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Cellulose phosphates as biomaterials. Mineralization of chemically modified regenerated cellulose hydrogels

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Femoral implantation of regenerated cellulose hydrogels revealed their biocompatible and osteoconductive properties, but a complete osseointegration could not be observed. Phosphorylation was therefore envisaged as the means to enhance cellulose bioactivity. Once implanted, phosphorylated cellulose could promote the formation of calcium phosphates, having therefore closer resemblance to bone functionality and assuring a satisfactory bonding at the interface between hard tissue and biomaterial. In the present work, regenerated cellulose hydrogels were surface modified via phosphorylation. Phosphorylated materials, having varying degrees of substitution, were soaked in a Simulated Body Fluid (SBF) solution in order to investigate their ability to induce the formation of a calcium phosphate layer. Mineralization was assessed by Scanning Electron Microscopy (SEM), Energy Dispersive Spectroscopy (EDS) and Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) spectroscopy. It was demonstrated that the calcium salt of cellulose phosphates mineralized at a higher extent than materials only phosphorylated. The degree of phosphorylation influenced the extent of surface mineralization. Moderate degrees of surface phosphorylation promoted the highest extent of mineralization. This was attributed to inadequate functionality of the surface in terms of density of PO_4 groups and overall surface charge, in the case of low and high phosphate contents.

1. Introduction

Mineralization of biomaterials for orthopedic applications is usually desirable. The term mineralization is used here as the ability of a material to induce the formation of an apatite layer, when immersed in a simulated plasma solution. A successful implantation of a material for orthopedic applications is dependent of a close apposition between bone and biomaterial [1, 2]. Since the mineral part of bone is mainly constituted by calcium phosphates, bone bonding to biomaterials surfaces can be improved by the existence of this mineral on the surface of implant materials. Coating of metallic prostheses with calcium phosphates is based on this assumption [3].

Synthetic calcium phosphates generally induce the formation of an apatite layer in simulated plasma solutions [4–8]. Some other synthetic biomaterials have also shown the capability of inducing the formation of a calcium phosphate mineral, like glass-ceramics [9–11], silicon-based materials [12–14], titanium-based materials [15–20], including polymers like poly(ethylene oxide)/poly(butylene terephtalate) [21, 22], and polyethylene glycol [23, 24]. Several techniques have also been developed in order to turn polymeric biomaterials with limited or no tendency to mineralize into mineralizing ones. Polymer/bioactive ceramic composites [25–27] and the so-called biomimetic coatings [28–31] are among the most well succeeded ones. Grafting of phosphorus-containing groups on polymer chains is another promising approach, and it is the object of the present work. Some works are available in the literature, concerning the mineralization of phosphorylated materials, namely self-assembled monolayers [32], and polymers [33–36], including cellulose derivatives [37–39].

Cellulose is the world's most abundant natural, renewable and biodegradable polymer. The biocompatibility of some cellulosics is well documented [40–46]. Oxidized cellulose is used as a wound dressing [47–49]. Cellulose regenerated by the viscose process (CRV) has been investigated as an implantable material in orthopedic surgery, as a sealing material for the femoral component in hip prostheses, in place of the acrylic cement. It was envisaged to take advantage not only of its good matching with mechanical properties of bone but also of its hydroexpansivity, therefore allowing a satisfactory fixation to hard tissue [50–52]. The osteoconduction of this material has been demonstrated [50, 51]. Nevertheless, a full bioactive character cannot be attributed to normally occurring cellulose due to lack of complete osseointegration.

To stimulate bone induction by CRV , its chemical modification via phosphorylation was envisaged. Once implanted, phosphorylated cellulose could promote the formation of calcium phosphates, having therefore closer resemblance to bone functionality and assuring a satisfactory bonding at the interface between hard tissue and biomaterial. In previous works, cellulose phosphorylation was carried out [53–55]. Reaction parameters were optimized using microcrystalline cellulose, and cellulose phosphates of high degrees of substitution were obtained [54, 55]. Phosphorylated materials were characterized and they were found cytocompatible, in cultured human osteoblasts as well as fibroblasts [55].

Phosphorylated CRV was previously characterized using X-ray Photoelectron Spectroscopy (XPS), Fourier Transform Infrared (FTIR) spectroscopy and contact angle measurements [53]. In the present work, phosphorylated materials at varying degrees of substitution were soaked in a Simulated Body Fluid solution (SBF) in order to study their ability to induce the formation of a calcium phosphate layer, in simulated physiological conditions. Mineralization was assessed by Scanning Electron Microscopy (SEM), Energy Dispersive Spectroscopy (EDS), and Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) spectroscopy.

2. Materials and methods

Cellulose Regenerated by the Viscose Process (CRV) was a generous gift from Hexabio (Bordeaux, France). All chemicals were of research grade purity, and used without further purification.

