Cell adhesion and proliferation on biomimetic calcium-phosphate coatings produced by a sodium silicate gel methodology

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The present study describes a methodology to produce bioactive coatings on the surface of starch based biodegradable polymers or other polymeric biomaterials. As an alternative to the more typical bioactive glass percursors, a sodium silicate gel is being employed as a nucleating agent, for inducing the formation of a calcium-phosphate (Ca-P) layer. The method has the advantage of being able to coat efficiently both compact materials and porous 3D architectures aimed at being used on tissue replacement applications and as bone tissue engineering scaffolds. This treatment is also very effective in reducing the incubation periods, being possible to observe the formation of an apatite-like layer, only after 6 h of immersion in a simulated body fluid (SBF). The influence of the SBF concentration on the formation of the apatite coating was also studied. The apatite coatings formed under different conditions were analyzed and compared in terms of morphology, chemical composition and structure. After the first days of SBF immersion, the apatite-like films exhibit the typical cauliflower like morphology. With increasing immersion times, these films exhibited a partially amorphous nature and the Ca/P ratios became very closer to the value attributed to hydroxyapatite (1.67). The obtained results are very promising for pre-calcifying bone tissue engineering scaffolds. Therefore, in order to study cell behavior and response to these apatite coatings, adhesion, morphology, and proliferation of a human osteoblast cell line (SaOS-2) was also analyzed after being cultured in the coatings formed after 15 days of immersion in SBF. Results indicate a good correlation between crystallinity of the apatite like coatings formed in these conditions and respective cell spreading and morphology. In general, higher cell proliferation was observed for higher crystalline Ca-P coatings.

1. Introduction

Biodegradable materials are a promising alternative for a range of bone related applications. Starch-based biodegradable polymers are particularly interesting for bone replacement. Besides being biodegradable, inexpensive (when compared to other biodegradable polymers) and available in large quantities [1, 2], starch-based polymers can be converted into complex geometries that exhibit interesting mechanical properties, by using standard equipment developed for the processing of synthetic polymers [3] or by means of using distinct innovative methodologies [4, 5]. Furthermore, in addition to their processing versatility, they exhibit a biocompatible behavior, already demonstrated by *in vitro* [6–8] and *in vivo* studies [9]. Therefore, they are under consideration for a wide range of biomedical applications like bone replacement/fixation [10, 11], novel hydrogels and partially degradable bone cements [12], drug delivery carriers [4] or temporary scaffolds for tissue engineering applications [4, 5].

In case of bone related applications like tissue replacement/fixation, or tissue engineering scaffolds to be applied in load-bearing sites, these systems must exhibit mechanical properties that match those of human bone, associated to degradation kinetics adequate to the healing of the tissues to be replaced or fixed [10, 11]. Additionally, when coated with a bone-like apatite layer, they can also exhibit a bone-bonding behavior. In the recent years, there has been an increasing interest in the so-called biomimetic preparation of calcium phosphate coatings on implant materials. A biomimetic process for coating an apatite layer on organic polymers or other

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types of materials was first developed by Kokubo and coworkers [13, 14]. In this methodology, the materials were immersed in a simulated body fluid (SBF) with a composition similar to the human blood plasma, in order to promote the nucleation and growth of a calcium phosphate (Ca-P) bone-like layer on its surface. This was achieved using bioactive silica based glass particles as nucleating agents.

The Ca-P minerals found in natural hard tissues are fabricated in a physiological environment at low temperatures from moderately supersaturated mineralizing solutions [15]. Therefore, a biomimetic approach is particularly suitable to coat polymeric materials [14, 16], as it can be carried out at low temperature reaction conditions. Due to that fact it is expectable that these types of coatings would be very friendly surfaces for cell adhesion and proliferation, which is a key issue for bone regeneration. These types of coatings have been previously produced on the surface of biodegradable materials by using different biomimetic routes, in spite of all the difficulties arising from the pH changes and continuous degradation of substrates surface [17, 18].

The herein described biomimetic methodology reports on the use of a sodium silicate gel as an alternative nucleating agent applied before the subsequent immersion in a simulated body fluid (SBF) to promote apatite nucleation and growth. With this method, it was possible to reduce the incubation period for the formation of an apatite coating, when compared to the traditional biomimetic methodology, which uses bioactive glass particles as nucleating agents [17]. On the other had it can either be applied to coat compact or porous 3D architectures to be used on tissue replacement and in tissue engineering scaffolding [21]. The present study describes the influence of the SBF concentration on the formation of the apatite coatings for this recently developed biomimetic methodology. The apatite coatings formed under different conditions were analyzed and compared in terms of morphology, chemistry and structure. In order to study cell behavior and response to these apatite coatings, adhesion, morphology, and proliferation of human osteoblast-like cells (SaOS-2) culture on coating surfaces were also analyzed.

