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Evaluation of the influence of albumin on the mineralization of a glass by Atomic Force Microscopy

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Abstract Bioactive glasses have been used as a graft material that can stimulate the formation of a new bone. *In vitro* tests usually give sensible indications about the potential bioactivity of these glasses. In the present work the influence of egg albumin on the formation of a Ca-P precipitate on a glass of the system SiO_2-CaO-MgO-$P_2O_5$was evaluated. The samples were immersed in simulated body fluid (SBF) that simulates the composition of human plasma, with and without albumin. After immersion in this solution for 7 and 14 days, the glass was characterized by X-Ray Diffraction (XRD) and Atomic Force Microscopy (AFM). AFM results of the samples after immersion in SBF with albumin show the development of a precipiate formed from the solution/substrate reaction. Glasses immersed in albumin-free SBF exhibit the formation of a thin layer easily detached from the substrate. XRD results indicate that the precipitate is essentially amorphous, evolving to octacalcium phosphate. As the formation of an adherent precipitate on the glass samples only occurred when the substrate was immersed in SBF with albumin, it is suggested that albumin improves the mineralization on the glasses.

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1 Introduction

Biomaterials as aloplastic grafts (inorganic materials), have been extensivelly studied in the last 40 years [1–4]. The development of these biomaterials aims to help the regeneration of the tissue more eficientelly. They act as an alternative to the autogenous grafts because they can eliminate the necessity of the donor surgical site, decreasing the discomfort of patients. Bioceramics have a distinctive place among the biomaterials. Some of the most important bioceramics studied are bioactive glasses, glass-ceramics and synthetic hydroxapatite. Bioactive glasses were first developed by Hench [5]. A significant number of bioactive glasses is composed of SiO_2-CaO-MgO-P_2O_5 as proposed by Kokubo [6]. Bioglasses can create a layer rich in Ca and P at the surface when immersed in an acellular solution that simulates the body fluid (SBF). This layer leads to a significant adhesion of the graft to the bone and helps the bone regeneration.

The development of a Ca-P rich layer initially occurs due to an ionic exchange between the glass and the solution and finishes with the formation of an hydroxycarbonate apatite (HCA) layer on the surface of the material. This process starts with the leaching of the glass modifier ions and the formation of SiOH on the surface. A loss of soluble silica and further formation of SiOH occurs in a second stage. Subsequently, the SiOH condenses and produces a hydrated silica gel layer where the amorphous calcium phosphate precipitates, crystallizes and, finally, a HCA layer forms [7]. The integration of the implant into the bone depends on the formation of a well-adherent apatite layer. This adhesion initializes with the adsorption of an organic component into the HCA layer; many times the adhesion depends on the presence of human body extra cellular proteins.

The most abundant human body extracellular protein is albumin. This protein maintains colloidal osmotic pressure in

the plasma and carries the insoluble materials [8, 9]. Albumin is present in the human body at concentrations of 3.8 to 5.0 $g.dL^{-1}$. Therefore, the albumin is the protein that will have the major contact with the implant. Albumin also is, apparently, the main protein involved in the bioactive glass adsorption and the first to surround foreign bodies when they are in contact with the blood [10]. This sequence of happenings leads to significant modifications of the materials surface.

A number of sophisticated equipments is now available to visualize biomaterials surfaces. One of the most versatile techniques is Atomic Force Microscopy (AFM), which enables the production of three-dimensional images of the samples topography at a nanometric scale [11–14].

In this study, a powder of a glass, which has previously showed *in vitro* bioactivity, was immersed in an acellular SBF, with and without albumin. After immersion, the surface was characterized via AFM at non-contact mode to evaluate the formation of a surface precipitate. The non-contact mode was used due to the fragility of the samples and to the powder roughness. The crystalline phases in the precipitates were analyzed by X-Ray Diffraction (XRD) and the concentration of the SBF was followed by Inductive coupled plasma (ICP) emission spectroscopy. The aim of this study was to assess and discuss the influence of albumin on the formation of the precipitate on the surface of a SiO_2-CaO-MgO-P_2O_5 glass.