2.1. Synthesis of phosphorylated cellulose

Regenerated cellulose (CRV) discs were machined from blocs dried in air at room temperature. The obtained CRV discs (10×2 mm) were swollen consecutively in distilled water, ethanol and hexanol, for 24 hours, prior to chemical modification. Phosphorylation reaction was carried out in a four-necked round-bottomed flask equipped with a nitrogen inlet, a condenser, a thermometer and a mechanical stirrer. CRV discs were dispersed in 30 mL of hexanol and a solution of phosphorus pentaoxide (50 g) in 37 mL of triethyl phosphate and 42 mL of 85% phosphoric acid was added portionwise to the suspension. The reaction was allowed to proceed at room temperature, under constant stirring and a nitrogen stream, for 4 h (P4), 8 h (P8) and 24 h (P24). Phosphorylated CRV (CRV-P) thus obtained was rinsed with hexanol and ethanol, washed by soxhlet extraction and then dialyzed against distilled and deionized water, at least for 24 hours, in order to wash out the H_3PO_4 excess, until tests for inorganic phosphate were negative. The modified materials were then dried in air at room temperature. Prior to mineralization assays, discs of phosphorylated CRV were immersed for 3 days, at 37°C, in 50 mL of a 0.05 M $CaCl_2$ aqueous solution, which was renewed every 24 hours.

2.2. *In vitro* mineralization studies

Untreated (CRV), phosphorylated (CRV-P) and phosphorylated and pre-incubated in $CaCl_2$ (CRV-P-Ca) discs were used to assess the ability of these materials to induce the formation of a calcium phosphate mineral. Each disc was immersed in SBF [56] solution (pH 7.4) using a material surface to solution volume ratio of ca. 1 cm^{-1}. Polyethylene screwtop flasks were used. The SBF solution was renewed every 24 hours and kept at 37°C in a water bath, between renewals. The pH was monitored before and after materials immersion. Periods of immersion in SBF ranged from 12 hours to 15 days. Before further examination, all materials were rinsed in deionized water and dried at room temperature.

2.3. Surface characterization

Scanning Electron Microscopy (SEM) and Energy Dispersive Spectroscopy (EDS) analyses were carried out at 15 kV using a Hitachi S-2500 scanning electron microscope. Observations were made on sputtered carbon coated specimens. The deposited calcium phosphate films were characterized by Attenuated Total Reflectance-Fourier Transform Infrared (ATR-FTIR) spectroscopy with a Perkin Elmer 2000 FT-IR spectrometer, using the Split Pea accessory (Harrick Scientific Corporation), equipped with a silicon hemispherical crystal. All samples were run at a spectral resolution of 4 cm^{-1}.

2.4. Control sample

A commercial hydroxyapatite (HAp; from CAM Implants, Merck ref. 1432), previously heated at 1000°C and cooled in the oven, was used as a control sample. The HAp powder was analyzed by X-ray diffraction and the *d* (*hkl*) spacing and intensity values found matched the standard values for hydroxyapatite (file 9-432 JCPDS) [57].

3. Results

The formation of cellulose phosphate calcium salt can be observed in Fig. 1. Independently of the phosphate content, after 3 days of immersion in $CaCl_2$, the pH stabilized at ca. 5.0 and then it raised to approximately 5.5 after 6 days. Lower degrees of phosphorylation (4 hours comparing to 8 hours), seemed to allow a slightly higher

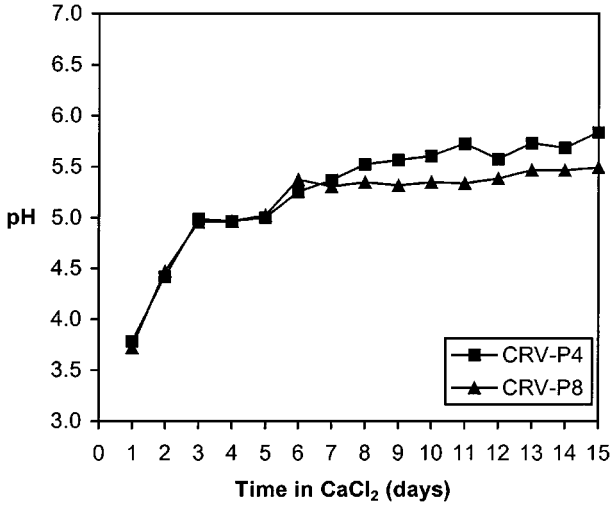


Figure 1 pH variation as a function of time of immersion of phosphorylated cellulose (CRV-P) in calcium chloride, at varying degrees of phosphorylation.

