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Effect of Protein Adsorption onto the Dissolution of Silicon-Substituted Hydroxyapatite

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Abstract

Several authors have shown the beneficial role of silicon incorporate into the hydroxyapatite lattice, although, the mechanism behind the enhanced bioactivity of this Si-hydroxyapatite (Si-HA) is poorly understood. The incorporation of Si into the HA lattice alters the surface charge of HA, leading to more negative values. Due to the importance of the surface properties on the interaction between biomaterials, physiological fluids, and the host tissue, it is important to further characterize the surface of Si-HA by determining its surface energy and wettability. Our results showed that the incorporation of Si increased the hydrophilicity of HA, leading to a higher interfacial tension. Another important property for osteointegration is the formation of an apatite layer. The dissolution of Si-HA in the presence of serum-free simulated body fluid (SBF) started at early time points and using atomic force microscopy (AFM) it was possible to observe the dissolution at the grain boundaries and grains. As the dissolutionprecipitation process is much more complex *in vivo*, we tried to mimic the initial stages of the *in vivo* reaction by incubating the Si-HA in serum-SBF. It was shown that the dissolution kinetics in serum-SBF was slower when compared to the dissolution in serum free-SBF. At the same time point, no significant dissolution features were observed or apatite layer was visualized. The phase imaging AFM indicated the presence of a layer on top of these materials that could be a proteinaceous layer, as XPS analysis detected an increase on the concentration of nitrogen on the surface of the samples incubated in the presence of proteins.

Keywords: Biomaterials, Silicon, Surface Properties, Proteins, Dissolution

1. Introduction

Since 1970's several studies demonstrated the positive effect of silicon on bone mineralization [1-3]. Later on, Patel *et al.* showed the beneficial role of the incorporation of silicon into hydroxyapatite (HA) lattice *in vivo* [4], where he demonstrated that silicon-substituted HA (Si-HA) enhanced bone regeneration. Although, the mechanism behind the enhanced bioactivity of this new biomaterial is still poorly understood. Due to the complexity of the *in vivo* model, several *in vitro* studies have been performed in order to clarify the mechanism behind the positive effect of silicon [5-11]. It has been shown that the incorporation of 0.8 wt% of silicon into the HA lattice decreases the time required for apatite formation in 64% (28 days for phase pure HA to 10 days for 0.8 wt% oSi-HA), when incubated in simulated body fluid (SBF) at physiologic conditions [8].

The ability of a biomaterial to form an apatite layer is considered to be an important characteristic for osseointegration [12]. Although, when a biomaterial is implanted there are several steps that will lead to the osseointegration, being the first event the adhesion of proteins to the surface, this step will take less than a second [13,14], following the formation of a protein monolayer. Only after the adsorption of the protein layer cells reach the implanted site. Therefore, cells will mainly respond to this layer instead of the biomaterial surface itself [14]. The integrin receptors present in most cells recognize the adsorbed proteins, being this mechanism responsible for the bioreaction to an implant [15]. In the presence of a solid phase proteins in solution have the tendency to adsorb [16] and change their conformation to maximize the energy gain. The adsorption will be irreversible if the conformational change required for desorption is energetically unfavourable [17], although, some proteins may be desorbed from the surface by other molecules in solution, as described by the "Vroman effect" [18]. This theory states that the more profuse and smaller proteins from blood plasma can be displaced by the fewer larger proteins that have higher affinities for the surface [18]. Several studies showed that determined proteins may induce (e.g. fibronectin) or inhibit (e.g. albumin) the nucleation of the calcium phosphate crystals [19], therefore a possible function of the specific crystal proteins could be to control the nucleation and growth of physiological apatite during the mineralization process [19]. The physicochemical properties of the material, such as surface chemistry, surface charge and surface energy, will influence protein adsorption and functionality, through changes in its orientation and conformation [16,20,21], and consequently cell behaviour [22-24]. It has been shown that HA and Si-HA have different physicochemical properties, namely surface charge [7], where the incorporation of silicon on the HA lattice induce a more negative surface charge.