2 Materials and methods

A bioactive glass named VH30 was produced with the composition $32.76SiO_2$-40.44CaO.P_2O_5-26.80MgO. The reagents SiO_2, MgO, $CaCO_3$ and $Ca(H_2PO_4)_2$ were mixed manually with 96% ethylic alcohol and put into the planetary mill (Fritsh Pulveresette) using an agate lined jar and agate balls for 45 minutes to make a homogeneous mixture. The mixture was put in the stove (Memmert) at 60 °C for 24 hours to be dried. Mixture batches were smashed to transform them into powder again. Batches with 80 g were melted in platinum crucible at a temperature of 1500 °C in a cylindrical oven (Termolab) for one hour. The melts were poured quickly to a bucket with water to promote a fast cooling. The glass samples were dried in a stove (Memmert) for 24 hours. The samples were ground in an agate mill (Retsh MR 100) for one hour and sieved manually in a 30-μm mesh. Particles retained on the sieve were separated. The prepared material was a SiO_2-CaO-MgO-P_2O_5 glass powder.

Coulter (LS Particle Size Analyzer) tests were used to obtain the medium size of the glass particles using a Fraunhofer optical model in water as fluid. BET (Gemini 2370 V5.00) test was carried out in a sample of 0.7275 g of glass at 300 mm Hg/min of evacuation rate and under saturation pressure of 786 mm Hg to obtain the surface area of the powder.

Table 1 Ions concentration (mM) in plasma and in SBF [15]

	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	HCO_3^-	HPO_4^-	SO_4^{2-}
Plasma	142.0	5.0	2.5	1.5	103.0	27.0	1.0	0.5
SBF	142.0	5.0	2.5	1.5	148.8	4.2	1.0	0.5

The preparation of the SBF was carried out mixing the reagents NaCl, $NaHCO_3$, KCl, $K_2HPO_4.3H_2O$, $MgCl_2.6H_2O$,HCl, $CaCl_2.6H_2O$, Na_2SO_4, $NH_2C(CH_2OH)_3$ in 500 mL of ultra pure water in a beaker according to the method proposed by Kokubo et al. [15]. The solution had a pH = 7.25 corrected by HCl 1N. The solution was transferred to a chemical flask and ultra pure water was added to complete 1 L. The SBF ionic concentration simulates the human plasma and has the chemical composition presented inTable 1.

The *in vitro* tests were performed under static conditions. All flasks contained 100 mL of solution. The SBF with albumin (SBFA) was obtained by adding 2 g of albumin to the initial SBF. Each flask containing SBF or SBFA received 2 g of bioactive glass VH30. These flasks were sealed and put in a stove (Heraeus Function Line) at 37 °C for 7 and 14 days. After the immersion in the synthetic solutions, the samples were filtered and the retained solids were dried at 60°C.

The preparation of samples for observation by AFM was carried out using a drop of polyvinyl acetate over glass laminas, sliding each one with another lamina, to scatter the glue. The powders of glass from the SBF or SBFA were dispersed on the slides and the polymerization of the glue was awaited. Atomic Force Microscope (Park Scientific Instruments) was used to observe the morphology of the precipitates on the surface of the glass samples. Si_3N_4 cantilever with a spring constant of 0.26 N/m in a non-contact mode was employed.

X-Ray Diffraction (Rigaku PMG-VH), at Cu Kα radiation (40 kV, 30 mA, step size of 0.02 sec and a counting time of 3° 2θ/min) was used to confirm the amorphous nature of the initial glass and to analyze the crystalline phases in the precipitated layer.

The solutions of the *in vitro* tests were analysed by inductively coupled plasma (ICP) emission spectroscopy, to assess the evolution of ionic concentrations of Ca and P.