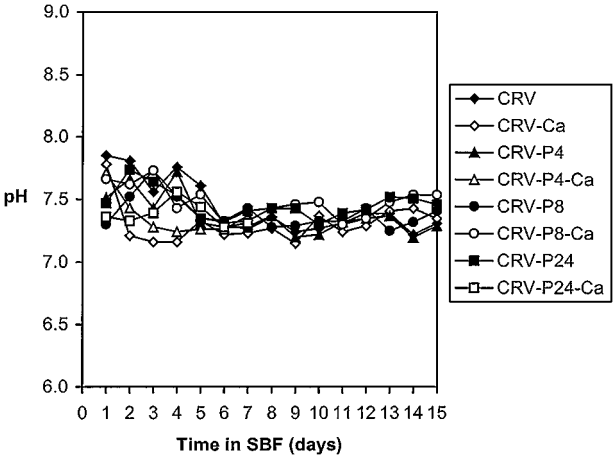


Figure 2 pH variation as a function of time of immersion of unmodified cellulose (CRV), and cellulose at varying degrees of phosphorylation (CRV-P), and their calcium salts, in SBF.

pH of the calcium salt. In Fig. 2 the pH variation as a function of immersion time in SBF can be observed. In the present case, SBF solution was renewed and its pH measured every day. Fig. 2 shows that 1 day after immersion in SBF all solutions had a pH between 7.0 and 8.0. Unmodified materials and calcium salts resulted in values closer to 8.0, whereas phosphorylated materials resulted in values closer to 7.0. Some higher variation can be observed between the first and the sixth day, but from then on all samples promoted stabilization of solution pH around 7.4, until the fifteenth day.

Fig. 3 shows SEM micrographs of the calcium phosphates formed over phosphorylated materials (CRV-P) and their calcium salts (CRV-P-Ca), after immersion in SBF. It can be observed that phosphorylated materials promoted only a residual formation of calcium phosphates. CRV-P24 seemed to induce a higher extent of mineralization and a more homogeneous distribution. On the contrary, materials phosphorylated and then pre-incubated in $CaCl_2$, induced the formation of calcium phosphate particles all over the surface. CRV-P8-Ca promoted the best surface coverage, and CRV-P24 the worst, among the phosphate contents tested.

SEM observations on cross-sections (Fig. 4), showed the interface between the calcium phosphate layers formed and materials surface. Materials phosphorylated and then pre-incubated in $CaCl_2$ seemed to promote a continuous interface. Materials only phosphorylated did not seem to exhibit such a good integration with the calcium phosphate layer, since a clear separation between the two materials could be observed.

EDS microanalyses results (Figs 5 and 6) showed that the calcium phosphates formed became thicker since the carbon and oxygen contents from the polymer backbone decreased in relative intensity from 2 to 15 days of immersion in SBF (Fig 5). Furthermore, the Ca/P ratios obtained increased from ca. 1.26 after 2 days in SBF to ca. 1.66 after 15 days in SBF (Fig. 6).

ATR-FTIR analyses of the calcium phosphates formed are shown in Fig. 7, compared to a standard hydroxyapatite. New peaks at near 557 and 595 cm^{-1} were found for materials immersed in SBF in comparison to control CRV and CRV-P8. These peaks, which are characteristic of the ν_4 vibration of PO_4 groups in apatitic structures [13, 30, 58–61], were found in the control hydroxyapatite, as well as in every material immersed in SBF, but not on untreated neither on phosphorylated cellulose. Phosphorylated materials, pre-incubated in $CaCl_2$ and immersed in SBF, showed the better-defined peaks. There is also evidence of carbonate bands at 867 and 1407 cm^{-1}, which are typical of carbonated biological apatites [4, 60–66].

4. Discussion

In a previous work, the surface of phosphorylated cellulose hydrogels was characterized by several techniques demonstrating that increasing reaction time promoted higher phosphate contents on the surface [53].

Phosphorylated cellulose is an acidic material, as it has been previously described [53, 54]. Its neutralization via the formation of the calcium salt was demonstrated here. After immersion in SBF, all materials had a pH between 7.0 and 8.0, which stabilized near the physiological pH from the sixth day on. Materials pre-incubated in $CaCl_2$ stabilized at slightly higher pH values than materials only phosphorylated, indicating that there is probably an exchange of calcium ions with the medium in the case of pre-incubated materials.

It must be stressed that although the SBF technique has been the most widely spread in the literature for investigating biomaterials mineralization, it should be analyzed very carefully. During the present work this technique was observed to lack reproducibility. All results presented were averaged from three samples for every material and every condition tested. Nevertheless, considerable differences were found for the same combination of parameters. The stability of the SBF solution must also be carefully monitored by measuring its pH at least every day and, preferably, it should be stored at 4°C between renewals, in order to assure a higher degree of reproducibility.

SEM micrographs of the calcium phosphates formed, after immersion in SBF, showed that phosphorylated materials promoted only a residual formation of apatitic

mineral, whereas materials phosphorylated and pre-incubated in $CaCl_2$ induced the formation of apatite particles all over the surface. In the case of materials only phosphorylated, higher phosphate contents seemed to favor increasing mineralization. In the case of materials pre-incubated in calcium, higher phosphate contents seemed to promote a higher extent of mineralization until 8 hours of phosphorylation treatment. The calcium salt of materials phosphorylated for 24 hours, which had the highest phosphate content tested, promoted a lower mineralization extent than the one observed with lower phosphate contents. This fact seems to be in accordance with current theories of biomineralization, where it is described that several factors play important roles, namely adequate spatial arrangements of surface functional groups and surface charge [67–72]. The concentration of phosphate groups on the surface of materials treated for 24 hours may be too high to allow the adequate fixation of calcium ions, and thus the formation of the calcium phosphate layer.