In order to better understand the biological behaviour of the Si-HA two additional physical-chemical properties were determined, surface wettability and interfacial tension. These properties can be determined through the measurement of contact angle, being the most common experimental technique the sessile drop [25]. In this method a droplet is placed very slowly onto the surface of the material, originating three-phase equilibrium onto the surface of the material, originating three-phase equilibrium. The contact angle formed by the drop and the interfacial tension of the solid-vapour, liquid-vapour and solid-liquid interface can be related through the Young's [26] equation (Equation (1)):

$$\gamma_{LV}\cos\theta = \gamma_{SV} - \gamma_{SL} \quad (1)$$

where γ_{LV} is the interfacial tension of a liquid in equilibrium with its vapour, γ_{SV} is the solid-vapor interfacial tension and γ_{SL} is the solid-liquid interfacial tension and θ represents the contact angle of a liquid drop on a solid surface. This equation has two unknowns variables, therefore another equation is required. Several approaches have been proposed, for example the geometric mean method proposed initially by Scatchard and Hildebrand [27,28] and later on adapted by Owens and Wendt's [29], the harmonic mean proposed by Wu [30] and the van Oss, Good and Chaudhury method [31]. The Owens and Wendt's method is the most used and it states that the interfacial tension between two phases in contact, particularly between a solid and a liquid can be described by the following equation (Equation (2)):

$$\gamma_{SL} = \gamma_L + \gamma_S - 2\sqrt{\gamma_S^d \times \gamma_L^d} - 2\sqrt{\gamma_S^p \times \gamma_L^p} \quad (2)$$

This method assumes that the dispersive (γ^d) and polar (γ^p) intermolecular forces operate across the interface and therefore the total surface tension is the sum of the two components (Equation (3)):

$$\gamma = \gamma^d + \gamma^p \quad (3)$$

The combination of Equation (1) and (2) leads to a forth equation that will allow the determination of γ_{SV} and γ_{LV}:

$$\gamma_{LV}(\cos\theta + 1) = 2\sqrt{\gamma_{SV}^d \times \gamma_{LV}^d} + \sqrt{\gamma_{SV}^p \times \gamma_{LV}^p} \quad (4)$$

Using these equations and experimental values of contact angles measured with a pair of testing liquids of known dispersive and polar surface tension components, γ_{SV} and γ_{SL} can be determined.

In order to determine if there is a relationship between the incorporation of silicon into the HA lattice and the time required for the formation of an apatite layer, 1.5 wt% Si-HA was incubated in SBF for a period up to 10 days, at 37˚C. To study the effect of human serum proteins (HS) on the surface dissolution and consequently apatite formation, different compositions of Si-HA were incubated in SBF with HS (serum SBF). In this study several techniques were used: environmental scanning electron microscopy (ESEM), atomic force microscopy (AFM), phase imaging AFM, X-ray photoelectron spectroscopy (XPS) and inductively couple plasma spectroscopy (ICP).

2. Materials and Methods

2.1. Materials Preparation

The preparation of Si-HA was performed through an aqueous precipitation chemical route between calcium hydroxide, orthophosphoric acid and silicon tetraacetate as a source of silicate ions and it can be described by the following equation:

$$10\mathrm{Ca}^{2+} + (6-x)\mathrm{PO}_4^{3-} + x\mathrm{SiO}_4^{4-} + (2-x)\mathrm{OH}^- \rightarrow Ca_{10}(\mathrm{PO}_4)_{(6-x)}(\mathrm{SiO}_4)_x(\mathrm{OH})_{(2-x)}$$

where, x is the number moles of silicon. A detailed protocol is fully described elsewhere [5,7,32].

2.2. X-Ray Diffraction (XRD); X-Ray Fluorescence

To determine the phase purity of the precipitated material used in this study X-ray diffraction (XRD) was performed using a Philips PW1710 X-ray diffractometer (PANalytical Inc., Almelo, The Netherlands), being the data collected between 25˚ and 40˚ 2θ using a step size of 0.02˚ and a count time of 2.5 seconds. Phase identification was carried out by comparing the peak positions of the diffracttion patterns with ICDD (JCPDS) standards. The X-ray fluorescence was used to confirm the incorporation of 0.8 wt% Si, 1.2 wt% Si and 1.5 wt% Si into the HA lattice using a Philips PW1606 spectrometer.