2. Materials and methods

2.1. Materials

Natural origin starch based polymers exhibit a great potential to be used in several applications. In this particular work, the studied material is based on a blend of starch/ethylene vinyl alcohol (SEVA-C) supplied by Novamont, Italy. The blend contains around 50% starch by weight, while the copolymer discloses a composition of 60/40 mol/mol. Compact samples were produced by injection molding on a Klockner Desma FM-20, using a nozzle temperature of 170 °C. All samples were standard ASTM tensile test bars, with a cross section of $2 \times 4\,mm^2$, being produced on previously optimized processing conditions.

2.2. Sodium silicate methodology

To produce the bioactive coatings a commercially available sodium silicate gel from SIGMA-ALDRICH ($Na_2SiO_3 \cdot H_2O$, containing $\sim$ 14% NaOH and $\sim$ 27% SiO_2, pH $\approx$ 13) was used. The viscosity of the gel is $6 \times 10^{-2}\,Pa\,s^{-1}$. Other authors have used before solutions based on sodium silicate gels [19, 20]. However, to design this methodology as simple as possible (and that means to look at it as a possible future easy to apply industrial technology), the sodium silicate gel was herein used with a concentration and SiO_2/Na_2O molar ratio as received. On the other hand the relatively high viscosity of the gel allows for the impregnation of the materials instead of gel uptaking, which would seriously affect the bulk mechanical and degradation behavior of the material. The materials were ''impregnated'' for 24 h with the gel, in a controled atmosphere (23 ± 1 °C; 55% RH). After this treatment the solution, containing the samples, was diluted with 50% vol. of distilled water and stirred for 5 min. This procedure was aimed at diminishing the viscosity of the gel in order to produce a homogeneous and thin layer of vitrified sodium silicate in the surface of the materials. The samples were then removed from the solution and then dried in controled atmosphere (23 ± 1 °C; 55% RH).

2.3. Water-uptake ability

Water-uptake studies are of a great importance for a biodegradable material, because when implanted, it will be inevitably in the presence of the body fluids that will diffuse into the bulk of the polymer as degradation is taking place. For water-uptake measurements all the samples were weighted before being immersed on distilled water (at room temperature) and then each 2 h, during the first 12 h of immersion. After that period, the weights started to be registered each 24 h, until the end of the experiment time (7 days). The samples were carefully removed from the water containing flasks and immediately weighted for the determination of the wet weight as function of the immersion time. Water-uptake is given by:

$$\text{Water absorbed} = \left[(m_f - m_i)/m_i\right]^{*} 100 \qquad (1)$$

where m_i is the initial weight of the sample, and m_f is the sample weight after a given time of immersion.

2.4. Contact angle measurements

In previous works SEVA-C has demonstrated to be a quite hydrophilic substrate [18, 22]. It is also known that the presence of OH^- groups on the surface seems to facilitate the connection with an apatite layer, when it is nucleated by a biomimetic treatment [18, 22]. Alkali attack, namely with KOH has already proved to be very effective in increasing the number of OH^- groups at the surface [18]. Since the used sodium silicate gel contains $\sim$ 14% of NaOH, it is expected an increase in the hydrophilicity of the surface which will have an important role during apatite formation. Contact angle measurements were performed, for compact SEVA-C samples that were submitted to the sodium silicate gel treatment for 24 h, in order to detect any eventual modification in the surface of the material. After treatment, the samples were washed in distilled water

TABLE I Ion concentrations and ionic activity products of the human blood plasma and SBF solutions in the present study [11, 21]

Solution	Concentration/mM								
	Na^+	K^+	Ca^{2+}	Mg^{2+}	Cl^-	HCO_3^-	HPO_4^{2-}	SO_4^{2-}	Log IP
Human plasma	142.0	5.0	2.5	1.5	103.0	27.0	1.0	0.5	– 96.6
SBF 1.0 ×	142.0	5.0	2.5	1.5	147.8	4.2	1.0	0.5	– 96.6
SBF 1.5 ×	213.0	7.5	3.8	2.6	223.0	6.3	1.5	0.8	– 93.8
SBF 2.0 ×	284.0	10.0	5.0	3.0	297.6	8.4	2.0	1.0	– 91.9