Grafting of phosphate groups for inducing mineralization was inspired in several works describing the

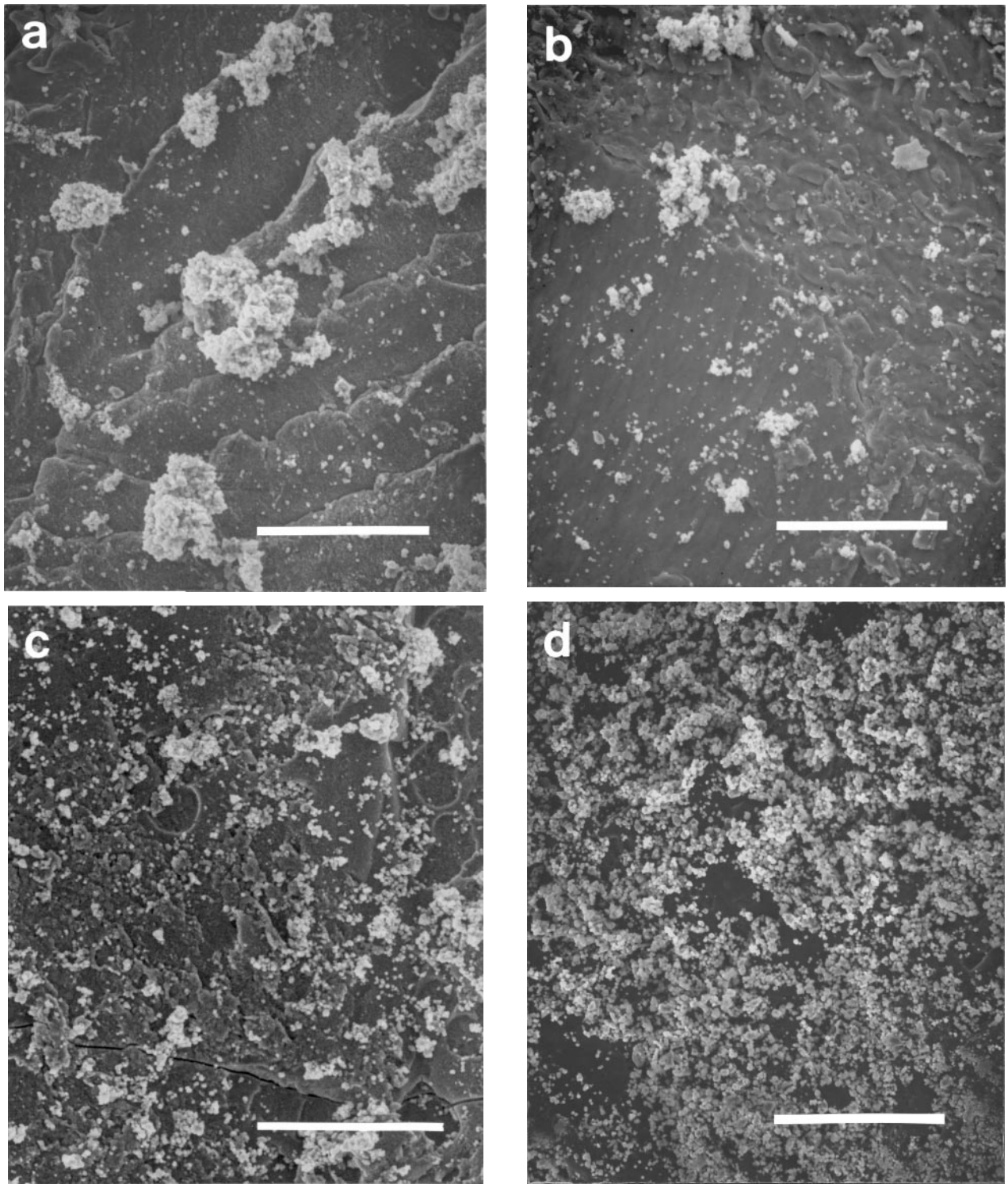


Figure 3 SEM micrographs of the calcium phosphates formed on phosphorylated cellulose (CRV-P) and its calcium salts, after immersion in SBF for 7 days: a) CRV-P4, b) CRV-P8, c) CRV-P24, d) CRV-P4-Ca, e) CRV-P8-Ca and f) CRV-P24-Ca. White bars in the pictures correspond to 100 μm. *(Continued)*

