersion/withdrawal rate was 0.038 mm s^{-1}; the surface pressure (π) was kept at 30 mN m^{-1}. For this study, LB films with 2, 4, or 6 DHP layers were obtained. The DHP mass deposited per layer was 147 ± 10 ng, as measured using a quartz crystal microbalance. All the films ended with the hydrophilic group; i.e., the phosphate group of the phospholipid was exposed in the top layer (Fig. 1B). Because the latter layer displayed a hydrophilic end, the films were kept in a recipient containing pure water, to avoid the "flip-flop" of the phospholipid molecules before exposure to the calcium phosphate precursor solutions.

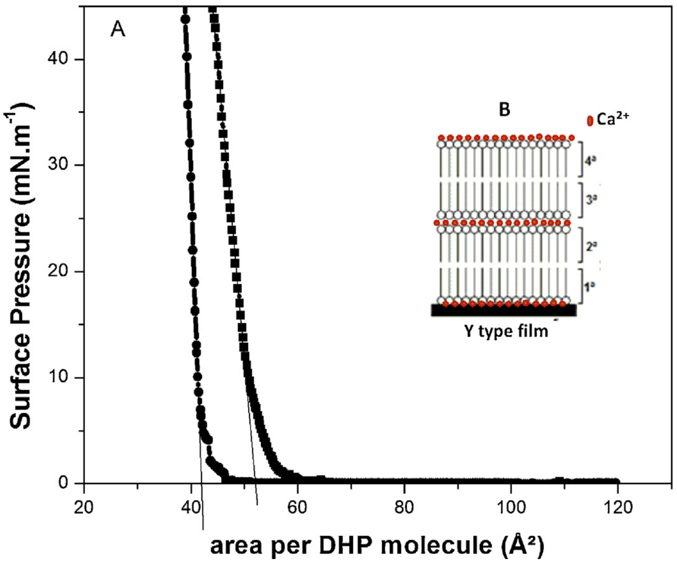

Fig. 1. (A) Surface pressure–surface area isotherm obtained for DHP on (■) water and on (●) 1×10^{-4} mol L^{-1} $CaCl_2$ aqueous solution at 24 ± 1 °C; (B) schematic representation of the Y-type LB film consisting of DHP and Ca^{2+} ions.

2.4. Mineral coating

Coating of the metallic surfaces (modified with the DHP–LB films) with the minerals involved two steps. In the first step, the supports were immersed in a 1.0 mmol L^{-1} Ca^{2+} aqueous solution for 12 h, and in KH_2PO_4/NaOH phosphate buffer (pH 7.5) for additional 12 h. This procedure was designated Ca^{2+}/phosphate buffer exposure cycle and was conducted four consecutive times, to guarantee maximum calcium ions binding to the negative charges of the phospholipid in the LB films and increase the local supersaturation at the interface. In the second step, the metallic surfaces were immersed in the SBF solution, which is a well-known procedure to evaluate surface bioactivity: it reproduces in vivo apatite layer formation on various materials surfaces after implantation [21]. The temperature of the solutions was kept at 37 °C during all the procedure.

2.5. Films characterization

The surface morphology of the gold-coated films was investigated by scanning electron microscopy (SEM) using a Zeiss-EVO 50 microscope. The Ca/P molar ratio in the films was evaluated by X-ray dispersive energy (EDX) (IXRF system 500 Digital Processing spectrometer). Atomic force microscopy (AFM) (Shimadzu-SPM 9600) in the contact mode was used to explore film roughness. The chemical groups were identified by Fourier-transform infrared spectroscopy (FTIR) coupled with an attenuated total reflectance (ATR) accessory (Shimadzu-IRPrestige-21). The surface free energy (SFE) of the samples was determined by measuring the contact angles (θ) (DataPhysics OCA20) of five test liquids (diiodomethane, water, hexadecane, formamide, and toluene) on