TABLE II Surface tension (γ_l) of water and the methylene iodide and its polar (γ_l^p) and disperse (γ_l^d) components

	γ_l	γ_l^d	γ_l^p
Water	72.8	21.8	51.0
Methylene iodide	50.8	50.8	0.0

and dried in a controled atmosphere (23 ± 1 °C; 55% RH). Contact angle measurements were obtained by the sessile drop method using a standard contact angle apparatus (Krüss, Hamburg, Germany). The measurements were performed with the aid of an image analysis system (G2/G40) installed in the apparatus. Duplicate measurements were recorded for each drop deposition. The average values were recorded after 5 s of the drop deposition (settling time). Ten average values were recorded for each condition and a final average value was calculated. All the measurements were made at room temperature and the probe liquids were water and methylene iodide. The obtained results from both liquids were used to calculate the surface tension (γ) according to a method proposed by Owens and Wendt [23]. According to this method the surface tension of each phase can be resolved into a polar (γ_l^p) and dispersive (γ_l^d) parts, as presented in Table I. Using these two test liquids the resulting values of the surface tension were calculated by this method, which is particularly useful for determination of the surface energy of low energy surfaces (such as polymers).

2.5. Apatite formation

To produce control biomimetic apatite coatings the substrates were submitted to a procedure inspired in the so-called biomimetic treatment, previously described by Kokubo and co-workers [13, 14] and adapted by Reis *et al*. [17, 18]. The procedure is schematized in Fig. 1. This standard biomimetic methodology was used as a control – reference for comparative purposes. In this way it was possible to evaluate the efficacy of the hereby newly proposed methodology. For the standard biomimetic coatings production, the treated samples were:

1. First soaked in a 1.0 × simulated body fluid (SBF) with an ion concentration nearly equal to human's blood plasma (Table II) in order to form apatite nuclei.
2. After 7 days, soaked in another solution, with ion concentrations 1.5 × SBF for making apatite nuclei to grow. Another group of samples was submitted to step (1) but, after 7 days they were soaked in a solution with ion concentrations of 2.0 × SBF.

In fact, it is believed that the concentration of SBF solution influences the nature of the apatite formed during the growth stage [24]. Increasing the SBF concentration in 1.5 or 2 ×, after the first week of apatite formation will raise the ionic activity product from $10^{-96.6}$ to $10^{-1.9}$, respectively. In this way it is expectable that it might be possible to produce different (tailored) apatite coatings with different Ca-P ratios and relative crystallinities. In both cases, studied times were an incubation period up to 7 days (to understand the nucleation process) and a growing period up to 15 days.

2.6. Scanning electron microscopy, energy dispersive spectroscopy, and X-ray diffraction

The morphological characterization was carried out by Scanning Electron Microscopy (SEM) analysis, in a JEOL JSM 7301 F. All the samples were coated with a thin film of carbon, by ion sputtering, prior to any observation. The electron beam energy was changed between 10 and 12 keV.

A half-quantitative characterization, using well established calibration sub routines for analyzing the atomic

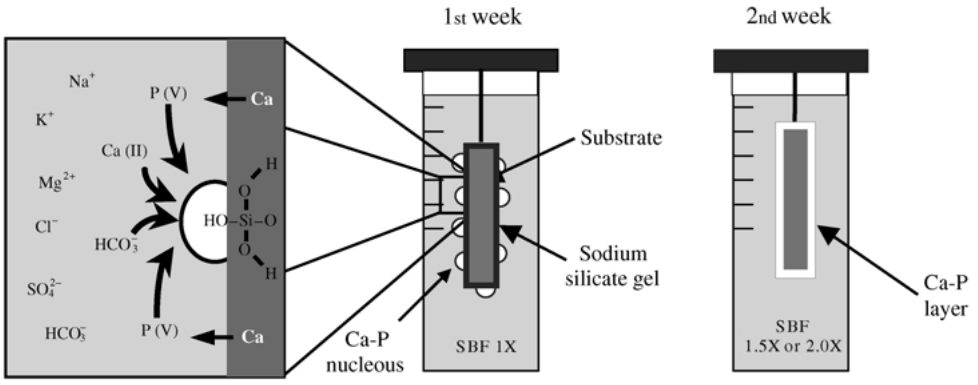

Figure 1 Schematic representation of the sodium silicate gel biomimetic methodology. Adapted by Reis *et al*. [17, 18] from the procedure developed by Kokubo *et al*. [13, 14].

concentrations of Ca and P within the coatings was performed by means of Energy Dispersive Spectroscopy (EDS), in a Rontec spectrometer. Ca, P, Mg, K, and Na amounts were quantified. From these results Ca/P ratios were calculated. Considering the possibility of Mg^{2+}, Na^{+}, and K^{+}, present in the SBF, substituting the Ca^{2+} in the crystalline network of the apatite, the following relation was also calculated: $(Ca + Na + Mg + K)/P$.

