Mineralization of regenerated cellulose hydrogels

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Due to their high water swelling, regenerated cellulose hydrogels and sponges were pre-incubated in a Ca-containing solution, and their mineralization was investigated. Results obtained demonstrate that a simple pre-incubation treatment in a Ca containing solution can induce mineralization in materials with limited or no tendency to mineralize. The minerals formed had an apatitic carbonated and poorly crystalline structure, resembling carbonated hydroxyapatite found in bone mineral. The apatitic layer formed showed a relatively accelerated growth using this technique, exhibiting nodules in their macroscopic structure, which seem to indicate lateral growth. The porous structure of regenerated cellulose sponges was also homogeneously mineralized using this technique.
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1. Introduction

Synthetic calcium phosphates generally mineralize [1–4]. Some other synthetic biomaterials have also shown the capability of inducing the formation of an apatite layer, like glass-ceramics [5–7], silicon-based materials [8–10], titanium-based materials [11–15], and some synthetic polymers [16–19]. 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 [20–22] and the so-called biomimetic coatings [23–25] are among the most well succeeded ones. Grafting of silanol groups [26, 27] or polyethylene glycol [28, 29] showed to be adequate techniques to induce mineralization.

Grafting of phosphate groups on polymer chains is another promising approach, which has been reported in a previous work by the present authors [30]. In another work, we reported that surfaces bearing phosphate groups induce the formation of a calcium phosphate layer [31]. However, in order to obtain an homogeneous calcium phosphate layer, phosphorylated materials must firstly be pre-incubated in a calcium-containing solution. Other independent investigations have reported similar observations [32–36], indicating that phosphate groups induce mineralization to a lower extent when a calcium salt is not previously formed.

Based on our previous findings, a simpler technique has been developed, in order to promote the mineralization of polymeric materials with limited or no tendency to mineralize. Regenerated cellulose hydrogels and sponges were selected for this purpose [37–41]. Due to their high water swelling, regenerated cellulose hydrogels and sponges were pre-incubated in a calcium-containing solution and their mineralization was investigated. Scanning electron microscopy (SEM), energy dispersive spectroscopy (EDS) and attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy were used to characterize the minerals formed.

2. Materials and methods

Regenerated cellulose hydrogels (CRV®) and sponges (Cellspon®) were gently offered by Hexabio (Bordeaux, France) and Cellomeda (Turku, Finland), respectively.

2.1. In vitro mineralization studies
2.1.1. Regenerated cellulose hydrogels

Prior to mineralization assays, disks of regenerated cellulose hydrogels (10 mm diameter) were immersed for 3 days, at 37 °C, in 50 mL of a 0.05M $CaCl_2$ solution, which was renewed every 24 h. Each disk was then rinsed with deionized water and immersed in simulated body fluid (SBF) [42] solution (pH 7.4) using a material surface to solution volume ratio of ca. 1 cm^{-1}. Polyethylene screwtop flasks were used. The SBF

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solution was renewed every 24 h and kept at 37 °C in a water bath, between renewals. Periods of immersion in SBF ranged from 4 to 15 days. Before further examination, all materials were rinsed with deionized water and dried at room temperature.

2.1.2. Regenerated cellulose sponges

Prior to mineralization assays, strips (30 × 30 mm) of regenerated cellulose sponges were immersed for 24 h, at 37 °C, in 50 mL of a saturated calcium acetate solution. Each strip was then immersed in SBF solution, using the same procedure described above. Periods of immersion in SBF ranged from 1 to 4 days. Calcium acetate was used in order to check if another calcium-containing solution would promote similar results.

2.2. 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 $4 \, cm^{-1}$.

2.2.1. Control sample

As a control sample, a commercial hydroxyapatite (HAp; from CAM Implants, Merck ref. 1432) was used, previously heated at 1000 °C and cooled in the oven. 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) [43].

3. Results
3.1. Regenerated cellulose hydrogels

Regenerated cellulose hydrogels swell in water up to approximately 60 wt %, as shown in Fig. 1. Taking advantage of this property, the material was left to swell in a calcium chloride solution and the mineralization of the untreated material in SBF was assessed, in comparison with materials previously incubated in calcium chloride.