2.3. Contact Angle Measurements

The powder containing the intermediate level of silicon 1.2 wt% Si-HA and HA were uniaxially pressed and sintered at 1300˚C for 2 hours, with a heating rate of 2.5˚C /min and cooled down at a rate of 10˚C/min. The samples were polished until a final Ra of (0.5 ± 0.1) μm. The contact angle was measured on the materials surface (HA and 1.2 wt% Si-HA) by the sessile drop method, at 25˚C in a saturated chamber. Pictures of the drops were taken as a function of time, at regular intervals using a videocamera mounted on a microscope to record the drop image. The program used to analyse the profile of the drop was Axisymmetric Drop Shape Analysis-Profile (ADSA-P) and it was developed by Neumann and co-workers [33,34]. The interfacial tension was assessed by the Owens-Wendt using as testing liquids deionised water and diiodomethane (>99%, Merk Schuchardt), doubly distilled under vacuum. The polar and dispersive components of the surface tension of the liquids at 25˚C were taken from the literature [35], the γ_{LV} of water and diiodomethane are 72 mJ/m^2 (γ^d = 21.3 mJ/m^2, γ^p = 50.7 mJ/m^2) and 50.3 mJ/m^2 (γ^d = 49.9 mJ/m^2, γ^p = 0.4 mJ/m^2), respectively.

2.4. Si-HA and Human Serum Proteins Interaction

2.4.1. Dense Discs

The powders containing the lower and upper levels of silicon, 0.8 wt% Si and 1.5 wt% Si, respectively, were uniaxially pressed into dense discs. The samples were incubated in different solutions: SBF (serum free-SBF) and SBF with 10% (v/v) of HS (serum-SBF) (HD Supplies, UK) at 37˚C for different periods of time to analyse the effect of protein on the surface dissolution. The ratio of surface area per volume used was 0.1 cm^{-1}. The surface of the materials was characterized before and after incubation by AFM and ESEM. AFM imaging was performed on a Digital Instruments NanoScope III. The topographic image on the AFM was obtained by the tapping mode using the changes in the cantilever oscillation amplitude, but in the case of the phase image, the phase lag of the cantilever motion relative to the driving oscillator was also registered and used to generate images [17,36-39]. ESEM signals were collected in low vacuum mode, with an off axis gaseous secondary electron detector. In order to access the presence of proteins on the ceramic surface, the atomic percentage of nitrogen was analysed by XPS. The Si-HA samples were incubated in SBF, SBF with 10% (v/v) HS and in an additional solution with 0.1% (v/v) of HS for 5 days.

2.4.2. Powders

To determine the effect of human serum proteins on silicon release to the surrounding medium Si-HA powders were immersed in SBF, serum SBF (10% (v/v) HS). The surface area was measured by the Brunauer-Emmett-Teller method (BET) (Micromeretics TriStar 3000) and the ratio surface area per volume was increased 100 times (10 cm^2/mL), and the silicon concentration in solution measured by inductively couple plasma spectroscopy (ICP).

2.4.3. Statistical Analysis

All results were statistically evaluated by ANOVA and post-hoc testing with Bonferroni's correction on SPSS statistical software. Significance was set at the 5% level ($p < 0.05$).

3. Results

The XRD analysis to HA and Si-HA did not detected secondary phases, such as tricalcium phosphate (TCP) or calcium oxide (CaO), indicating a phase pure material. The XRF results confirmed the presence of 0.8 wt% Si, 1.2 wt% Si and 1.5 wt% Si in the Si-HA material. Contact angle measurements showed that Si-HA has an initial contact angle of (64.9 ± 6.1˚, n = 10) that is lower than HA (71.8 ± 4.1˚, n = 10). As the water droplet sat on the surface of both materials the contact angle undergone a small decreased, although after that period of time the contact angle tended toward a constant value (**Figure 1**). When using the diiodomethane as a testing liquid, contact angles reached a plateau in a few seconds, (42.8 ± 5.8˚, n = 6) for HA and (44.1 ± 3.3, n = 6), for Si-HA (**Figure 1**). According to the OWRK approach it was possible