Thin-film X-ray Diffraction (TF-XRD, 1° incidence angle) spectra were acquired in a Rigaku equipment at 60 kV at 30 mA in order to identify the crystalline phases present in the several Ca-P layers, and their relative crystallinity.

2.7. Cell adhesion and proliferation

In order to study the morphology, attachment and proliferation of osteoblast-like cells over pre-calcified SEVA-C surfaces, a human osteoblasts SaOS-2 cell line obtained from the European Collection of Cell Cultures was selected. Dulbecco's Modified Eagle's Medium (DMEM, Sigma, USA), supplemented with 10% Foetal Bovine Serum (FBS, Biochrome, Germany) and 1% antibiotic/antimicotic solution (Sigma, USA) was used as cell incubation medium and growth to confluency in controled atmosphere conditions (37 °C, 5% CO_2, 100% humidity). Detachment of confluent low passage cells was performed using 0,25% Trypsin/EDTA solution (Sigma, USA). Subsequently, osteoblast-like cells were cultured over samples in a concentration of 3.3×10^4 cell/ml and incubated for 24 h and 7 days in controled atmosphere conditions. Tissue culture grade polystyrene was used as control and replicates were prepared. After each incubation period, samples were washed with Phosphate Buffer Saline (PBS, Sigma, USA) solution and fixed in Gluteraldehyde 2.5% (V/V) for observation by means of Scanning Electronic Microscope. After fixation, samples were dehydrated in crescent ethanol concentrations (50%, 70%, 90% and 100%), air-dried and sputter coated with gold.

3. Results and discussion

3.1. Water-uptake

Fig. 2 shows the water-uptake versus time for the SEVA-C material, untreated and treated with sodium silicate.

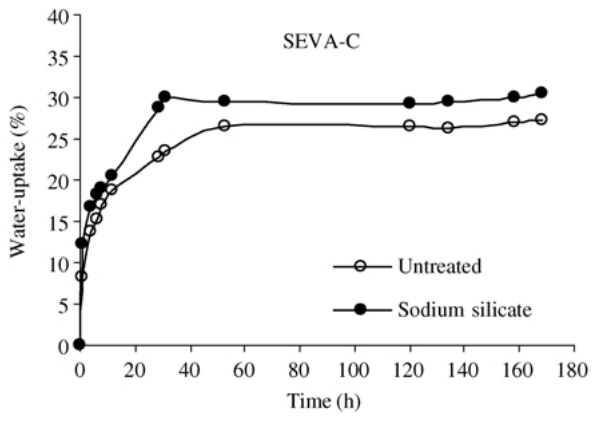

Figure 2 Water-uptake (%) versus time for untreated and sodium silicate treated SEVA-C samples.

After 50 h, the equilibrium hydration degree of untreated samples is about 25%. This hydrophilic behavior is mainly a result of the presence of starch and vinyl alcohol hydroxyl groups, as it has been previously described [25]. With sodium silicate treatment the amount of water uptake increases around 5%. This result can be justified by the fact that the commercially available sodium silicate that was used contains 14% of NaOH in its composition and discloses a pH around 13. This type of alkali attack can be compared to the previously studied KOH surface pre-treatment performed on SEVA-C [26]. The increase of hydrophilicity after this treatment can be justified by the molecular hydroxy-NaOH hydrogen bonding complexes, which are easily solvated by water molecules.

3.2. Contact angle measurements

Understanding the surface behavior of a biomaterial in contact with hydrated media is of great importance to predict interactions of a certain material with the surrounding tissues, when it is applied in a particular biomedical application. In this work, contact angle measurements were performed on compact SEVA-C samples. Table III presents the results concerning the average water/methylene iodide contact angle (θ_w/θ_m) and the average surface tension (γ_s) of the sodium silicate treated surfaces. This treatment, performed on the SEVA-C samples did modify the surface parameters. The average water contact angle decreased from 63.4° to 29.7°. An increment in the total surface energy, as a consequence of the increase of the polar contribution, was observed. Values changed from 45.9° to 68.0° after treatment. These significant changes can mainly be attributed to an increase in the polar contribution γ_s^p (from 13.4° to 30.32°) that resulted from an alkali attack by the sodium silicate gel, which can be compared to the previously studied KOH surface pre-treatment performed on SEVA-C substrates [18].