3 Results and discussion

The analysis of the glass powders by Coulter gave a medium size of 10.50 μm and a standard deviation of 9.42 μm. The tests carried out by BET indicated that the surface area of the particles is 0.7583 $m^2.g^{-1}$ in the multipoint mode. In *in vivo* situations, the size and shape of the particles influence the mineralization process and the formation of the new tissue. Additionally, extra cellular matrix proteins control

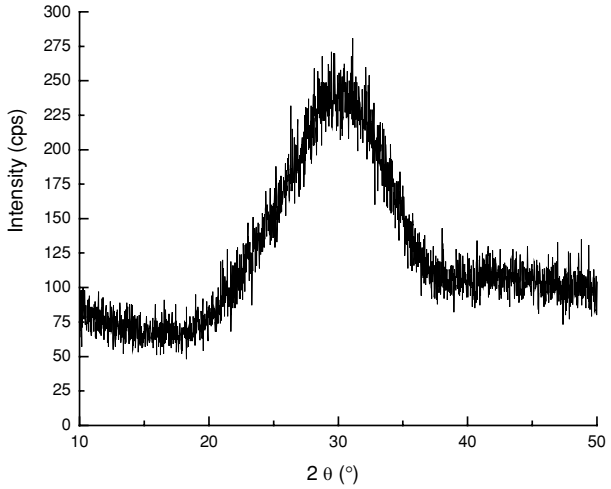

Fig. 1 XRD of VH30 bioactive glass indicating its amorphous condition

the deposition and growth of the hydroxyapatite crystals on the hard tissues[11]. The surface area values obtained in this work are lower than those tested by Rosengren et al. [16] with zirconiua, alumina, and hydroxyapatite powders. According to Zhong et al. [17] only bioactive glasses with surface areas larger than 40–80 $m^2.g^{-1}$ can induce HCA formation. However, Krajewski et al [18] studied glasse powders with surface areas between 0.083 and 0.255 $m^2.g^{-1}$ and concluded that they still exhibited bioactivity.

Figure1 shows the X-Ray Diffraction results for the starting glass powder, where it is clear its amorphous condition.

Images obtained by AFM are depicted inFigs. 2 to 4. Figure 2 (a and b) refer to the glass VH30 as prepared. The VH30 samples after immersion in SBFA during 7 and 14 days are shown in Fig. 3 a–b and 3 c–d, respectively, being clear the formation of globular precipitates at the surface of the glass. The average size of the globular formations is around 121.45 ± 17.2 nm after 7 days in SBFA (Fig. 3–a) and around 239.27 ± 4.0 nm after 14 days (Fig. 3–c). Figures 3–a and c also show a clear tendency for the precipitate to grow and become thicker and more uniform with increasing immersion times. However, when the samples were soaked in SBF alone, the precipitate was not observed, but a highly corroded surface was detected (Fig. 4–a and b). The composition of the glass probably explains the observed behaviour because other authors [19], working with different glass compositions in SBF have detected by AFM the formation of a surface precipitate.

XRD of the samples after immersion in SBFA and in SBF are shown in Figs. 5 and 6. There is no significant difference between both sets of results, which indicates that the globular precipitates at the surface of the samples immersed in SBFA are essentially amorphous. However, taking into consideration the indications of incipient crystallization in Fig. 5, it seems likely that further heat treatment of the