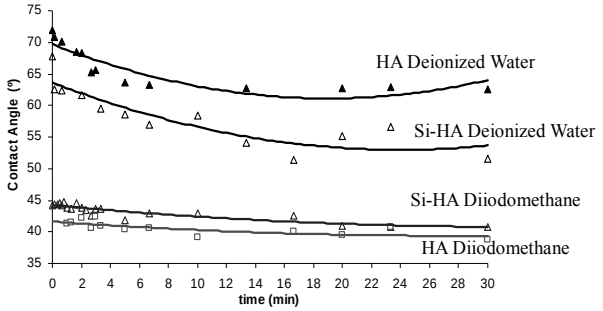


Figure 1. Variation of the contact angle with time for 1.2 wt% Si-HA and HA.

to determine the interfacial tension of the substrates; 42.9 mJ/m^2 (γ_{SV}^d = 35.1 mJ/m^2 and γ_{SV}^p = 7.82 mJ/m^2) for HA and 45.5 mJ/m^2 (γ_{SV}^d = 33.8 mJ/m^2 and γ_{SV}^p = 11.80 mJ/m^2), for Si-HA. The dispersive component of the interfacial tension is predominant in both materials, although the polar component gives a significant contribution. The non-dispersive component or polarity ($\gamma_{SV}^P / \gamma_{SV}$) can be used as a quantitative indicator of than HA, $\gamma_{SV}^P / \gamma_{SV}$ = 0.18 and $\gamma_{SV}^P / \gamma_{SV}$ = 0.25, for HA and SiHA, respectively.

The surface of the materials was analysed by ESEM, AFM and phase imaging AFM before incubation. Prior to incubation no specific features or deposits were observed, the grains and grain boundaries were clearly visible (**Figure 2**).

On the ESEM images it is possible to observe the formation of dissolution features (white arrow) on several grains, after incubation in serum free-SBF for a period of 1 day (**Figure 3**). These features grew in size and number with incubation time leading to the formation of calcium phosphate deposits, in particular on the surface of 1.5 wt% Si-HA.

After 7 days a confluent apatite layer was observed at the surface 1.5 wt% Si-HA (**Figure 4**). Thin-film XRD (Th-XRD) corroborated this data (data not shown). So, the incorporation of higher concentration of silicon decreases the time required for apatite formation in 30% (from 10 days to 7 days) and 75% (from 28 days to 7 days) when compared to 0.8 wt% Si-HA and HA respectively.

The differences on the behaviour of the samples incubated in serum free-SBF and SBF with proteins started at day one; they are more evident on the AFM images. On the phase image it is clear that the surface has different characteristics, due to significant differences on the contrast (**Figure 5**). As reported in a previous paper the dissolution of 0.8 wt% Si-HA starts as early as day 1 [8]. Similar results were observed for 1.5 wt% Si-HA where extensive dissolution was observed at day 1 (data not shown). At day 2, the samples showed clear signs of dissolution, as it can be seen at the topographical AFM images and phase image AFM (**Figure 5**). Dissolution at the grain boundaries and on the surface of the grains was clear, it seems that the grain boundary is completely dissolved (white arrow) (**Figure 5**).

Different features were observed on the samples surface when incubated in serum-SBF. Through the ESEM imaging it was not possible to observe significant dissolution features on the surface of these samples. When