3.3. Apatite formation

Previous studies [21] have demonstrated that a very cohesive apatite-like layer can be formed when using the sodium silicate gel methodology only after 6 h or immersion in SBF. This indicates that the number of the nucleating sites on the surface of the substrates during the first hours of immersion is rather high, allowing for the formation of a dense and uniform apatite layer [21]. SEVA-C is a polar and quite hydrophilic substrate, where the presence of OH^- groups in the surface tends to facilitate the connection with the apatite layer. It seems that a fairly strong bond could be formed between the polar groups and calcium ions of the apatite layer.

It has been shown by Li *et al*. [27], and Cho *et al*. [28] that heterogeneous nucleation of apatite can be induced from metaestable solutions, including physiologic solutions on those specific superficial sites, where there are OH^- containing groups. The proposed biomimetic methodology leads to an increase of the hydrophilicity (Table III) and water-uptake ability (Fig. 2) of the polymers, resulting in the increase of polar groups in the

TABLE III Water (θ_w) and methylene iodide (θ_m) contact angle and surface energy measurements of SEVA-C untreated and after sodium silicate treatment for 24 h

	$\theta_w(^\circ)$	$\theta_m(^\circ)$	γ_s(mN/m)	$\gamma_s{}^d$(mN/m)	$\gamma_s{}^p$(mN/m)
Untreated SEVA-C	63.4 ± 1.85	53.2 ± 0.67	45.9 ± 1.09	32.5 ± 0.38	13.4 ± 1.08
Treated SEVA-C	29.7 ± 3.79	43.8 ± 2.02	68.0 ± 2.02	37.6 ± 1.07	30.3 ± 1.83

surface. In this way, the polymers tend to absorb higher quantities of Ca^{2+} ions from the SBF solution. As a consequence, the Ca^{2+} ion concentration at the surface will be increased, leading to the formation of additional nucleating sites for the Ca-P coating formation. This phenomenon has already been reported before for PEO/PBT copolymers [29], being related to a chelation effect. On the other hand, the formation at the surface of silanol groups (Si-OH), will act as favorable sites for the apatite nucleation. As a result, a large number of apatite nuclei are rapidly formed on the surface. Subsequently, they grow spontaneously by consuming the calcium and phosphate ions from the surrounding fluids (the body fluid is already saturated with respect to the apatite).

Degradation of the starch-based polymers induces a greater degree of complexity to the reactions since it is continuously changing the composition of the solution adjacent to the interface, namely the pH and the polymer surface itself. This phenomenon has been previously described by Reis *et al*. [17]. Nevertheless, the apatite-like layer could be formed in the proposed polymeric material by means of adapted biomimetic coating routes

Fig. 3 presents the evolution of the Ca/P and (Ca + Mg + Na + K)/P ratios of the apatites formed on SEVA-C after immersion in SBF (1.5 × or 2.0 × after 7 days) for different periods up to 30 days. Apatites formed in a solution often replace the Ca^{2+} ion site with small amounts of Na^+, Mg^{2+}, or K^+ ions. The presence of these ions in the crystalline network of the apatite was evaluated. In fact, for the first stages of immersion in SBF there is a considerable difference between Ca/P and (Ca + Mg + Na + K)/P, being Na^+ and Mg^+ the main contributors for the presented values (data not shown). The presence of considerable amounts of Na^+ in the apatites formed in the first hours can be justified by the existence of these ions at the surface, resulting from adsorption during impregnation with sodium silicate gel.

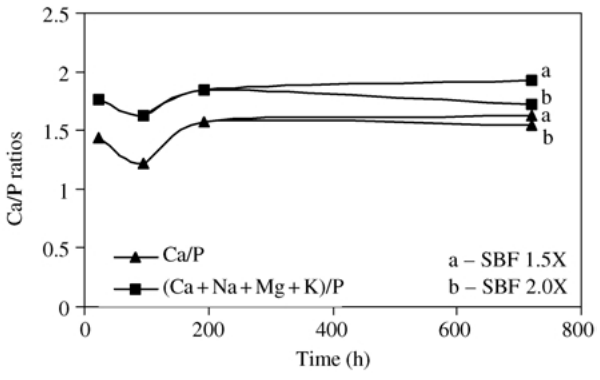

Figure 3 Ca/P and (Ca + Mg + Na + K)/P ratios of the apatites formed on compact SEVA-C substrates, after immersion in SBF 1 × and after 7 days in SBF 1.5 × or 2.0 × .

