HA and double-layer HA-P$_2$O$_5$/CaO glass coatings: influence of chemical composition on human bone marrow cells osteoblastic behavior

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Human osteoblastic bone marrow derived cells were cultured for 28 days onto the surface of a glass reinforced hydroxyapatite (HA) composite and a commercial type HA plasma sprayed coatings, both in the “as-received” condition and after an immersion treatment with culture medium during 21 days. Cell proliferation and differentiation were analyzed as a function of the chemical composition of the coatings and the immersion treatment.

Cell attachment, growth and differentiation of osteoblastic bone marrow cells seeded onto “as-received” plasma sprayed coatings were strongly affected by the time-dependent variation of the surface structure occurring during the first hours of culture. Initial interactions leading to higher amounts of adsorbed protein and zeta potential shifts towards negative charges appeared to result in surface structures with better biological performance. Cultures grown onto the pretreated coatings showed higher rate of cell proliferation and increased functional activity, as compared to those grown onto the corresponding “as-received” materials. However, the cell behavior was similar in the glass composite and HA coatings.

The results showed that the glass composites present better characteristics for bone cell growth and function than HA. In addition, this work also provide evidence that the biological performance of the glass composites can be modulated and improved by manipulations in the chemical composition, namely in the content of glass added to HA.

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1. Introduction
Plasma sprayed hydroxyapatite (HA) coatings applied onto metallic substrates (stainless steel or titanium alloy) are currently being used as implants and prostheses in many dental and orthopedic applications. These coatings combine high strength and fracture toughness of the metallic substrate with the bioactivity of the HA coatings. Several studies have shown that this system is more effective than metallic substrates [1–6].

Multilayered coatings composed of mixtures of P$_2$O$_5$ glasses and HA may present several advantages than HA coatings alone [7–9]. Although being bioactive, HA presents slow osteoconduction in vivo, requiring long-term immobilization periods after surgery. Due to the high solubility induced by the P$_2$O$_5$ glass, the use of multilayered coatings of HA/P$_2$O$_5$ glass composite enhances a faster Ca and P exchange with the local environment after implantation. The presence of a stable HA underlayer facilitates long-term osteointegration and stabilization of the prosthesis, while the high soluble phase is expected to facilitate the mineralization process.

Cell culture studies have shown that “as-received” plasma sprayed HA coatings hardly supported osteoblastic cell growth [6, 7, 10, 11]. However, an immersion pretreatment with culture medium greatly improved cell growth and function [7, 10]. A comparative study showed that glass reinforced HA plasma sprayed coatings presented a better performance concerning human osteoblastic cell proliferation and differentiation, as compared to a simple HA plasma sprayed coating, both at the “as-received” condition and after an immersion pretreatment [7]. In vitro bioactivity testing using simulated body fluid-SBF also showed that during the immersion of glass reinforced HA coatings, dissolution of the coating surface occurred and apatite layer formation on the surface took place faster than on the HA coatings [8]. This observation suggested higher bioactivity for the composite coatings.

Cell growth and function are significantly affected by the surface characteristics of the biomaterial such as morphology, roughness, chemical composition and pretreatments [12–19]. In a previous work, authors

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reported the proliferation–differentiation behavior of human osteoblastic bone marrow cells cultured onto a surface of a glass reinforced HA plasma sprayed coating prepared with CaO-P2O5 glass addition of 2 wt % to HA (HA/G4 2%) [7]. In this work, the behavior of the same bone cell system was studied culturing the cells onto a glass reinforced HA plasma sprayed coating prepared with CaO-P2O5 glass addition of 4 wt % to HA (HA/G4 4%) and also onto a simple HA plasma sprayed coating, both at the “as-received condition” and after an immersion pretreatment with culture medium. Osteoblastic cell proliferation and differentiation on the glass reinforced HA composites were analyzed as a function of the chemical composition of the coatings and the immersion treatment with the culture medium. Protein adsorption, zeta potential measurements and phase identification and quantification using XRD analysis were performed to try to explain the in vitro biological behavior of studied materials.