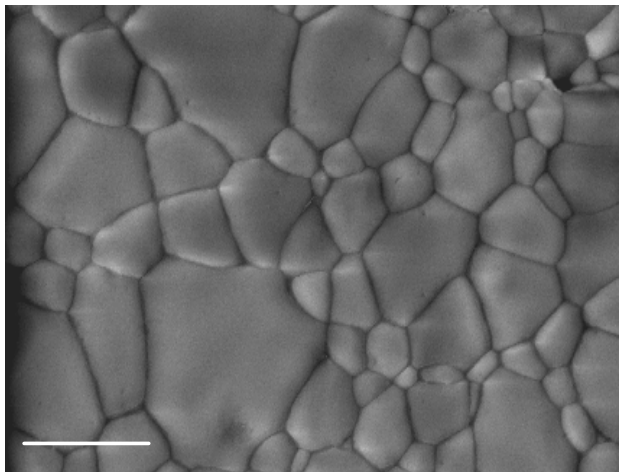
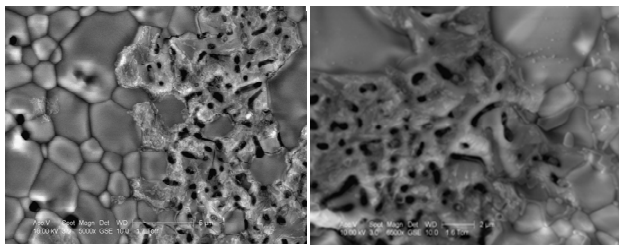
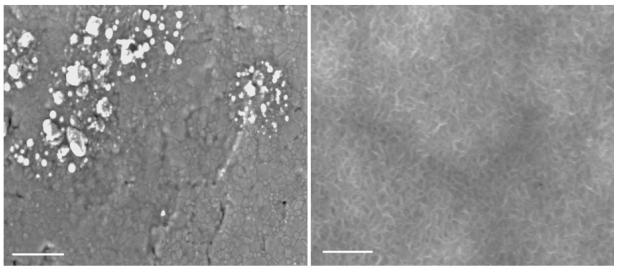


Figure 2. Environmental Scanning Electron Microscopy image of 1.5 wt% Si-HA before incubation. Similar image was obtained for 0.8 wt% Si-HA (data not shown). Scale bar 5 µm.



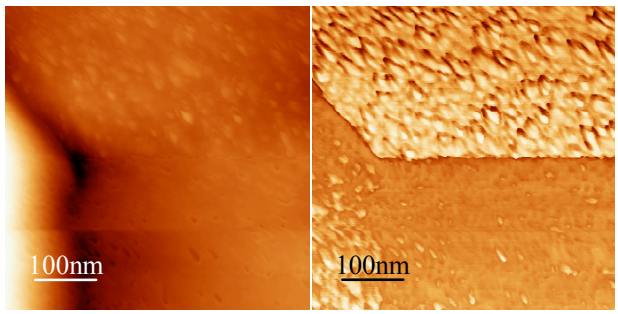
(a) (b)

Figure 3. Environmental scanning electron microscopy images of 1.5 wt% Si-HA after incubation in SBF for 1 day, showing extensive dissolution features (white arrow). Scale bar (a) 5 µm and (b) 2 µm.

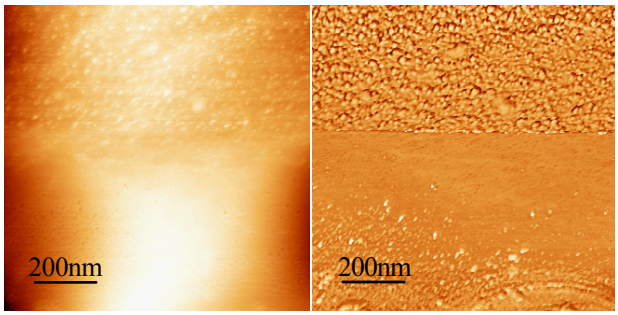


(a) (b)

Figure 4. Environmental scanning electron microscopy images of 1.5 wt% Si-HA after incubation in SBF for 7 days. Scale bar (a) 20 µm and (b) 1 µm.