Considerable amounts of Mg^{2+} were also detected for the first hours. This phenomenon is presently under study. The Ca/P ratios, tend to increase with immersion time in SBF, to values in the range between tricalcium phosphate (TCP, 1.5) and HA (1.67). When an apatite layer is formed from an aqueous solution, some sites for the PO_4^{3-} are partially substituted by HPO_4^{2-} and CO_3^{2-} ion substituting for the PO_4^{3-} when comparing to the currently found for bone apatite (5.80 wt %) [24]. This result can be justified by a lower concentration of $HCO_3{}^-$ in the produced SBFs when compared to human blood plasma (Table II). After 30 days it is possible to observe a decrease in the Ca/P values of the apatites formed in the materials immersed in SBF 2.0 × after 7 days, when comparing with values resulting from a second immersion in SBF 1.5 × (from a to b). This decrease with increasing of the ionic activity product (IP) of the SBF solution was already reported by Kim *et al*. [24], that calculated $H_2PO_4{}^-$, HPO_4^{2-} and PO_4^{3-} concentration of SBF with different IPs. Since $H_2PO_4{}^-$ ion concentration increases appreciably with increasing IP, comparing with the other phosphate ions, it is possible to assume that the degree of substitution of these ions for the PO_4^{3-} ions also increases, leading to an increase in the Ca^{2+} deficiency [24]. This deficiency results from vacancies produced in the sites for the Ca^{2+} ions to maintain electrical neutrality.

Fig. 4 presents the TF-XRD patterns of the surface of SEVA-C, before and after immersion in SBF, which was raised to (a) 1.5 × or (b) 2.0 × after the first week. These patterns evidence poorly crystalline peaks mainly corresponding to hydroxylapatite on a mainly amorphous calcium-phosphate coating, for the longer SBF immersion periods. Nevertheless, when increasing the IP to 2 × SBF, the crystallinity of the formed layers seems to be higher. It is well known that bone apatite also presents a highly amorphous content. The layers formed are consequently approaching bone apatite structure and are expected resorbed faster than highly crystalline coatings. The obtained results seem to indicate that it will be possible to tailor the coating relative crystallinity (and consequently its resorbability) by means of playing with the gel composition and the SBF concentration.

Figs. 5 and 6 show the SEM micrographs of the morphology of SEVA-C surface after immersion in SBF for 15 days and subsequent SaOS-2 cell culturing for 1 and 7 days.

From these figures, it is possible to observe that the apatite layer is formed by aggregates of nucleus that have grown into the so-called *cauliflower* morphology, after 15 days of immersion in SBF (please remember that SBF concentration was raised to 1.5 × after 7 days). This type of morphology was already reported for biomimetic coatings on the surface of similar polymers nucleated

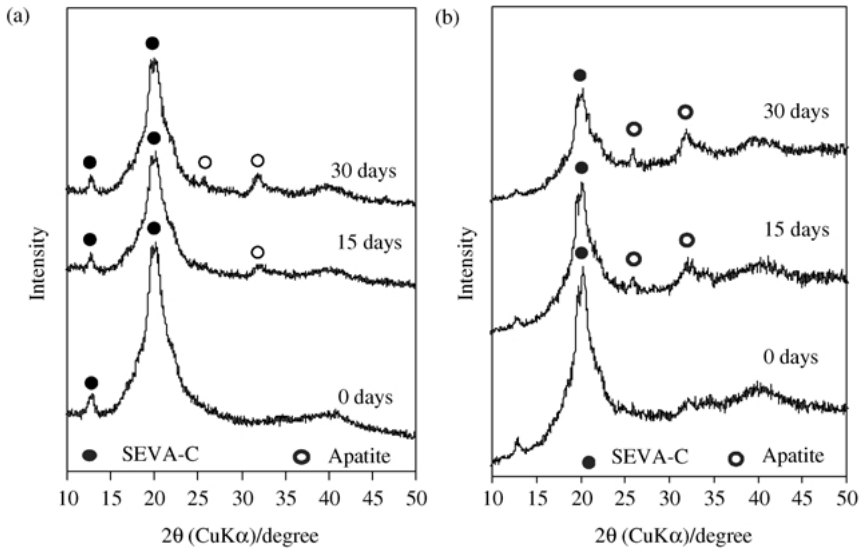

Figure 4 Thin-film X-ray diffraction patterns of the films formed on SEVA-C after treatment with sodium silicate and after 15 and 30 days of immersion in SBF. In (a) the SBF solution was raised to 1.5 × after 7 days, while in (b) it was raised to 2.0 × after the same period.