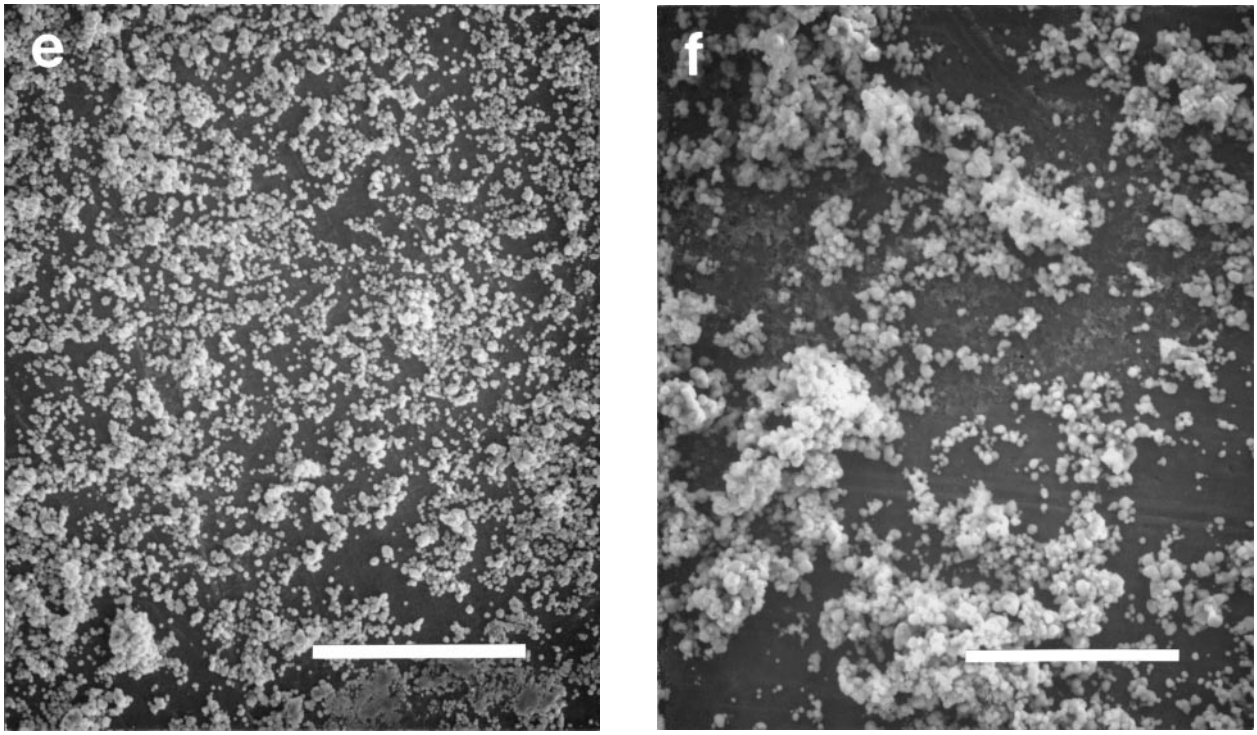


Figure 3 (Continued).

key role played by phosphoproteins in the biomineralization phenomenon [73–78]. Phosphorylated species have been described to promote mineralization when bound to a substrate, but to inhibit mineralization when in solution. In the present case, only materials phosphorylated and pre-incubated in $CaCl_2$ promoted an adequate mineralization at the surface. Phosphate ions on the surface of cellulose thus seem to act as phosphoproteins in solution. On the contrary, calcium ions bound to phosphate functionalities seem to constitute adequate nuclei for the formation of calcium phosphates, which is in accordance with previous findings from independent investigations [33, 34, 37–39]. There is some controversy concerning the capability of phosphorylated materials to induce apatite formation. Phosphorylated materials have been previously described as capable of inducing apatite formation [32–39], as well as inhibitors of this phenomenon [79–81]. This is probably related to what was previously discussed. When phosphorylated materials inhibited mineralization they were not bound to a substrate, as was the case of water soluble cellulose phosphates, or their calcium salt was not previously formed. Thus, several factors seem to be relevant so that mineralization can occur on phosphorylated materials, namely binding of phosphate groups to a substrate, and their binding to cations capable of nucleating calcium phosphates, namely calcium. This latter observation also seems to be in agreement with the most widely accepted mineralization theories, which describes mineralization as a two-step and not a merely physico-chemical process, where cells play a key role [76, 82–85]. Matrix-vesicles are responsible for providing the adequate physico-chemical environment for the formation of the first nuclei, which then grow to the extracellular matrix. The required supersaturation in calcium and phosphate ions is assured by the selective membrane. In the present case, this supersaturation seems to be assured only by the pre-incubation in $CaCl_2$. When phosphorylated materials were not pre-incubated in $CaCl_2$, probably the sodium salt was more readily formed, thus inhibiting the formation of the calcium phosphate mineral.

SEM observations on cross-sections showed that the interface between the calcium phosphate layers formed on the surface of phosphorylated materials pre-incubated in $CaCl_2$ was continuous, indicating that the mineral was integrated with the surface. Materials only phosphorylated showed a clear separation between the two materials.

The composition of the calcium phosphates formed was investigated by EDS microanalyses, which showed their apatitic nature. Resulting Ca/P ratios were characteristic of octacalcium phosphate after the initial stage in SBF, and of hydroxyapatite after 7 and 15 days.