(a) (b)

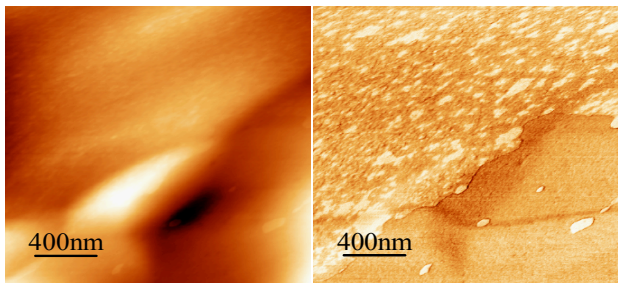


(c) (d)

Figure 5. Atomic force microscopy and phase maging of (a), (b) 0.8 wt% Si-HA and (c), (d) 1.5 wt% Si-HA after incubation in SBF for a period of two days. (a,c)—Topographical images; (c,d)—Phase image.

these samples were analysed by AFM and phase imaging AFM, the presence of a second layer was detected (**Figure 6**). The contrast observed on the surface shows that this layer has different characteristics from the underlying surface, which could indicate the presence of a protein layer on top of the samples. This layer may prevent the release of the ions into the surrounding medium and consequently delaying the formation of the apatite layer. At day 7 none of the substrates presented an apatite layer on their surface (data not shown).

In order to verify if the layer on the surface of the samples incubated on serum-SBF is a protein layer XPS analysis were performed. Proteins are biological macromolecules, formed by specific co-polymerization of up to 20 different amino acids [40]. The amino acids have a central or α carbon, to which four groups are bounded, a hydrogen, an amino group, a carboxyl group and a side chain usually designated by R. Nitrogen from the amino group (NH_3^+) and carbon from the carboxyl group (COO^-) can be measured and used as an indicator of the presence of proteins. At the surface of both materials an increase in the atomic percentage of nitrogen was observed with the increase of proteins concentration in solution, which indicates that the layer observed on the surface of the materials is indeed a protein layer (**Table 1**). The small concentration of nitrogen detected on the surface of the samples

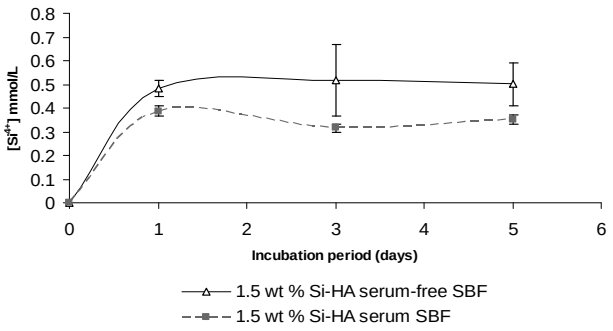


(a) (b)

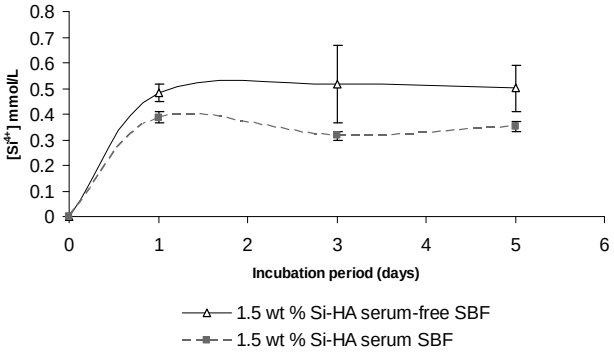
Figure 6. Atomic force microscopy and Phase Imaging of 1.5 wt% Si-HA after incubation in serum—SBF for a period of one day. (a)—Topographical image; (b)—Phase image.

Table 1. Atomic percentages of nitrogen at the surface of Si-HA after incubation for a period of five days.

	0.8 wt% Si-HA	1.5 wt% Si-HA
Solutions	N (At %)	N (At %)
SBF	0.8	0.5
SBF + 0.1 % (v/v) HS	6.5	9.1
SBF + 10 % (v/v) HS	10.4	10.8



(a)



(b)

Figures 7. Profile of silicon release from a) 1.5 wt% Si-HA and b) 0.8 wt% Si-HA, after incubation in serum free-SBF and serum SBF.

incubated in serum free-SBF is related with the presence of tris-hydroxymethyl amino-methane buffer (TRIS) in the SBF solution that adsorbed onto the surface of the sample.

