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HA and double-layer HA-P_2O_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.

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_2O_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_2O_5 glass, the use of multilayered coatings of HA/P_2O_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-P_2O_5 glass addition of 2 wt % to HA (HA/G_1 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-P_2O_5 glass addition of 4 wt % to HA (HA/$G_1$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 P_2O_5-based glass (G_1), with the following chemical composition (in mol %): 35 P_2O_5-35 CaO-20 Na_2O-10 K_2O, 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/$G_1$4% composite (double-layer composed of 60 μm HA, followed by a 60 μm HA/$G_1$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° 2θ 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-6A1-4V 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:

$$ZP = 4\pi \frac{\eta}{D} EM$$

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 ° 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% CO_2 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 2×4 cells/cm^2. 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/$G_1$4% composite and HA coated disks in ''as-received'' conditions; (ii) HA/$G_1$4% composite and HA coated discs pretreated with complete culture medium for 21 days at 37 °C and in a humidified atmosphere of 95% air and 5% CO_2. 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

calcium (Ca) and phosphorus (P) throughout the incubation time. For biochemical data each point represents the mean ± standard deviation of three replicates.

2.4.1. ALP activity and total protein content

At the end of each culture period, cultures were washed twice with phosphate-buffered solution (PBS) and stored at −20 °C until the end of the experiment. ALP activity was determined in cell lysates (obtained by treatment of the cultures with 0.1% triton) and assayed by the hydrolysis of p-nitrophenyl phosphate in alkaline buffer solution, pH 10.3, and colorimetric determination of the product (p-nitrophenol) at $\lambda = 405$ nm (hydrolysis was carried out for 30 min at 37 °C). Results are expressed in nanomoles of p-nitrophenol produced per min per cm^2 (nmol/min/cm^2).

Protein content was determined in 0.1 M NaOH cell lysates according to the method of Lowry using bovine serum albumin as a standard. Results are expressed as μg/cm^2

2.5. Scanning electron microscopy

SEM observation was performed on the ''as-received'' material samples, pretreated material samples, seeded materials and control cultures. Samples were fixed with 1.5% glutaraldehyde in 0.14 M sodium cacodylate (pH 7.3), then dehydrated in graded alcohols, critical-point dried, sputter-coated with gold and analyzed in JEOL JSM 6301F scanning electron microscope equipped with X-ray EDS microanalysis capability, Voyager XRMA System, Noran Instruments.

2.6. Calcium and phosphorus measurements

Culture media from control cultures and also from cultures growing on the surface of the material samples were collected twice a week, at each change of medium, and analyzed for ionized calcium and phosphorus concentration throughout the 28 days culture period. Quantification of these ions in the medium collected from the material samples during the 21 days treatment with culture medium was also performed.

Ionized calcium and phosphorus were measured using, respectively, a calcium kit (Sigma no. 587M) and the inorganic phosphorus kit (Sigma no. 670-C). The levels measured were not cumulative as the culture medium was totally replaced twice a week, so the values determined reflected the changes occurring in intervals of 3–4 days throughout the incubation period.

3. Results

Human bone marrow cells were grown in the presence of ascorbic acid, β-glycerophosphate and dexamethasone, experimental conditions described to favor the formation of osteoblast-rich cell cultures [20–24]. Cells were cultured for periods up to 28 days on the surface of a glass reinforced HA composite, HA/$G_1$4%, and a commercial type HA plasma sprayed coatings. The coatings were seeded both in the ''as-received'' condition and after an immersion treatment with culture medium during 21 days. As a reference control, bone marrow cells were cultured in parallel on tissue culture plastic plates.

3.1. Materials characterization studies

3.1.1. Quantitative phase analysis

Results from quantitative phase analysis of HA and HA-glass composite coatings obtained by XRD patterns using the Rietveld method are shown in Table I.

The relative proportion of β-$Ca_3(PO_4)_2$(β-TCP) in the structure of the composite coatings increased as the level of glass content added to HA increased. β-TCP was formed by reaction of P_2O_5 glass with HA. However, HA coatings were also partially converted into β-TCP, due to phase transformations taking place during thermal cycle imposed by plasma spraying.

3.1.2. Zeta potential and protein adsorption measurements

Fig. 1 shows the time-dependent variation of the zeta potential of HA, HA/$G_1$2% and HA/$G_1$4% materials. The zeta potential of the powder coatings was −9.9, −12.5 and −14.7 mV for HA, HA/$G_1$2% and HA/$G_1$4%, respectively. However, after immersion in α-MEM/10% FBS the zeta potential shifted negatively. The shifting rates were different and after 7 days of immersion the zeta potential of the coatings were −22.9, −26.2 and −35.8 mV for HA, HA/$G_1$2% and HA/$G_1$4% respectively, being the shift of HA/$G_1$4% higher than 20 mV from the initial potential.

Fig. 2 shows the time-dependent profile of the adsorbed amount of protein. The adsorption was more pronounced on the first hours and progressed gradually only for HA/$G_1$2% and HA/$G_1$4% being HA/$G_1$4% the powder that adsorbed a higher amount of protein.