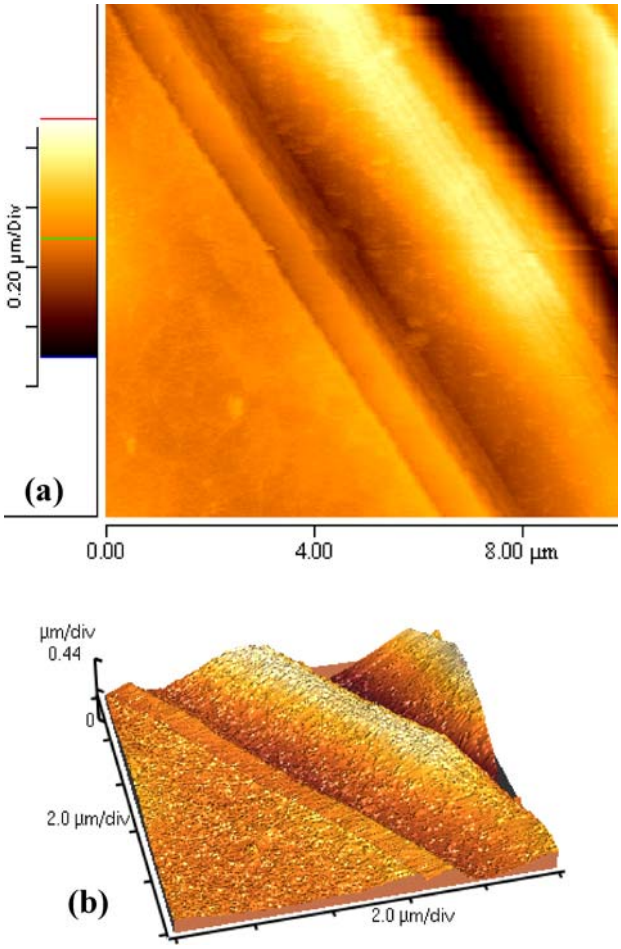

Fig. 2 AFM images of bioactive glass as prepared (a) 2D and (b) 3D

globular precipitate can give rise to an octacalcium phosphate precipitate.

Calcium phosphate presents 5 distinct phases: DCPD or bruxite ($CaHPO_4.2H_2O$), OCP or octacalcium phosphate [$Ca_8H_2(PO_4)_6.5H_2O$], β-TCP or β-tricalcium phosphate or β-whitlokite [$Ca_3(PO_4)_2$], HA or hydroxyapatite [$Ca_{10}(PO_4)_6(OH)$] and ACP or amorphous calcium phosphate. HA is the main phase of the mineral part of hard tissues of the human body. Variations in the composition of HA can occur due to the presence of CO_3^- substituting OH^- or PO_4^-. According to Le Geros [20], the globular morphology is related to the presence of hydroxycarbonate apatite (HCA). The concentration of ion carbonate (CO_3^-) in the molecule of HA defines the morphology; it varies from acicular to equi-axed crystals, which have around 12.5% of CO_3^-. The author states that all biologic apatites show a percentage of carbonate, CO_3^-. In the present work, the morphology of the precipitates found on the glass surface after immersion in SBFA is essentially spherical and similar to HCA precipitates. However, it was confirmed that the globular formations were mainly amorphous, possibly evolving to an HCA layer or to any other Ca-P crystalline phase. Hopefully the newly formed