using bioactive silica glasses, produced in this type of materials [17, 18]. For higher magnifications it is possible to observe a needle-like structure, which is typical of this type of coatings. This morphology is particularly evident after immersing in SBF 2.0 ×.

On what concerns to the study of cell adhesion and proliferation it is possible to observe, on the same SEM micrographs (Figs. 5 and 6), that these preliminary results indicate good cell attachment to both types of coated surfaces. In addition, cell morphology was shown to be influenced by the type of coating produced, as the formation of characteristic lamellipodium structures was mainly detected for samples immersed in 1.5 × SBF (please compare Fig. 5(a) and (b) that are typical reproducible examples of cells behavior on these surfaces). Even though 24 h of incubation was not enough to detect differences in cell number and distribution over both surfaces, after 7 days higher proliferation was observed for 2.0 × SBF samples (Fig. 6). In fact, the crystallinity of a biomaterial surface is known to determine specific cell responses like the organization of cytoskeleton filaments and cell proliferation mechanisms [30, 31]. More specifically, results reported indicate that osteoblast spreading/proliferation is slower for more amorphous surfaces [30, 31] mainly due to the development of less organized cytoskeleton [30]. Without minimising the influence of other factors, these results although still non-conclusive present a good reproducibility and a good correlation between crystallinity of apatite like coatings (see Fig. 4) and cell spreading (see Fig. 5) and morphology (see Fig. 6).

4. Conclusions

Sodium silicate gel methodology proved to be a very effective and simple methodology to reduce the induction period for the formation of a well-defined apatite-like layer on the surface of starch based biodegradable polymers. Furthermore, when raising the concentration of the SBF solution during the growing period, it was possible to produce an apatite like layer more similar to bone apatite. Nevertheless in both cases a very good osteoblast adhesion and an undisrupted morphology were obtained, corresponding to a preliminary indicator of the biocompatibility of the apatite coatings. Furthermore, it can be said that SBF treatment

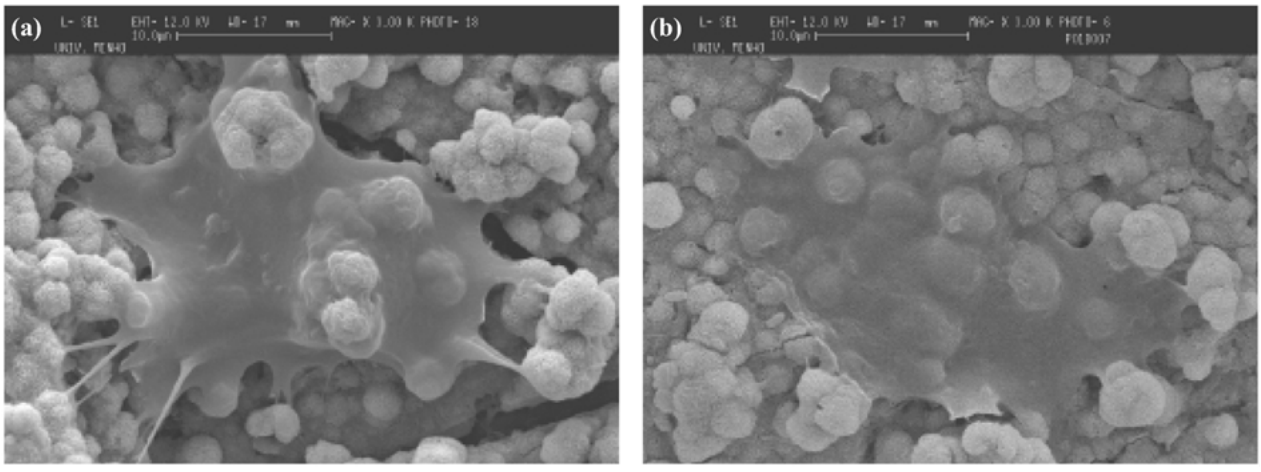

Figure 5 SEM micrographs showing the morphology of the apatite coatings formed on the surface of SEVA-C and the typical aspect of the SaOS-2 cells adhered to it: (a) 1.5 × SBF or (b) 2.0 × SBF.