3.1.3. Pretreatment of the material samples with culture medium

HA and HA/$G_1$4% were immersed during 21 days in culture medium using the same experimental conditions as the cell cultures and the levels of ionized calcium and

TABLE I Quantitative phase proportions of coatings determined by Rietveld analysis (%)

	Quantitative phase proportions		
	HA	β-TCP	CaO
HA	87.2 ± 0.5	6.4 ± 1.3	6.4 ± 1.8
HA/$G_1$2	84.7 ± 0.9	10.9 ± 0.8	4.4 ± 0.4
HA/$G_1$4	75.3 ± 0.3	22.5 ± 3.7	2.2 ± 0.1

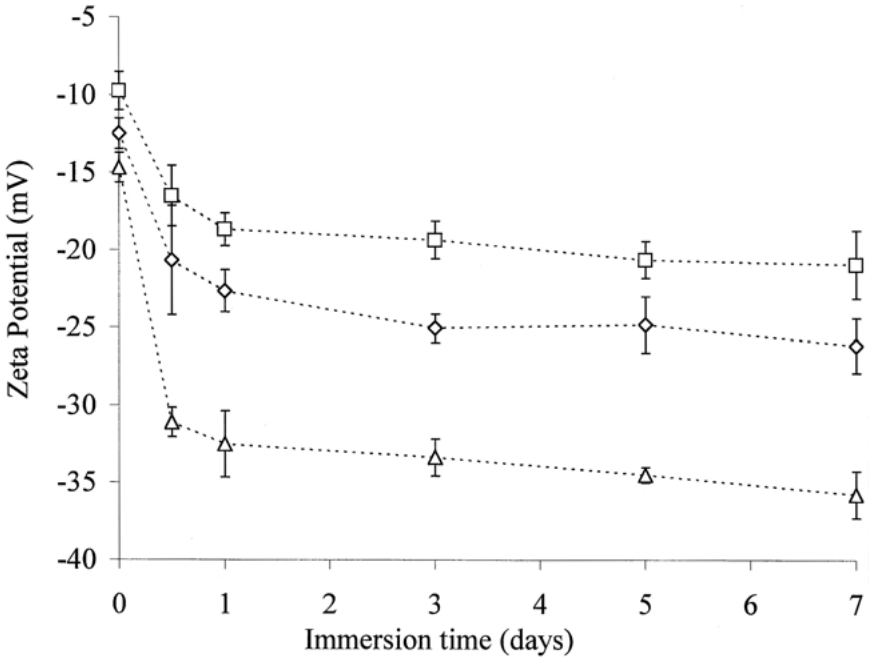

Figure 1 Time-dependent profile of zeta potentials of HA, HA/$G_1$2% and HA/$G_1$4% during a seven days immersion in α-MEM/10% FBS. (□) HA, (◇) HA/$G_1$2% and (△) HA/$G_1$4%.

phosphorus in the medium were measured throughout this period. Results are shown in Fig. 3.

Immersion of the ''as-received'' HA/$G_1$4% and HA coated samples resulted in an increase in the levels of ionized Ca and P in the culture medium during approximately the first 2 weeks of incubation and, from then onwards, levels were similar to those measured in the culture medium before any contact with the samples. The two materials presented a similar behavior concerning the levels of ionized P, that is a small and regular increase in the levels of this ion. Incubation of the HA glass composite also resulted in a small increase in the levels of ionized Ca, whereas, immersion of the HA coating caused a significantly higher increase in the levels of this ion, specially around days seven and ten.

Plasma sprayed HA and HA/$G_1$4% coated disks were observed by SEM in the ''as-received'' condition and after the 21 days immersion treatment with culture medium. In both material samples, the plasma sprayed surface was very rough with patches of smooth and shiny amorphous phase film. After immersion in culture medium, an apatite layer was observed throughout the coating surfaces in both materials. These observations were similar to those reported previously [7].

3.2. Seeded materials

3.2.1. Total protein content and ALP activity

Results concerning total protein content and ALP activity measured in control cultures and in the seeded material coatings are presented in Fig. 4.