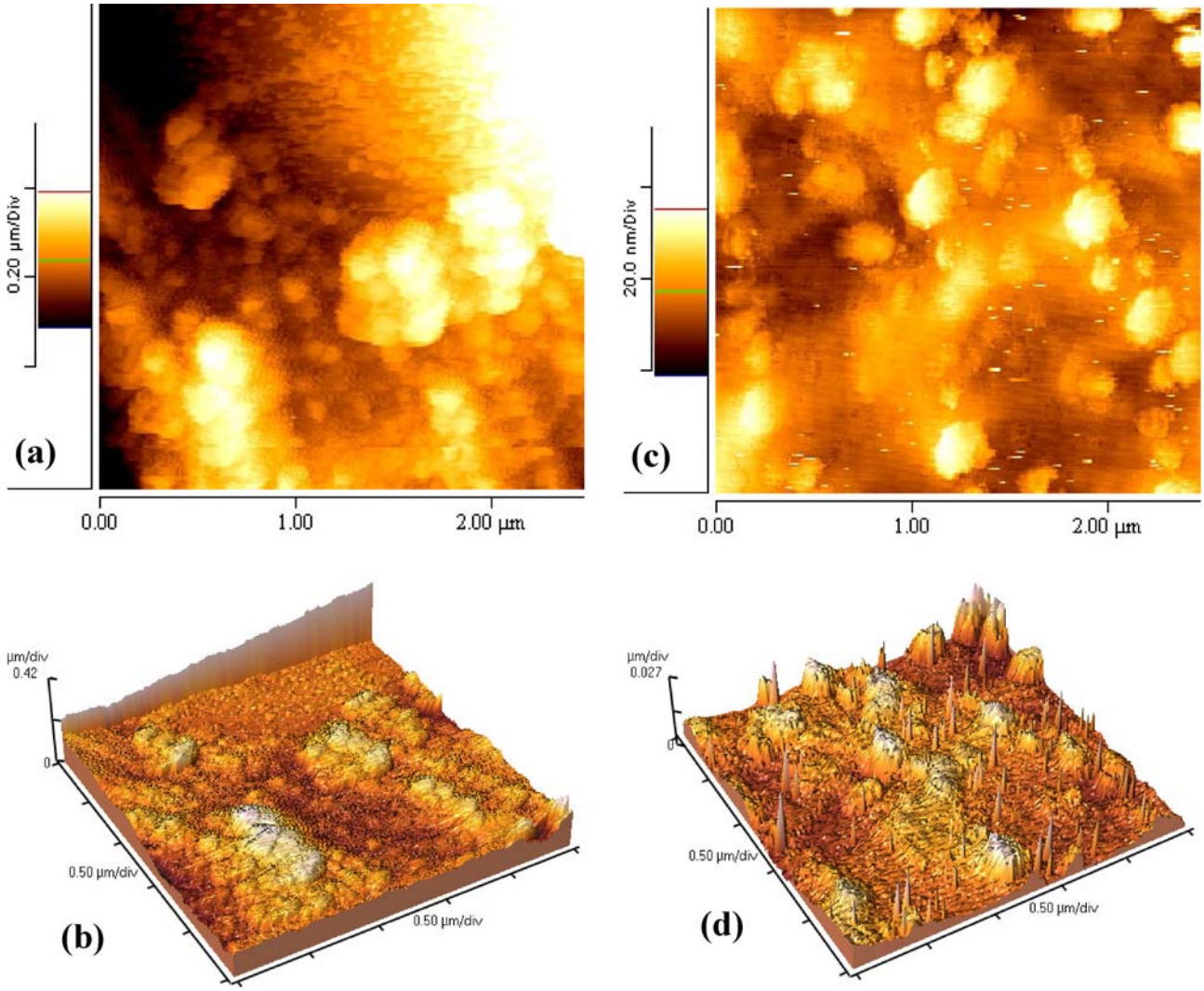

Fig. 3 AFM images of bioactive glass after soaked in SBFA: (a) 7 days 2D; (b) 7 days 3D; (c) 14 days 2D; (d) 14 days 3D

HCA layer should grow up to an adequate thickness and stability, thus influencing the adhesion of the cells, orientating, for example, the formation of the conjunctive tissue [21].

The surface of the glass after immersion in SBF without albumin presented in Fig. 4a and b. shows that it continuously degrades with time. This finding does not exclude the possibility of formation of a surface precipitate on the glass substrate that further detaches from it due to low strength adhesion. Pereira et al. [22] confirmed the presence of Ca-P layers on glasses of the same system, although weakly attached to the base flat glass samples. Krajewski et al. [10] and Rosengren et al. [16] suggest that the addition of albumin to SBF seems to provide stability to the solution and pushes it to a composition that is closer to the one of the human plasma.