ATR-FTIR analysis, in conjunction with previous evidences, showed the difference in the structures obtained when materials were pre-incubated in calcium. A standard hydroxyapatite was used for comparison, where its main bands could be observed. Fig. 7b, c and d show the spectra of untreated CRV and CRV-P8, and CRV-P24 immersed in SBF for 15 days (SBF15), respectively. The latter, among materials only phosphorylated, was the one exhibiting the highest mineralization extents. The comparison between the spectra of these three materials showed that calcium phosphates could be easily identified by ATR-FTIR, namely recurring to the peak at 557 cm^{-1}, which appeared as a weak band on CRV-P24 SBF15. The weak nature of this band is related to the fact that surface coverage by the calcium phosphate layer was neither homogeneous nor at a great extent. Consequently, the cellulose backbone was still perfectly evident, as could be

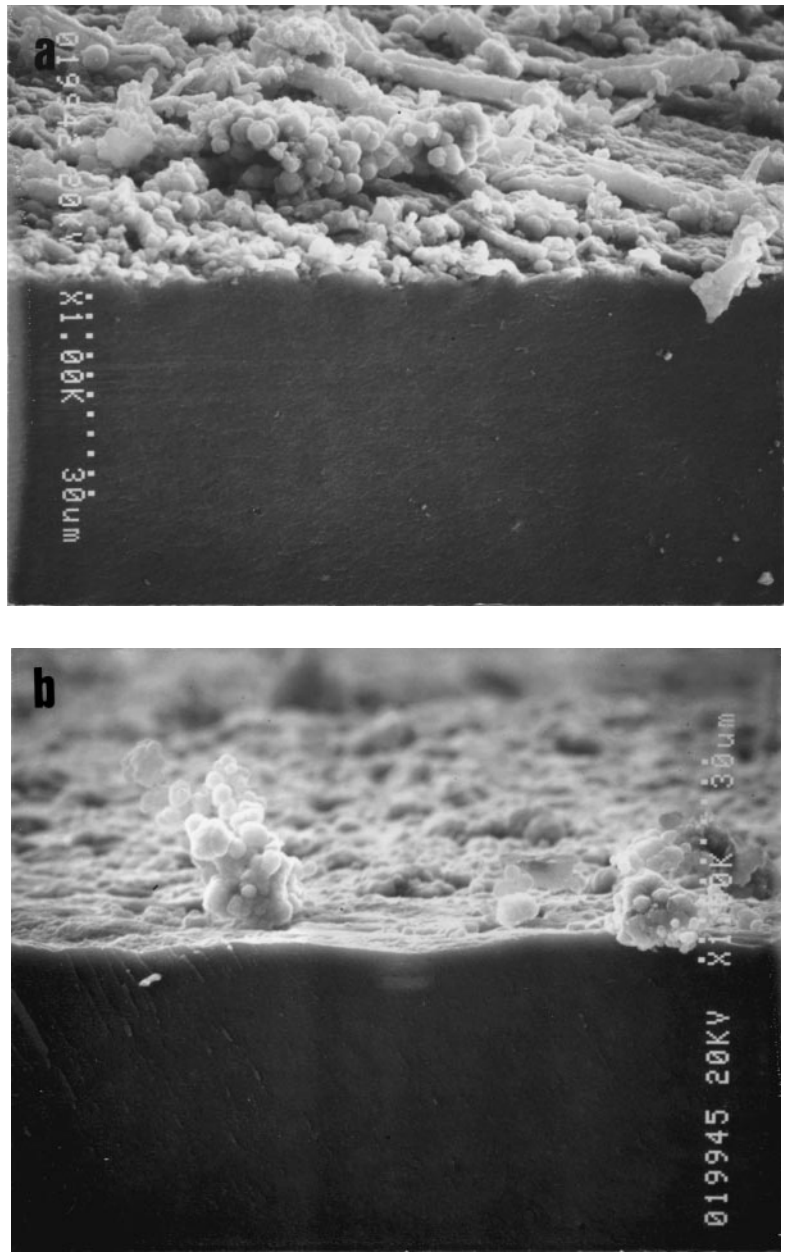
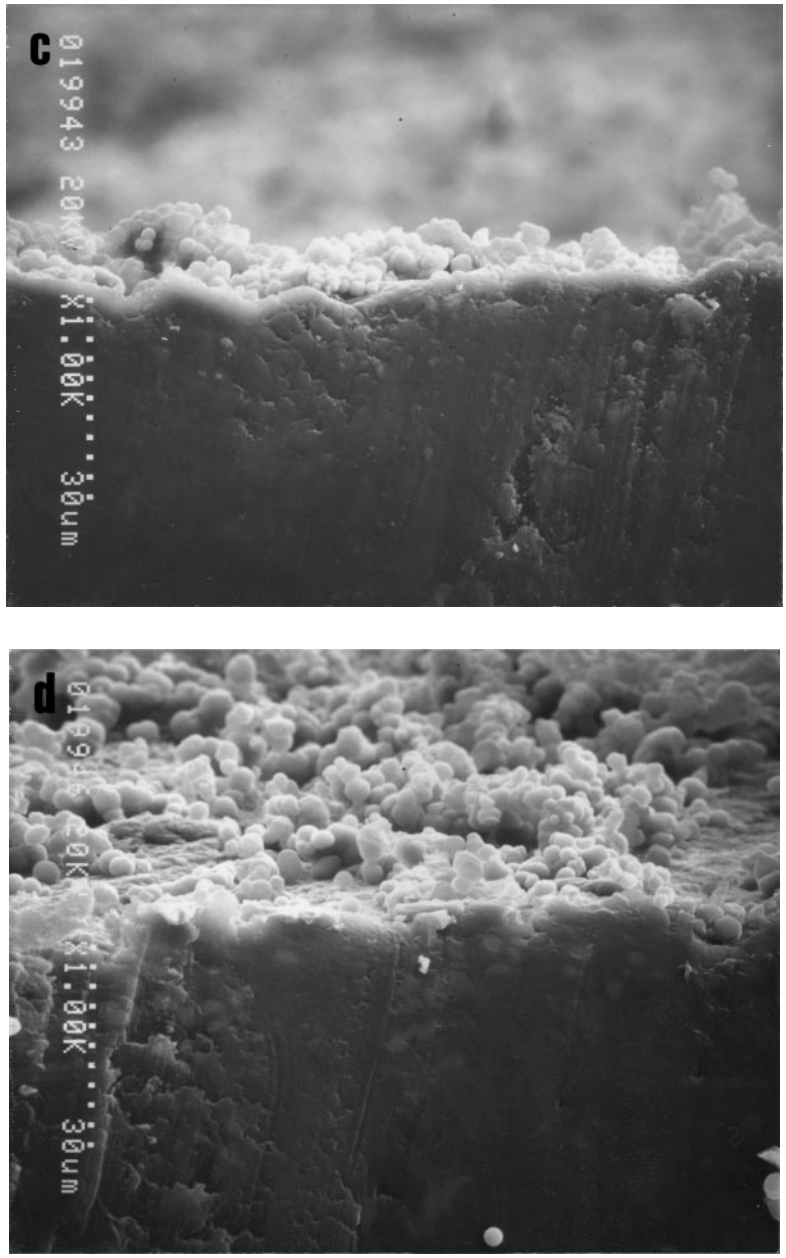