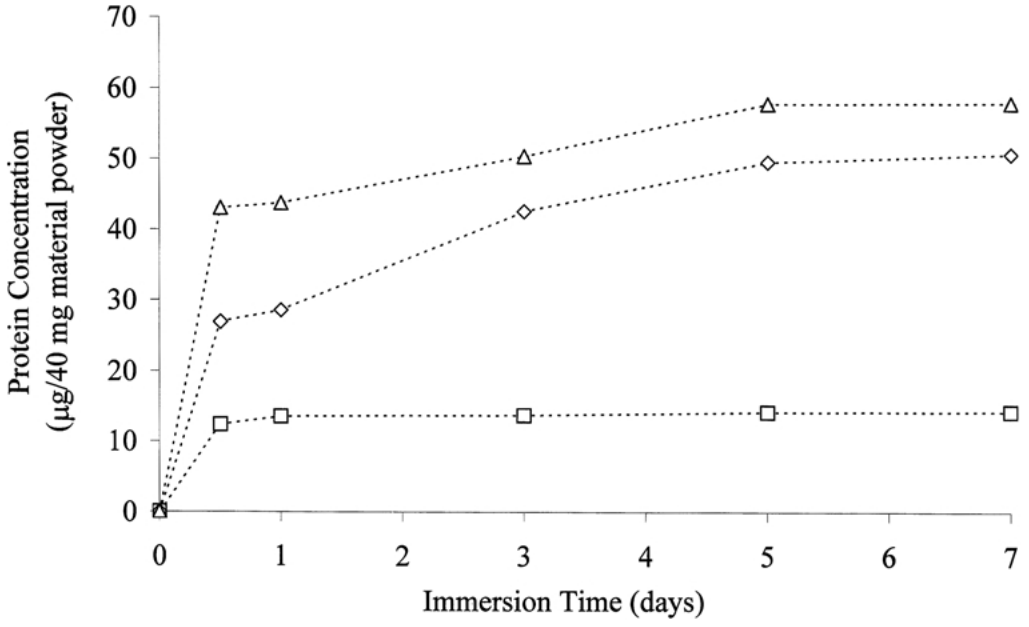

Figure 2 Time-dependent profile of adsorbed amount of protein to HA, HA/$G_1$2% and HA/$G_1$4% during a 7-day immersion in α-MEM/10% FBS. (□) HA, (◇) HA/$G_1$2% and (△) HA/$G_1$4%.

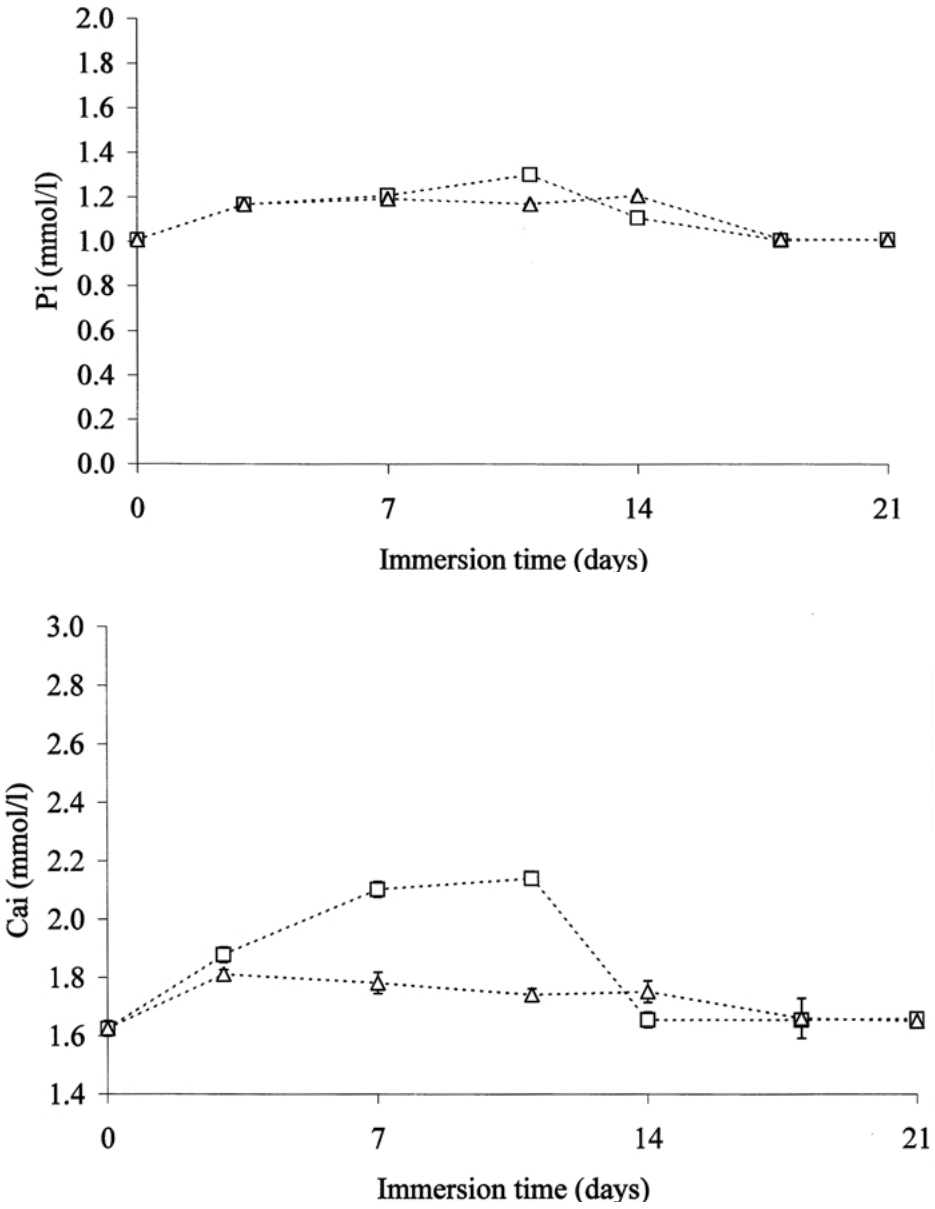

Figure 3 Levels of ionized phosphorus and calcium in the medium collected from the plasma sprayed coated samples during the 21 days pretreatment with culture medium. (□) HA and (△) HA/$G_1$4%.