The human plasma has many proteins [23]. By convenience in the present work only albumin was used. The presence of different proteins in the solution can change the behavior of a particular protein, specifically regarding to prosthesis surface adhesion [10].

The albumin adsorption should have an optimum value. A very intense protein adsorption would inhibit the formation of the Ca-P rich layer, affecting the bioactive properties of the glass. The specific adhesion is mostly influenced by the pH of the solution. Lower pH leads to a higher albumin adsorption [10].

Albumin, after adsorbed onto bioactive glass surfaces will be used like a bridge between $Ca^{2+}$and PO_4^{-3} ions, helping the connection between glass and the Ca-P layer. This connection stabilizes Ca-P layer. Because of this phenomenon, the glasses immersed in SBFA show higher stability at the Ca-P layer than those immersed in SBF alone.

Figure7 shows the variation with time of Ca and P concentrations in SBFA and in SBF after immersion of the glass powder samples. While P concentration drastically increases in SBF, denoting a severe dissolution of the glass at least up

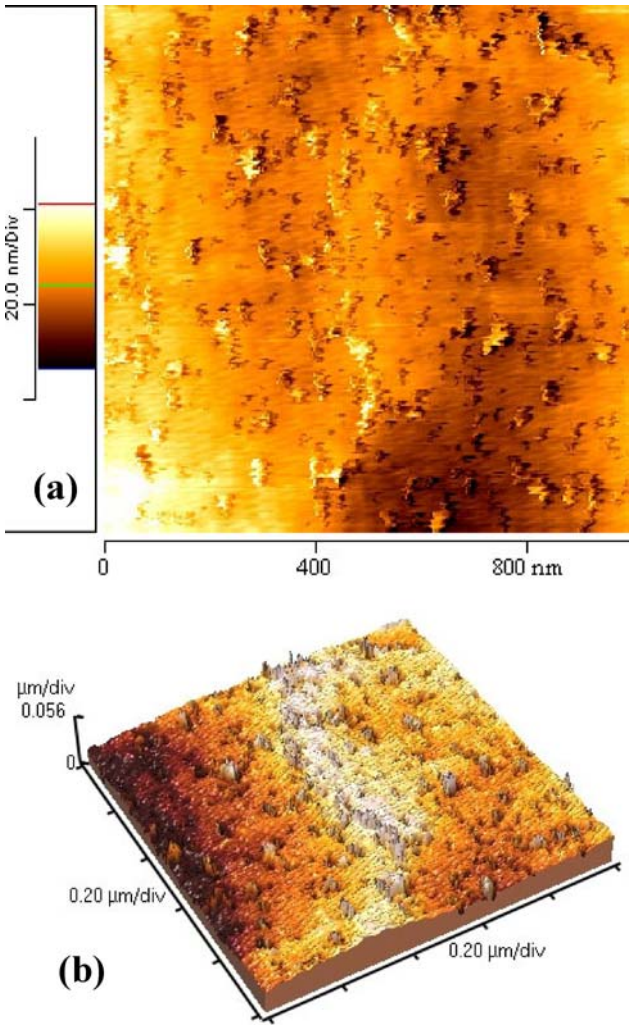

Fig. 4 AFM images of bioactive glass after 14 days in SBF: (a) 2D; (b) 3D

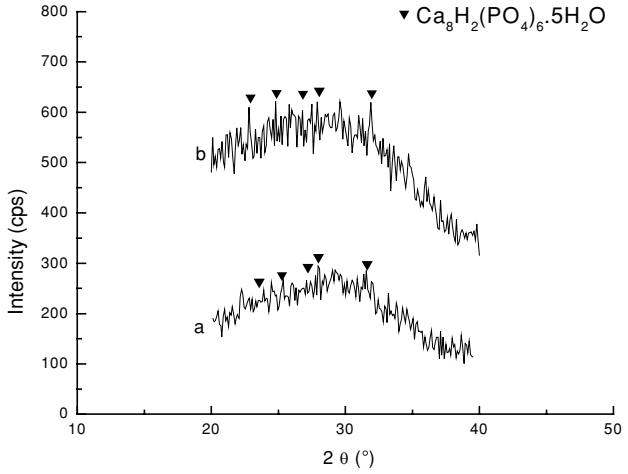

Fig. 5 XRD of bioactive glass immersed in SBFA: (a) 7 days, (b) 14 days

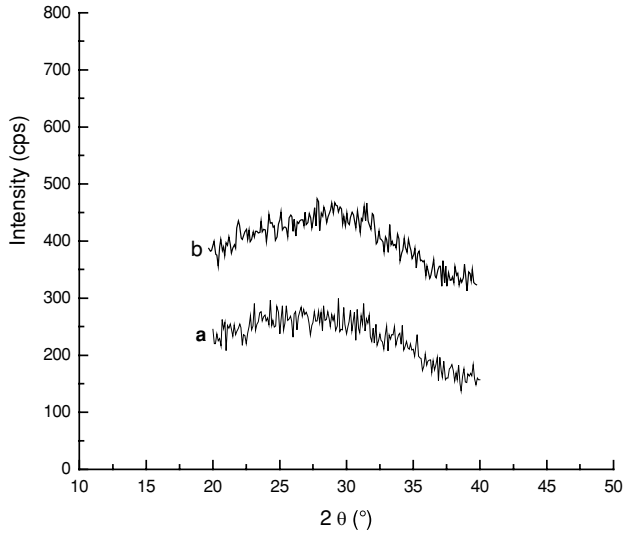