Figure 4 SEM micrographs of cross-sections showing the calcium phosphates formed on phosphorylated cellulose (CRV-P) and its calcium salts, after immersion in SBF for 15 days: a) CRV-P8, b) CRV-P24, c) CRV-P8-Ca and e) CRV-P24-Ca. White bars in the pictures correspond to 30 μm. *(Continued)*

seen in comparison with CRV and CRV-P8. Fig. 7e, f and g show the spectra of materials phosphorylated, pre-incubated in $CaCl_2$, and immersed in SBF, namely CRV-P4-Ca SBF7, CRV-P8-Ca SBF7 and CRV-P8-Ca SBF15, respectively. Major differences could be observed, in comparison with the previously discussed spectra. The cellulose backbone is now hardly distinguishable, indicating the good extent and homogeneity of mineralization on the surface. Furthermore, peak resolution was greatly enhanced, allowing the distinction of the ν_3 PO_4 band, centered at 1020 cm^{-1}, and the ν_4 PO_4 bands at 557 and 595 cm^{-1}. Untreated cellulose gives a main peak centered at 1019 cm^{-1}, which clearly interferes with ν_3 PO_4 vibration, the most prominent band of calcium phosphates. As a consequence, the analysis of calcium phosphates deposited on cellulose must be carefully performed in this region. It should be emphasized that the characterization of the calcium phosphate layers formed by X-ray diffraction was not carried out due to the irregular nature of the surface of dried materials. Regenerated cellulose hydrogels swell considerably in solution, and consequently the surface becomes irregular upon drying. Comparison of Figs. 7d and g clearly shows that, for the same immersion time in SBF (15 days), cellulose phosphates pre-incubated in $CaCl_2$ promoted a much better resolved spectrum. Furthermore, peaks at 866 cm^{-1} and 1406 cm^{-1} could also be observed, which can be attributed to the ν_2 CO_3 vibration of carbonate groups, usually found in carbonated and biological apatites [4, 60–66]. The ν_1 PO_4 peak at 962 cm^{-1}, appeared in the form of shoulder on the ν_3 PO_4, instead of the very narrow band found in hydroxyapatite, which seems to indicate the presence of a poorly crystalline apatite, resembling bone mineral [63]. During the immersion period in SBF there was a gradual intensification of absorption in the ν_1, ν_3 and