In control cultures, total protein content increased with incubation time specially during the first week and a tendency for a stationary state seems to have been achieved by the third week. ALP activity was relatively low in the first 3 days, then increased to a peak level of response until around day 14 and dropped significantly during the fourth week. The significant increase in the levels of this enzyme during the second week suggested that the cells were shifting to a more differentiated state [25, 26]. These results are in agreement with those reported previously for this culture system [27, 28].

Fig. 2 showed that milled samples of HA and HA/$G_1$4% adsorbed significant amounts of protein after being immersed in culture medium, in particular the HA/glass composite. This observation suggests that the amount of protein measured in the seeded material coatings includes the protein adsorbed by the material itself and also that resulting from the cell growth, which does not allow for a comparison with the results observed in the control cultures. However, as protein adsorption appeared to occur mainly in the first hours of incubation, determination of the protein content in the seeded materials would provide a relative measure of the cell proliferation in the various samples. Because protein adsorption was higher in the glass composite than in the HA, the only observation that can be drawn from the results presented in Fig. 4 is that bone marrow cells proliferated better in the pretreated material coatings than in the corresponding ''as-received'' samples.

Expression of ALP appeared to be delayed in the cultures grown on the plasma sprayed coatings. In the seeded pretreated materials, levels of the enzyme increased from approximately day 7 and maximal levels were attained by day 21 decreasing significantly after that. ALP activity was significantly higher in the cultures growing in the pretreated materials than in the ''as-received'' coatings. In the seeded ''as-received'' HA/$G_1$4% ALP activity increased specially from day 14 onwards but the levels of this enzyme measured on the seeded ''as-received'' HA were negligible.