2. Materials and methods

2.1. Preparation of the materials
A P2O5-based glass (G4), with the following chemical composition (in mol %): 35 P2O5-35 CaO-20 Na2O-10 K2O, was prepared from reagent grade chemicals using a conventional melting technique. Glass and HA powders (P120 batch, Plasma Biotal, Tideswell, UK) were wet-mixed in methanol and a content of 4 wt % of glass was added to HA. The method used to prepare the glass reinforced HA composites has been fully described elsewhere [7]. Mixed powders were then dried, isostatically pressed at 200 MPa and sintered. Samples were then milled and sieved to provide a particle size distribution between 53 and 150 μm.

Commercially available titanium alloy (Ti-6Al-4V) was used as substrate and discs with 14 mm diameter and 3 mm thick were prepared. Ti-6Al-4V disks were plasma sprayed with HA powder (120 μm) and with the HA/G4 4% composite (double-layered composite of 60 μm HA, followed by a 60 μm HA/G4 4% composite).

2.2. Quantitative phase analysis
X-ray diffraction (XRD) analysis was performed on coated samples, in a Siemens D5000 diffractometer. Using flat geometry, data were collected from 5° to 110° 20 values, with a step size of 0.02° and a count time of 12 s/step. Quantitative phase analysis was performed by Rietveld method using General Structure Analysis Software (GSAS; Los Alamos National Laboratory).

2.3. Zeta potential and protein adsorption measurements
Coatings were detached from the Ti-6Al-4V substrate and milled. Particle size and zeta potential (ZP) were measured in a Brookhaven ZetaPlus instrument. Particle size was bellow 1.3 μm, with 90% of the particles below 1 μm. The ZetaPlus instrument automatically calculates ZP according to Smoluchowski’s equation:

\[ ZP = 4\pi \eta \frac{EM}{D} \]

EM is the electroforetic mobility, η is the viscosity of the suspending liquid, D is the dielectric constant of the suspending liquid.

The initial zeta potential was measured after drying the powders at 60°C.

For measurement of the time-dependent variation of the zeta potential and protein adsorption, each 400 mg of the dry powder was immersed in 10 ml of α-minimal essential medium (α-MEM) containing 10% fetal bovine serum (FBS), 50 μg/ml gentamicin and 2.5 μg/ml amphotericin B. They were placed in an incubator at 37°C and shaken at 100 rpm for periods of 12 h, 1, 3, 5 and 7 days. At the end of each immersion time, the powder was obtained by centrifugation for 10 min at 4000 rpm. The centrifuged powder was then washed twice with 10 ml of distilled water and dried at 60°C. The zeta potential of the powder was measured at pH 7.2, in 10⁻³ M KCl in triplicate samples.

To evaluate the amount of adsorbed protein to the ceramic powder, 5 ml of 0.1 N NaOH was added to each 200 mg of the dried powder and samples were shaken for 6 h at 37°C to dissolve the adsorbed protein. The protein concentration in the alkaline solution was assayed by the Lowry’s method. The control solution was α-MEM/10% FBS incubated at 37°C in which no ceramics were immersed. All measurements were performed in triplicate.

2.4. Cell culture
Human bone marrow, obtained from surgery procedures on a 33-year-old woman, was cultured in α-MEM containing 10% FBS, 50 μg/ml gentamicin and 2.5 μg/ml amphotericin B and supplemented with ascorbic acid (50 μg/ml), β-glycerophosphate (βGp, 10 mM) and dexamethasone (10 nM). Incubation was carried out in a humidified atmosphere of 95% air and 5% CO2 at 37°C. Primary cultures were maintained until near confluency (10–15 days) and, at this stage, adherent cells were enzymatically released (0.04% trypsin and 0.025% collagenase) and seeded in 24-well dishes at a density of 2 x 10⁴ cells/cm². Bone marrow cells were cultured for periods up to 28 days, in the same experimental conditions as those used in primary cultures, on the surface of the plasma sprayed samples: (i) HA/G4 4% composite and HA coated disks in “as-received” conditions; (ii) HA/G4 4% composite and HA coated disks pretreated with complete culture medium for 21 days at 37°C in a humidified atmosphere of 95% air and 5% CO2. Also, bone marrow cells were cultured in parallel on standard plastic tissue culture plates as control cultures. Culture medium was changed twice a week in the cell cultures and also during the 21 days pretreatment of the material samples.

Control cultures and cultures growing on the surface of the coated discs were characterized to evaluate total protein content and alkaline phosphatase activity (ALP), and were observed by scanning electron microscopy (SEM); cultures were tested at days 3, 7, 14, 21 and 28. In addition, the culture medium was analyzed for ionized