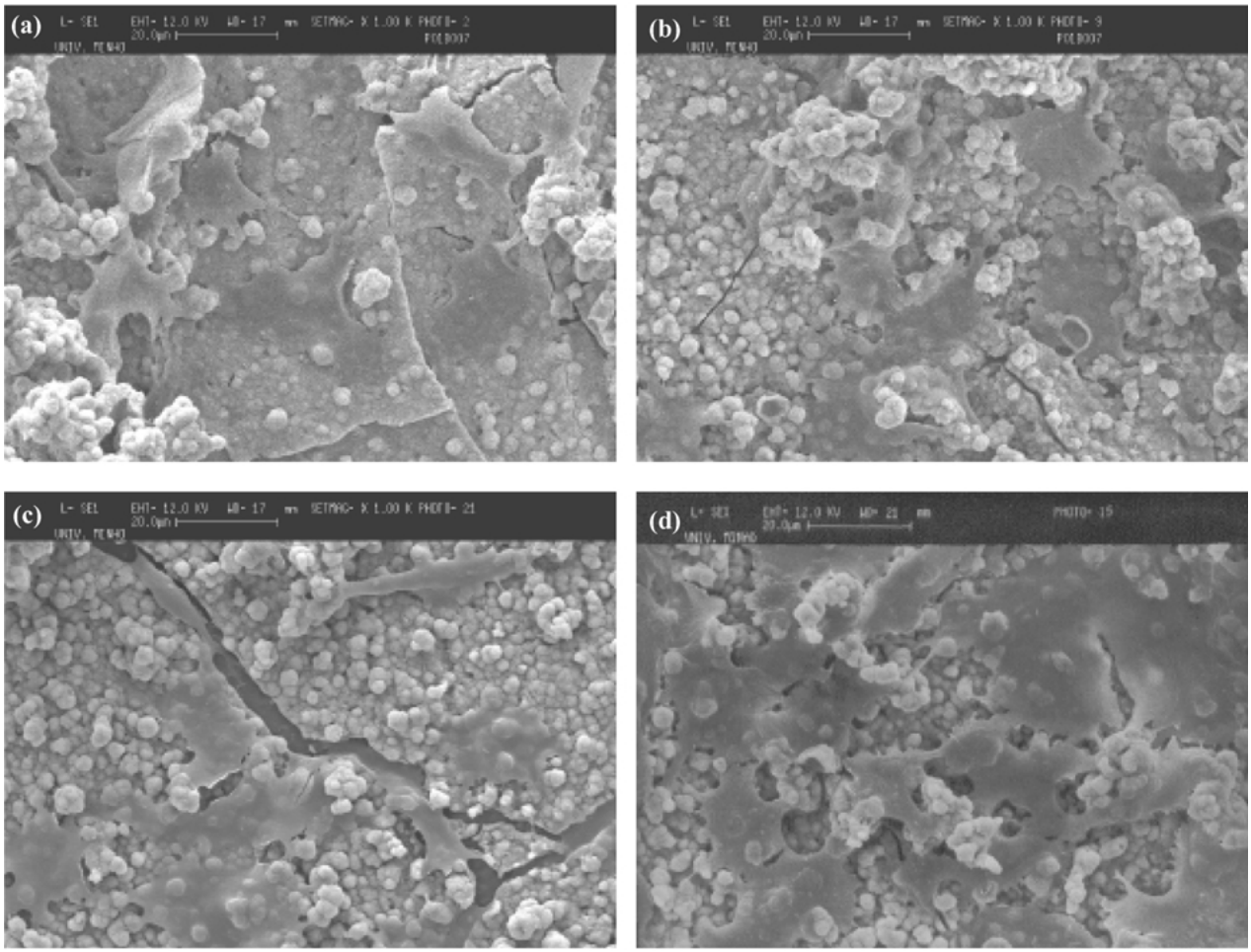

Figure 6 Proliferation of osteoblast-like cells after culture over the different surfaces: 1.5 × SBF for (a) 1 day and (b) 1 week; and 2.0 × SBF for (c) 1 day and (d) 1 week.

or more specifically, the obtained characteristics of the coatings, influence positively cell proliferation kinetics. Cell behavior seems to depend on the coatings crystallinity, being slower for more amorphous surfaces.

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