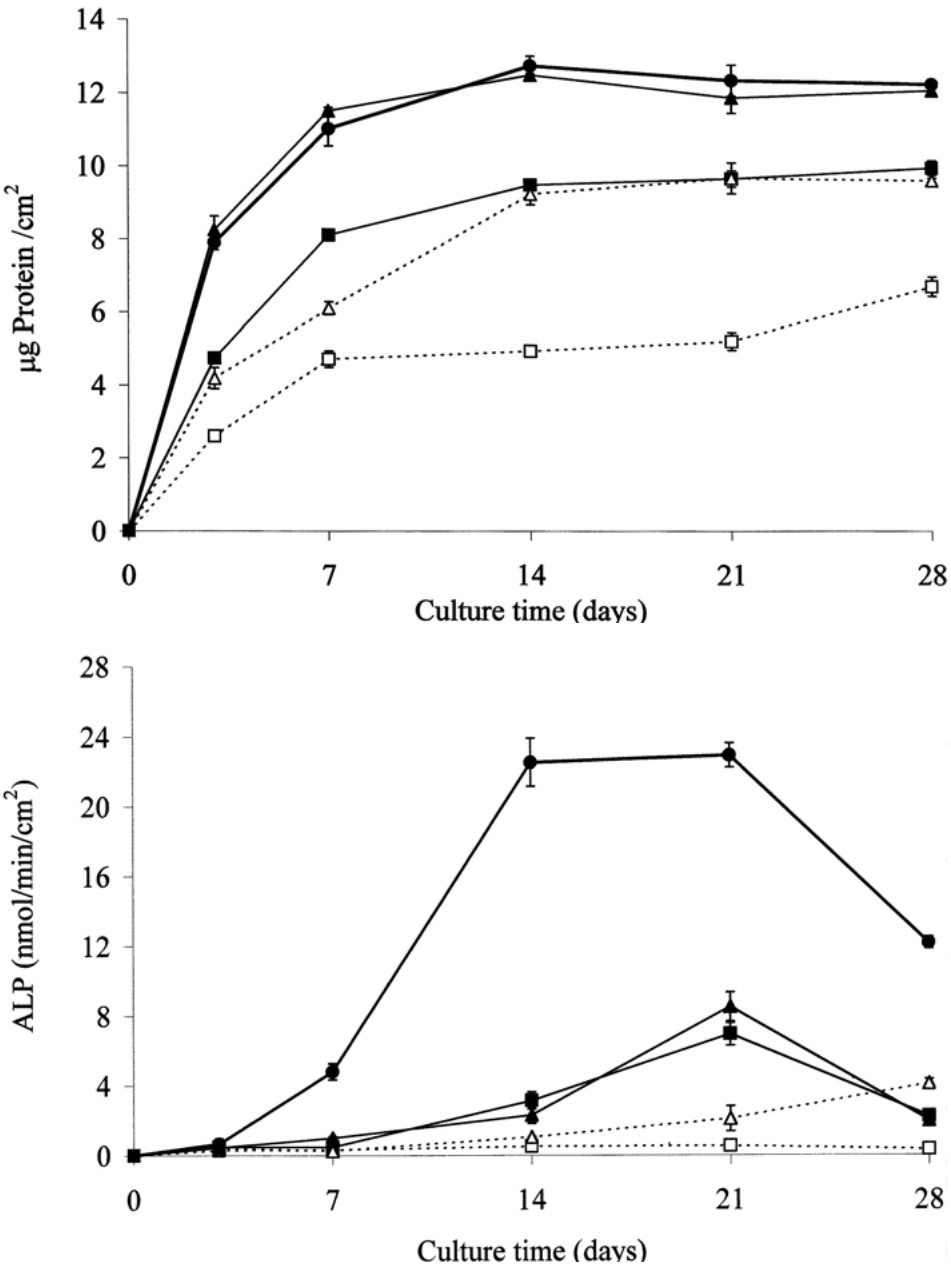

Figure 4 Total protein content and ALP activity measured in the control cultures and in the seeded plasma sprayed coated samples throughout the 28 days culture period. (●) control cultures; ''as-received'' HA (□) and HA/$G_1$4% (△); pretreated HA (■) and HA/$G_1$4% (▲).

3.2.2. Scanning electron microscopy

SEM observation of the control cultures at the various culture times showed that proliferation of bone marrow cells was accompanied by the production of a fibrilar extracellular matrix, and the 21 days cultures presented numerous mineral globular structures, shown by X-ray microanalysis to contain Ca and P (results not shown); this aspect is in accordance with other previously reported studies in this culture system [27, 28].

Bone marrow cells seeded in the pre-immersed HA and HA/$G_1$4% composite coatings have a similar behavior characterized by cell attachment, spreading and proliferation in an attempt to colonize the entire substrate surface. Also, elaboration of an abundant intercellular matrix was observed. At days 21 and 28, cultures showed the presence of numerous mineral globular deposits. X-ray microanalysis of this biological structures confirmed the presence of calcium phosphates, carbon (C) and oxygen (O) compounds, characteristics of an osteogenic tissue (results not shown). This behavior is exemplified in Fig. 5 for 28-day seeded pretreated HA coatings.

The ''as-received'' plasma sprayed coatings presented a less favorable behavior concerning cell proliferation and differentiation. SEM observation of the seeded HA coated disks showed that cell growth was observed only in some areas being most of the material surface covered by fragments of dead cells and fibrous seric proteins adsorbed on the coating (Fig. 6A, B), as found in previous studies [7]. In contrast, at day 28, the surface of the ''as-received'' HA/$G_1$4% composite was completely covered with cells and formation of some mineral globular structures were observed throughout the culture (Fig. 6C, D). X-ray microanalysis of this biological structures showed the presence of Ca, P, C and O peaks (results not shown).

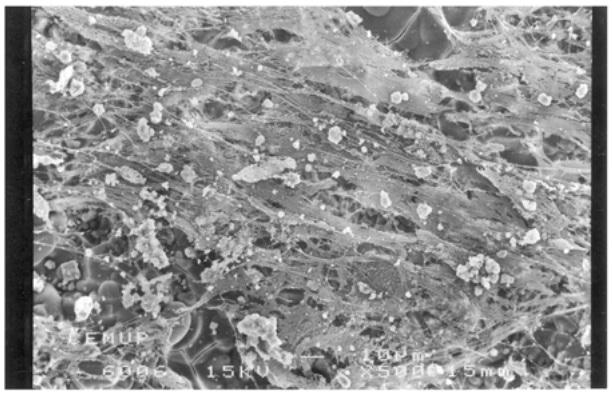

Figure 5 SEM appearance of 28-day cultures grown on the surface of pretreated plasma sprayed HA coatings (original magnification, × 500).

3.2.3. Quantification of ionized calcium and phosphorus in the culture medium

Concentrations of ionized calcium and phosphorus in the culture media collected from the seeded materials and also from control cultures were determined throughout the 28 days incubation period. Results are shown in Fig. 7.

Control cultures. Culture medium collected from control cultures (bone marrow cells growing on tissue culture dishes) showed an increase in the concentration of ionized P until approximately days 14–17. This increase resulted from the hydrolysis of β-glycerophosphate added to the culture medium (10 mM) most probably by ALP known to have high efficacy in hydrolyzing this ester phosphate [29]. Because the measured levels of Ca and P were not cumulative, results concerning the levels of ionized P suggested that the ability of bone cells to hydrolyze this compound increased with the incubation time. This observation seems to agree with the results observed for the pattern of variation of the ALP activity showing that the levels of this enzyme increased significantly during the second week of culture (Fig. 4). During the first 2 weeks, levels of ionized Ca were approximately constant. However, from then onwards, levels of ionized Ca and P decreased significantly. Consumption of these species from the culture medium reflected the formation of calcium phosphate deposits in these cultures. These results are in agreement with previous work showing that in this culture system, the mineralization process occurred from 2 weeks onwards [24, 27, 28].

Cultures grown on plasma sprayed coatings. The pattern of variation of ionized Ca and P in the culture medium collected from cultures growing on previously immersed HA/$G_1$4% composite and HA coatings was similar to that observed in control cultures, although some differences were evident. In the seeded pretreated materials, levels of ionized P began to increase few days later and attained lower maximal values, in agreement with the later expression of ALP in these cultures (Fig. 4). This effect was more pronounced in the cultures growing in the HA coated samples. Levels of ionized Ca and P measured in the culture medium decreased significantly from days 17 to 21, suggesting that the formation of biologically induced calcium phosphate deposits occurred in these cultures.