In order to study the effect of proteins on silicon dissolution a different experiment was performed. This experiment comprised the suspension of Si-HA powders, in serum free- SBF and serum-SBF. The powders had similar surface areas, 0.242 m^2/g, 0.250 m^2/g, for 0.8 wt% Si-HA and 1.5 wt% Si-HA, respectively. The surface area per volume ratio used was 100 cm^2/mL and the ionic release was measured by ICP. Silicon was released from both substrates during the incubation period to the surrounding media. A higher content of silicon on the solution containing 1.5 wt% Si-HA was measured when compared to 0.8 wt % Si-HA (**Figures 7(a)** and **(b)**). The kinetics dissolution of the materials incubated in serum free SBF and serum-SBF was different. In the presence of proteins a slower rate was observed. This result confirms that the proteins layer on the surface of the material delays the diffusion of the ions from the surface of the ceramic material to the surrounding solution.

4. Discussion and Conclusions

In previous studies it was demonstrated that the incorporation of silicon into the HA lattice alters the surface charge of HA [7], leading to more negative values. Due to the importance of the surface properties on the interacttion between biomaterials, physiological fluids, and the host tissue after implantation [41-43], it was important to further characterize the surface of Si-HA by determining its surface energy and wettability. Our results showed that the incorporation of silicon increased the hydrophilicity of HA, leading to a higher interfacial tension. Although these differences did not reach statistical significance, they corroborate the zeta-potential results due to the increase on the polar component on the Si-HA material, therefore the increase of the interfacial tension can be due to the presence of unsaturated Si-O bonds leading to the formation of Si-OH in the presence of an aqueous medium. Similar results are described in the literature for SiO_2-containing material and phase pure HA [44-46].

Another important property for osseointegration is the formation of an apatite layer. It has been reported that the incorporation of 0.8 wt% Si into the HA lattice decreased the time required for the formation of an apatite layer in 64% when incubated in SBF (28 days for HA and 10 days for 0.8 wt% Si-HA) [8]. A further increase on the concentration of silicon resulted in an additional decrease on the time required for the formation of the apatite (75% when compared to phase pure HA). These results support the mechanism proposed for the enhanced bioactivity of Si-HA on previous reports, where it was stated that this increase is due to a combination of its high dissolution rate and its physical-chemical properties. Its high solubility leads to a faster super-saturation of the serum free-SBF, and therefore a faster precipitation of an apatite layer. The more electronegative Si-HA surface can provide a preferential site for the nucleation of an amorphous calcium phosphate apatite layer than the HA surface, which can occur through the adsorption of Ca^{2+} ions onto the electronegative surface, resulting in an increase in surface charge and the attraction of phosphate groups [7].

The dissolution of Si-HA in the presence of serum-free SBF starts at early time points, on the AFM image the dissolution on the grain boundaries and grain are clear, therefore an apatite layer will form in a short period of time, as mentioned previously. Although, when a biomaterial is implanted in a living system, there are several proteins, enzymes and cell types that will influence its behaviour [47], therefore, the dissolution-precipitation process is much more complex *in vivo*. In order to mimic the initial stages of the *in vivo* reactions the materials were incubated in serum-SBF to assess its effect on the dissolution and apatite formation. The dissolution kinetics of the materials incubated in serum SBF was slower when compared to the dissolution in serum free-SBF. At the same time point, no significant dissolution features were observed or apatite layer was visualized. The phase imaging AFM indicated the presence of a layer on top on these materials that could be a proteinaceous layer. The XPS analysis confirms this result, due to the increase on the concentration of nitrogen on the surface of the samples incubated in the presence of proteins.

Several mechanism have been proposed to explain the mechanism behind the effect of proteins on the apatite formation, namely that proteins can bind to the surface of the ceramic and control secondary nucleation, affecting the dissolution reprecipitation process in the ceramic [47], according to Kaufmann *et al.* the protein layer will protect the surface and prevent early dissolution [48]. The ICP results demonstrated that in the presence of proteins a release of silicon into the surrounding medium was lower when compared to the samples incubated in serum free-SBF. Therefore, the results presented here are in agreement with Kaufman *et al.*, indicating that the presence of the protein layer will indeed protect the surface of the material, delaying the diffusion of the atomic species to the surrounding medium, delaying the supersaturation of the solution and consequent apatite layer.

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