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In vitro assessment of zinc apatite coatings on titanium surfaces

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ABSTRACT

In this paper, coatings of hydroxyapatite partially substituted with zinc (ZnHA) were produced on titanium substrates by a two-step hydrothermal process using a precursor solution rich in calcium, phosphate and zinc. Activation of titanium surfaces was performed by oxidation with an acidic HF/HNO_3 solution. The coated substrates were then converted into HA by immersion in an alkali 0.1 M NaOH solution. The ZnHA samples were characterized by several techniques and their *in vitro* behavior was studied in comparison to hydroxyapatite (HA) and titanium (Ti-control) samples. A uniform and homogeneous calcium-deficient carbonate apatite coating was obtained for all samples, both doped and undoped with zinc. The percentage of zinc incorporated in the coatings is 7 at%, and the Ca/P ratio is 1.61 ($\pm$0.01) for both types of samples, suggesting that Zn is incorporated substitutionally, replacing Ca atoms into the HA structure. The incorporation of Zn in the HA structure changed the crystals morphology, reduced crystals sizes and decreased the deposition rate showing that zinc is an inhibitor of the growth of HA crystal. X-ray diffraction showed that HA is the single crystalline phase present after alkali treatment. The coating adhesion strength was evaluated in terms of the critical load (Lc) obtained from scratch tests and no significant difference was found between the two tested groups, indicating the good adhesion of ZnHA to Ti substrates. The *in vitro* response of human osteoblasts (HOB) exposed to the surfaces of HA and ZnHA coatings was evaluated. The results of Live/Dead tests showed cell viability for all samples surfaces, but the adhesion and proliferation tests showed that ZnHA samples presented better adhered and spread cells compared with HA. ZnHA coatings presented cells with elongated or polygonal shapes and clearly more spread than HA. Quantitative analysis showed that there was a significantly higher number of cells adhered to ZnHA coatings compared to HA, indicating the zinc incorporation stimulates osteoblast proliferation.

1. Introduction

The search for optimization of coating processes and surface treatments of biocompatible metals like titanium aiming bone-bonding has been subject of many researches [1–4]. Hydroxyapatite (HA) is used as bone graft and bioactive coating on titanium implants because its chemical composition is similar to that of bone, and its bioactivity allows chemical connection to the bone tissue. HA stoichiometric formula is $Ca_{10}(PO_4)_6(OH)_2$, with a molar Ca/P ratio of 1.67. Different molar ratios are associated with the presence of other calcium phosphate phases or ionic substitutions in the HA structure [5]. Due to its complex structure, HA enables anionic and cationic isomorphic substitutions. Pure HA does not occur on a macroscopic scale in biological systems. Biological apatite contains impurities, is not stoichiometric and is mainly found in enamel, dentin and bone. The main impurity found is the carbonate group $(CO_3)^{2-}$. Other impurities include ions such as Na^+, Mg^{2+}, K^+, Cl^-, F^- and others [6]. Because of its deficiency in calcium of about 10 at%, natural HA is called calcium

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deficient hydroxyapatite, and when carbonate is present in the structure, the it is called carbonated hydroxyapatite [7].

In an attempt to mimic natural HA, recent studies have shown that the incorporation of other elements in the HA structure, like Zn and Sr, can improve some of its properties [6,8–11]. Zinc is a structural component of some important enzymes, proteins and bone. When incorporated into HA, zinc may have positive effects; at low concentrations it can stimulate osteoblasts proliferation, allowing the formation of bone matrix and increasing the alkaline phosphatase activity, besides inhibiting osteoclast action [10–12]. For this reason, coating titanium surfaces with hydroxyapatite partially substituted with zinc (ZnHA) may contribute positively to the successful bone-bonding of the implants.

Among the various methods for HA deposition, the biomimetic method, proposed by Kokubo et al. in 1990, tries to mimic the natural process of biological apatite formation and consists of the immersion of bioactive chemically treated titanium in a simulated body fluid (SBF) solution for a long period of time [13]. In order to reduce the deposition time, calcium and phosphate ion concentrations in the solution have been increased and different treatments to activate the substrate surface have been proposed [14–16]. Adhesion of the coatings can be enhanced by etching the substrate surface, thus increasing surface roughness. Recent studies show that when the titanium surface is chemically activated by acidic treatment, an intermediate oxide layer between the bioactive precipitated apatite and the metal substrate is created, improving the nucleation and growth of calcium phosphates. This procedure simulates the process of bone biomineralization [17].

The biological response of osteoblastic cells includes cell attachment, cell growth, proliferation and functional activity. Previous studies have demonstrated that ZnHA exhibits good biocompatibility [9,12] and good performance under *in vitro* and *in vivo* testing [11,18]. However, others authors reported that no significant differences resulted from the addition of Zn to calcium phosphate compounds. This may be due to the low Zn concentration incorporated into the material. Sogo et al. found no significant difference on osteoblastic cell proliferation on rat skulls between pure β-TCP and β-TCP with a zinc content of 0.11 wt% [9].