In the seeded ''as-received'' HA/$G_1$4% levels of ionized P in the culture medium increased from the

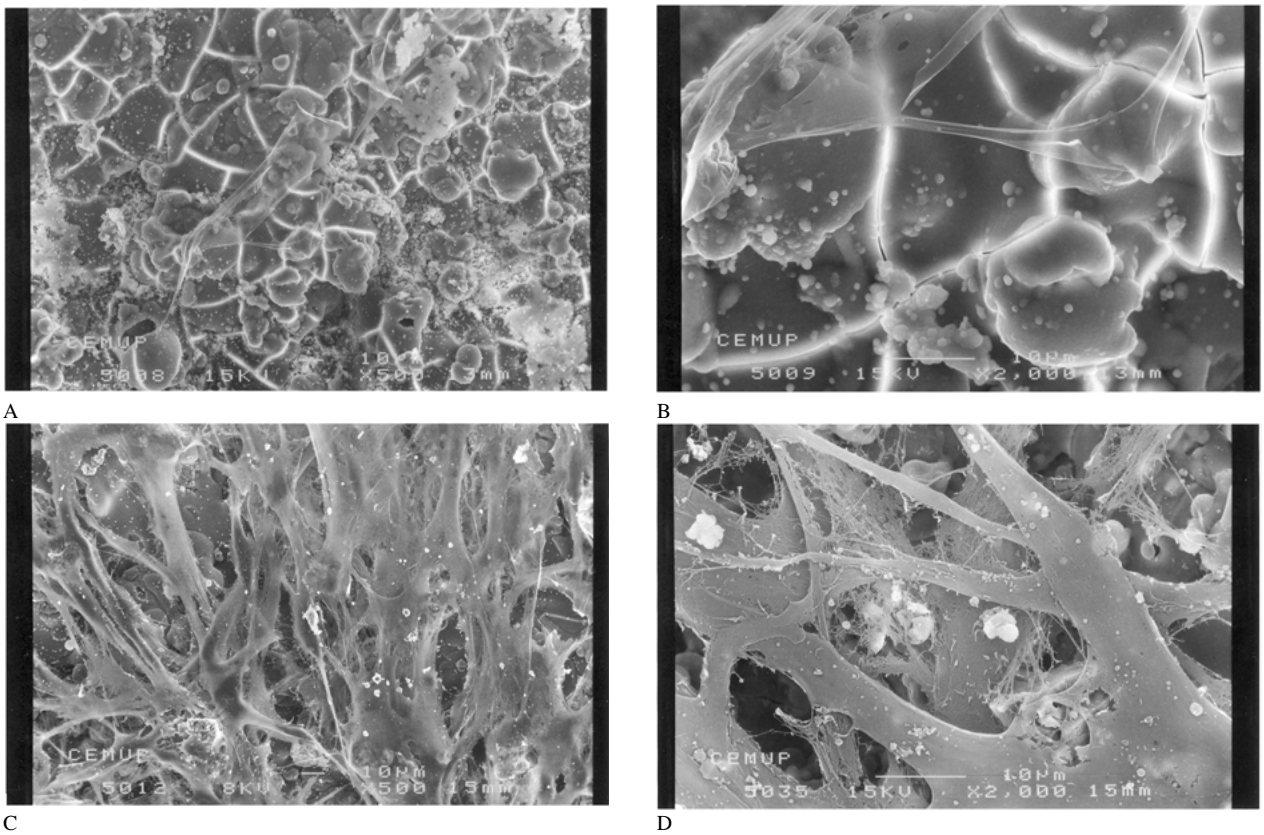

Figure 6 SEM appearance of 28-day cultures grown on the surface of ''as-received'' plasma sprayed HA (A, B) and HA/$G_1$4% (C, D) coatings. (A, C, original magnification, × 500; B, D, original magnification, × 2000).