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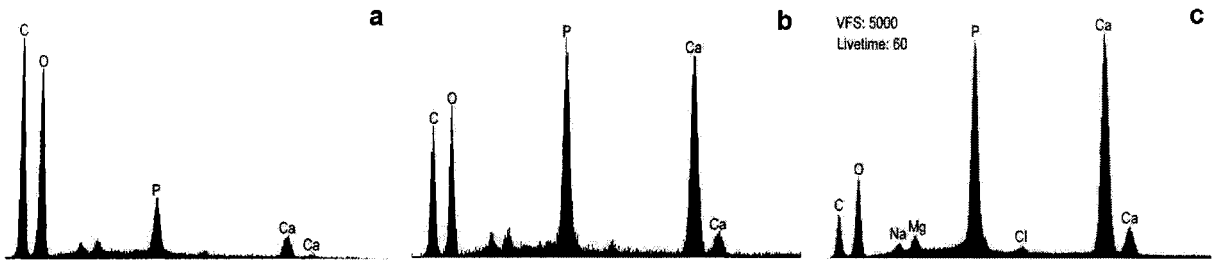

Figure 5 EDS spectra of the calcium phosphates formed on phosphorylated cellulose (CRV-P8) immersed in SBF, at varying immersion times: a) 2 days, b) 7 days and c) 15 days.

ν_4 PO_4 regions with time, indicating the change of the structure originally formed to another one, similar to hydroxyapatite. The doublets found in the ν_4 PO_4 region, indicate that the precursor phase of the calcium phosphate formed was octacalcium phosphate (OCP) and not amorphous calcium phosphate (ACP) [60, 63, 66, 72, 86]. When HAp is formed from ACP these bands are broad singlets, whereas in the case of OCP band splitting usually occurs, indicating that minerals formed were poorly crystalline but not amorphous

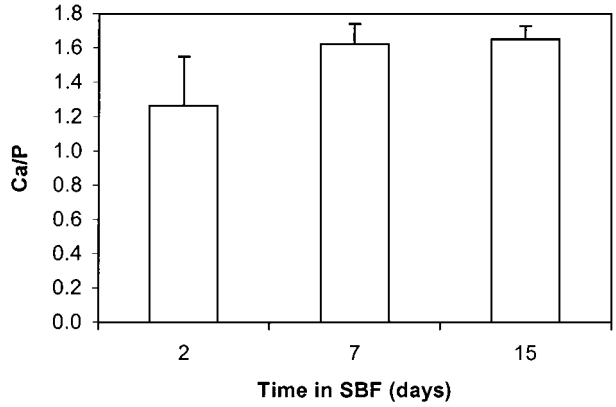

Figure 6 Ca/P ratio of the calcium phosphates formed on phosphorylated cellulose pre-incubated in $CaCl_2$ (CRV-P8-Ca) and immersed in SBF, at varying immersion times, as determined by EDS.

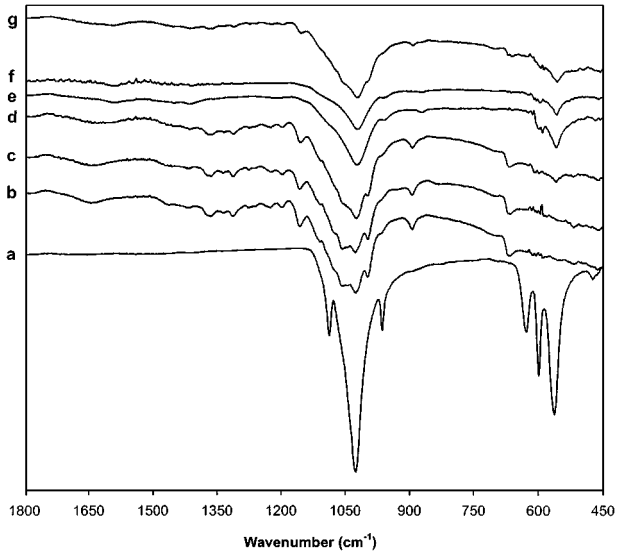

Figure 7 ATR-FTIR spectra of: a) HAp, b) CRV, c) CRV-P8, d) CRV-P24 SBF15, e) CRV-P4-Ca SBF7, f) CRV-P8-Ca SBF7, and g) CRV-P8-Ca SBF15.

[60, 63, 66, 72, 86]. ATR-FTIR results confirmed the apatitic nature of the calcium phosphates formed, similar to hydroxyapatite [4, 13, 58–66, 86–91].

5. Conclusions

Phosphorylated materials, having varying degrees of substitution, were soaked in Simulated Body Fluid (SBF) solution in order to investigate their ability to induce the formation of a calcium phosphate layer, in simulated physiological conditions. Mineralization was assessed by SEM/EDS and ATR-FTIR spectroscopy and it was demonstrated that the calcium salt of cellulose phosphates mineralized at a higher extent than materials only phosphorylated. Other independent investigations have reported similar findings, indicating that phosphate groups mineralize at a lower extent when the calcium salt is not previously formed. The degree of phosphorylation, whether the material is pre-incubated in calcium or not, influenced the extent of surface mineralization. Moderate degrees of surface phosphorylation promoted the highest extent of mineralization. This was attributed to inadequate functionality of the surface in terms of density of PO_4 groups and overall surface charge, in the case of low and high phosphate contents.

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