Fig. 6 XRD of bioactive glass immersed in SBF: (a) 7 days, (b) 14 days

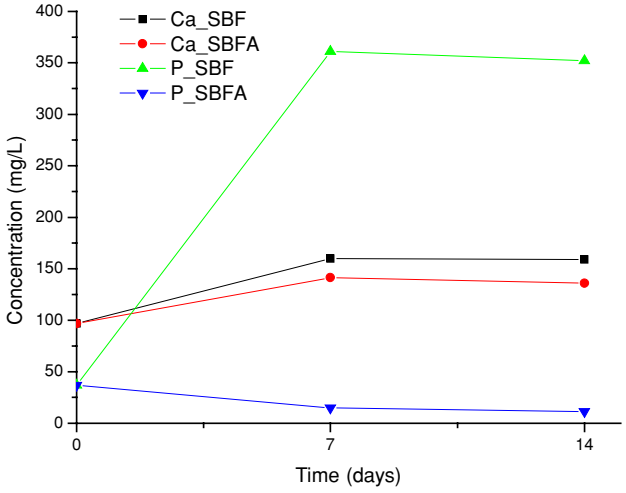

Fig. 7 Evolution of ionic concentration of Ca and P in SBF and SBFA after immersion of glass powders

to 7 days, the variation of Ca and P concentrations in SBFA is rather slight and keeps about constant after 7 days. These results, together with the AFM images and the XRD indications strongly suggest that the globular precipitates found on the glass surface immersed in SBFA are Ca-P formations, essentially amorphous. Subsequent heating of the precipitates would be useful to produce the crystallization of the globular formations and to help the identification of the final Ca/P ratio in the crystalline phase.

4 Conclusion

AFM appeared to be an efficient and powerful tool to observe the morphology of the bioglass surfaces, and it can provide the magnitude of the topographic features. Calcium

phosphate globular morphologies were found on the glass samples immersed in SBFA. The average size of the globules was 121.45 ± 17.2 nm after 7 days and 239.27 ± 4.0 nm, after 14 days. Ca-P layers grew more uniform and compact after 14 days when compared to the calcium phosphate grown after 7 days. Ca-P precipitates probably grow onto bioactive glass immersed in SBF but delaminate from the substrate. The albumin has an important effect on the formation of calcium phosphate layers on glasses.

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