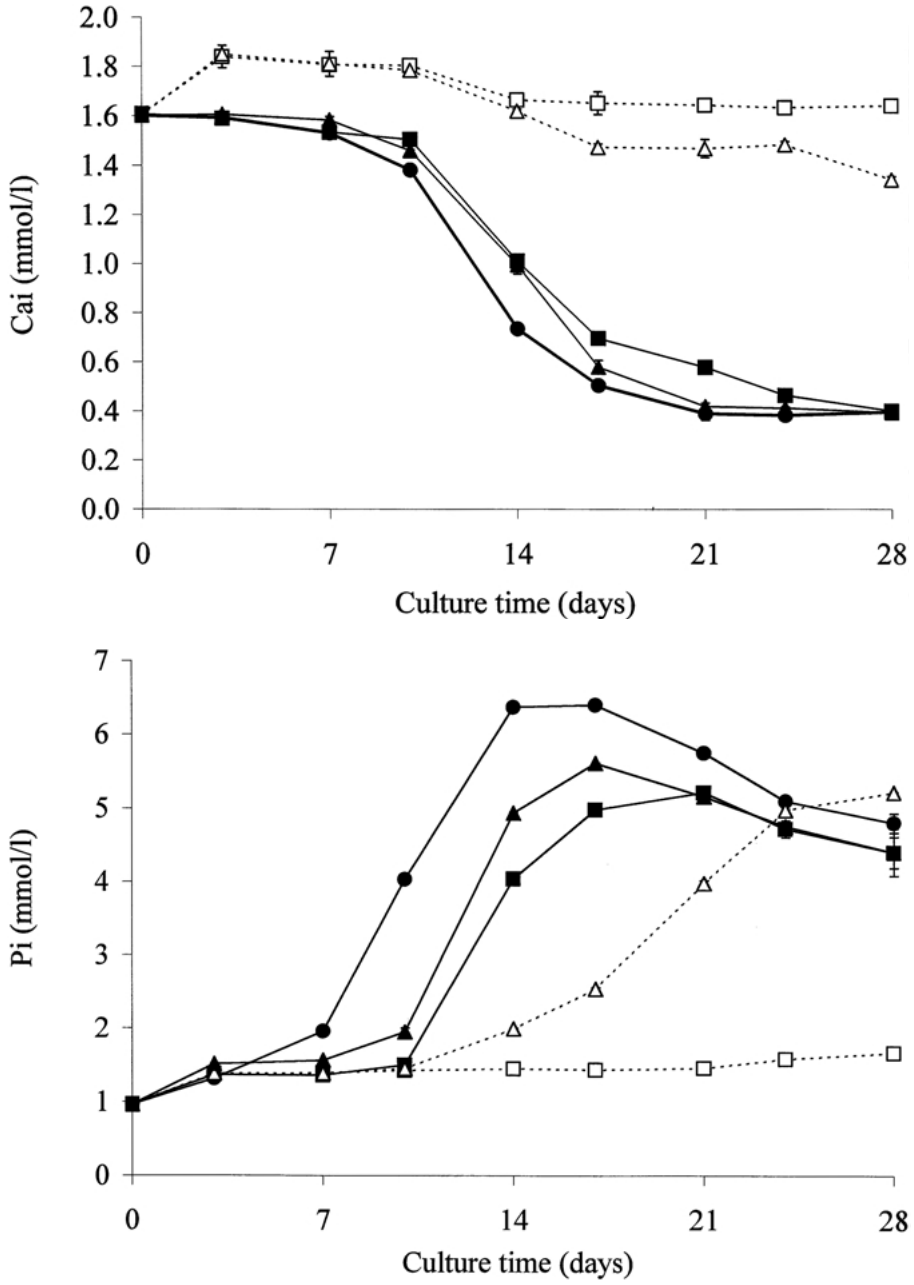

Figure 7 Levels of ionized calcium and phosphorus in the culture medium collected from control cultures and seeded plasma sprayed coated samples throughout the 28 days culture period. (•) control cultures; ''as-received'' HA (□) and HA/$G_1$4% (△); pretreated HA (■) and HA/$G_1$4% (▲).

second week of culture and reached significant levels during the third and fourth weeks of culture; this profile is in agreement with the results observed for the expression of ALP in these cultures (Fig. 4). During the first 2 weeks, levels of ionized Ca in the medium were higher than those found in the seeded pretreated materials (most probably due to dissolution reactions occurring during this period, Fig. 3) and then decreased for levels similar to those measured in the control culture medium; however, a decrease in the concentration of this ion was evident in the last days of incubation, suggesting a tendency for the formation of calcium phosphate salts.

By contrast, levels of ionized P in the medium from the seeded ''as-received'' HA remained constant and similar to those measured before any contact with the material. Levels of ionized Ca were higher than those measured in the medium from the control cultures and remained constant during the culture time. However, during the first 2 weeks levels of this ion were lower than those measured in the absence of cells (Fig. 3), suggesting that cell growth occurring on the surface of the ''as-received'' HA prevent, in some way, the dissolution reactions observed during the immersion of this material.

4. Discussion

Bone cell attachment, growth and function are significantly affected by the physical and chemical characteristics of the biomaterials surface [12–19]. In agreement with this, the results reported in the previous section showed significant differences between plasma sprayed HA and HA/$G_1$4% coatings concerning the proliferation–differentiation behavior of human osteoblastic bone marrow cells seeded onto the surface of these materials, both in the ''as-received'' condition and after an immersion treatment with culture medium. A

similar observation has been reported in a previous work that compared HA and a glass composite with a different composition, HA/$G_1$2% [7].

Time-dependent determination of the amount of protein adsorbed on milled HA, HA/$G_1$2% and HA/$G_1$4% during 7-day immersion in culture medium showed that protein adsorption occurred specially during the first hours and that the glass composites, in particular, HA/$G_1$4% adsorbed higher amounts of total protein as compared to HA. Measurements of the zeta potential during the same period showed, for the three materials, a shift toward negative charge. The decrease in the values of the zeta potential was observed specially during the first hours of immersion and after approximately 24 h values did not change significantly. As compared to HA, the glass composites presented higher negative shifts, specially HA/$G_1$4%. These results showed that the amount of adsorbed protein appears to correlate with the values of the zeta potential measured for these materials. The higher the amount of adsorbed protein is, the more negative the value of the zeta potential. These results are in agreement with previous studies concerning the adsorption of albumin to ceramic powders [30] and also to biological glasses [31].