In the present study, ZnHA coatings were produced on titanium by a hydrothermal process and their compositional, structural, morphological and adhesion properties were characterized in comparison to pure HA coatings. Then, *in vitro* tests were performed seeding human osteoblastic cells (HOB) on HA, ZnHA and Ti (control) surfaces in order to evaluate the cytocompatibility, cell attachment and cell proliferation.

2. Materials and methods

2.1. Treatment of titanium substrates

Commercially pure titanium (cp-Ti) substrates were cut into small squares of $10 \times 10 \times 1$ mm^3, grounded with 400–600 grit SiC sandpapers and then cleaned with deionized water. Substrates were subsequently immersed in a hydrofluoric and nitric acid solution [HF/HNO_3] for 30 s at a concentration of 70 ml H_2O, 28 ml HNO_3, 2 ml HF, followed by an ultrasonic cleaning with acetone and deionized water and then dried in an oven at 80 °C for 10 min.

2.2. Preparation of precursor solution

A saturated solution of Ca^{2+} and PO_4^{3-} ions (SSCP) was prepared for the deposition of the HA coatings on titanium substrates as described elsewhere [16]. During the preparation, 0.5 M calcium hydroxide ($Ca(OH)_2$), 0.3 M phosphoric acid (H_3PO_4) and 1 M lactic acid ($C_3H_6O_3$) were successively dissolved in 750 ml of deionized water at constant stirring. For the coatings containing zinc, a saturated solution of Ca^{2+} and PO_4^{3-} ions and Zn^{2+} (SSCP-Zn) was prepared replacing 10 mol% of calcium hydroxide for zinc nitrate as described by Prado et al. [19]. The solution was stirred for 24 h at room temperature, controlling the pH to be around 3.7.

2.3. Coating deposition process

For the deposition of HA coatings, pretreated titanium samples were immersed in 250 ml of SSCP and heated in a water bath at 80 °C for 1 h. In case of ZnHA coatings, the substrates were immersed in 250 ml of SSCP-Zn, heated in a water bath at 80 °C for 12 h. Crystals precipitated on the metal surface producing a continuous adhered coating. After deposition of the coatings, both types of samples were placed in a solution of 0.1 M NaOH at 60 °C for 24 h.

2.4. Characterization

Test materials were divided in three groups according to the different surface treatments: Ti coated with hidroxyapatite (HA), Ti coated with hydroxyapatite partially substituted with zinc (ZnHA) and polished cp-Ti (Ti).

The phases present in the coating materials were identified by X-ray diffraction in a PANalytical X'Pert X-ray diffraction system operating with CuKα ($\lambda = 0.1542$ Å) X-ray source with a grazing incident angle of 2.5° and a step rate of 0.05°/s, from 10° to 80°. The coatings morphology was observed by scanning electron microscopy (SEM-JEOL JSM 5800LV) operated at an accelerating voltage of 20 kV in the low vacuum mode. Semi-quantitative element analysis was obtained by energy-dispersive X-ray analysis (EDX). To identify the chemical groups present in the coatings, HA and ZnHA were scraped off the substrates for Fourier transform infrared spectroscopy (FTIR) analysis using the KBr pellet technique. Elemental quantitative analysis for the presence of Ca, P and Zn were made using X-ray fluorescence (XRF) spectroscopy (Vulcan X-ray Fluorescence Spectrometer).

Scratch tests were carried out in order to determine the adhesion of the HA and ZnHA coatings to the Ti substrates using a Universal Mechanical Tester (Bruker UMT-2). This test involves dragging a Rockwell diamond indenter across the surface of the sample while the load applied to the indenter is linearly increased. The lateral (friction) force F_f and the acoustic emission (AE) signal are continuously monitored during the experiment. A maximum normal load of 20 N was employed, and the loading rate and sliding speed were 5.3 N/s and 1.66 mm/s, respectively. The resulting scratch scars of approximately 6 mm in length were examined with an optical microscope and the coating adhesion strength was evaluated by the critical load (Lc) [20]. For each sample nine scratch tests were performed and the average values and standard deviations of Lc were obtained. Coating thickness was measured by stylus profilometry (Bruker Dektak XT). The film thickness was determined as the average value of five different scans.

2.5. In vitro evaluation

All experiments were performed using human osteoblasts cells (HOB). The cell cultures were maintained using Dulbecco's modified Eagle's medium (DMEM) low glucose (GIBCO) and supplemented with 10% fetal bovine serum (FBS, Soromed) maintained at 37 °C in a humidified atmosphere of 5% CO_2/air. The cells were lysed with 0.05% trypsin/0.02% EDTA in PBS solution and cultured on sample surfaces using 1×10^4, 5×10^4 or 1×10^5 cells/sample. All samples were identified and sterilized by gamma radiation (cobalt 60 source, 15 KGy, 19.72 Gy/min, total 12 h) before each analysis.