Immersion of the ''as-received'' HA and HA/$G_1$4% plasma sprayed samples in the culture medium for 21 days resulted in increased concentrations of ionized Ca and P in the medium during the first 2 weeks. After that, an equilibrium state seemed to have been reached as the levels of these species in the medium were similar to those measured before any contact with the material disks (Fig. 3).

The previous results suggested that the interactions occurring during the immersion of HA, HA/$G_1$2% [7] and HA/$G_1$4% in culture medium resulted in dynamic changes of the surface structure of these materials. Surface modifications occurred as a result of reactions involving Ca^{2+} and PO^{3-} ions and also adsorption of components dissolved in the culture medium such as peptides and proteins. These interactions appeared to be strongly dependent on the chemical composition of the material, namely the relative content of β-TCP, a compound far more instable than HA. The relative proportion of β-TCP in the structure of the composite coatings increased with the level of glass content added to HA and, accordingly, HA/G_1 4% was the more interactive material.

Most of the interactions involving the material surface and the culture medium occurred during the first hours of incubation, stage in which cells were adhering to the material surface. With regard to this, the three materials presented significant differences in behavior during the first hours of immersion concerning parameters as protein adsorption and zeta potential known to play an important role in the adhesion and spreading of a bone cell population to the material surface and, consequently, its growth and differentiation [16, 30, 32, 33].

In agreement with these observations, the ''as-received'' HA, HA/$G_1$2% [7] and HA/$G_1$4% plasma sprayed coatings presented significant differences concerning osteoblast cell growth and differentiation of human bone marrow cells. HA hardly supported cell growth (Figs 4 and 6; [7]), in HA/$G_1$2%, cell proliferation was observed but osteoblast parameters such as ALP and formation of cell mediated mineral deposits were not expressed [7] and HA/$G_1$4% presented surface characteristics that allowed cell growth and differentiation of osteoblastic bone marrow cells (Figs 4 and 6).

Cultures grown onto the plasma sprayed coatings previously immersed in culture medium for 21 days showed higher rate of cell proliferation and increased functional activity, as compared to those grown onto the corresponding ''as-received'' materials, in agreement with that observed in other similar studies [7, 10, 34]. Surface modifications occurred during the immersion period significantly improved bone cell growth and differentiation. This is probably related to the formation of a relatively stable calcium–phosphorus rich layer on the surface coatings [8] that is reported to increase the bioactivity of the material [35–38]. In addition, and as referred above, immersion of the material would promote adsorption of biological molecules from the culture medium which have important roles in the adhesion process and functional behavior of the osteoblastic cells [39–41].

It is interesting to observe that previously immersed HA, HA/$G_1$2% [7] and HA/$G_1$4% presented a similar behavior concerning cell growth and differentiation. Although the glass composites appeared to present a somewhat better biological performance, as suggested by the measured parameters. After a long immersion period in culture medium exchange reactions between the coatings and the medium reached an equilibrium, event that would contribute to an attenuation of the differences of the surface structure of the three materials. This probably explains that the pretreated materials showed minor differences concerning the cell growth and function of the osteoblastic cells. A similar observation was reported in a previous work showing that the pre-immersion of HA, HA/$G_1$2% and HA/$G_1$4% plasma sprayed coatings resulted in little if any influence on the cellular response of osteoblastic MG63 cells among the three materials [34].

The results reported in the present work are in agreement with previous *in vitro* studies suggesting that HA glass composites showed better characteristics for bone cell growth and function than HA, either using human bone marrow cells [7] or human osteosarcoma cell lines [9, 34] to evaluate the biological response of the materials. Also, an *in vivo* study performed in a rabbit model reported that sintered glass reinforced HA composites induced earlier new bone formation around implants than sintered HA [42]. In addition, the results of this and other studies [7, 9, 34, 42] strongly suggested that increasing the percentage of glass in the composite appeared to have a beneficial effect in the performance of these materials.

5. Conclusion

Cell attachment, growth and differentiation of human osteoblastic bone marrow cells seeded onto ''as-received'' plasma sprayed glass reinforced HA and HA 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 characteristics. Accordingly, the glass composites, and in particular, HA/G_1 4% presented better biological performance than HA.

By contrast, bone marrow cells seeded onto the pretreated glass composites and HA coatings presented a similar osteoblastic behavior. In this case, the long-term treatment of the coatings with culture medium would contribute to a ''stabilization'' of the surface structure and also to an attenuation of the surface differences among materials with different composition.

Reactivity of the glass composites would favor the initial interactions of the material surface with the physiological environment, namely molecules involved in the recruitment and adhesion of bone cells, a fundamental step for the growth and differentiation of an osteogenic population and consequent new bone formation at the bone tissue/material interface. Results reported in 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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