After incubation in SBF, the formation of calcium phosphates was observed on the surface of untreated regenerated cellulose hydrogels (Fig. 2), as well as on these materials previously incubated in $CaCl_2$ (Fig. 3). In the latter case, the calcium phosphate formed constituted a dense and organized layer, covering the surface homogeneously (Fig. 3). In the case of cellulose samples not previously incubated in $CaCl_2$, some agglomerates of globular calcium phosphate particles could be observed, neither homogeneously distributed nor covering the whole surface (Fig. 2).
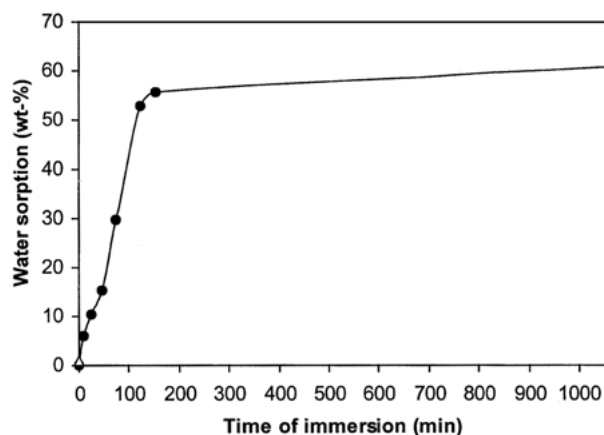
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Figure 1 Water swelling of regenerated cellulose hydrogels.

The evolution of the mineralization of materials preincubated in calcium chloride was investigated and is shown in Fig. 3. Only after 4 days in SBF, did a calcium phosphate layer already cover the surface. The upper layer of the calcium phosphate formed after 4 days (Figs. 3a and 3d) was similar to the one formed on untreated cellulose, i.e. round shaped agglomerates, randomly distributed. However, in materials pre-incubated in calcium chloride, the substrate was fully covered by the calcium phosphate. After 7 days in SBF (Figs. 3b and 3e) the structure observed was more organized and showed the presence of some nodules on the surface. After 15 days (Figs. 3c and 3f) this observation was confirmed, showing a nodular calcium phosphate structure.

The average Ca/P ratios obtained by EDS microanalysis (Figs. 4 and 5), for materials preincubated in $CaCl_2$, were in the range of the values expected for
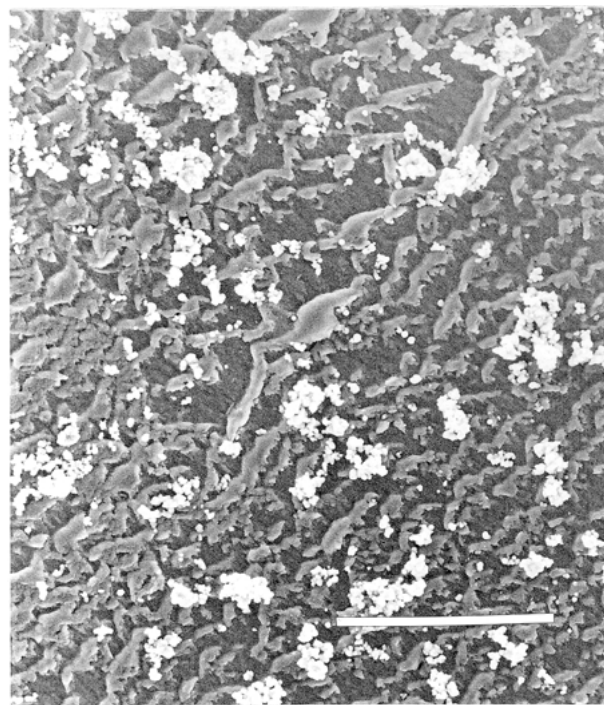

Figure 2 SEM micrographs of the calcium phosphates formed on unmodified regenerated cellulose hydrogels immersed in SBF for 15 days. The bar in the picture corresponds to 100 μm.
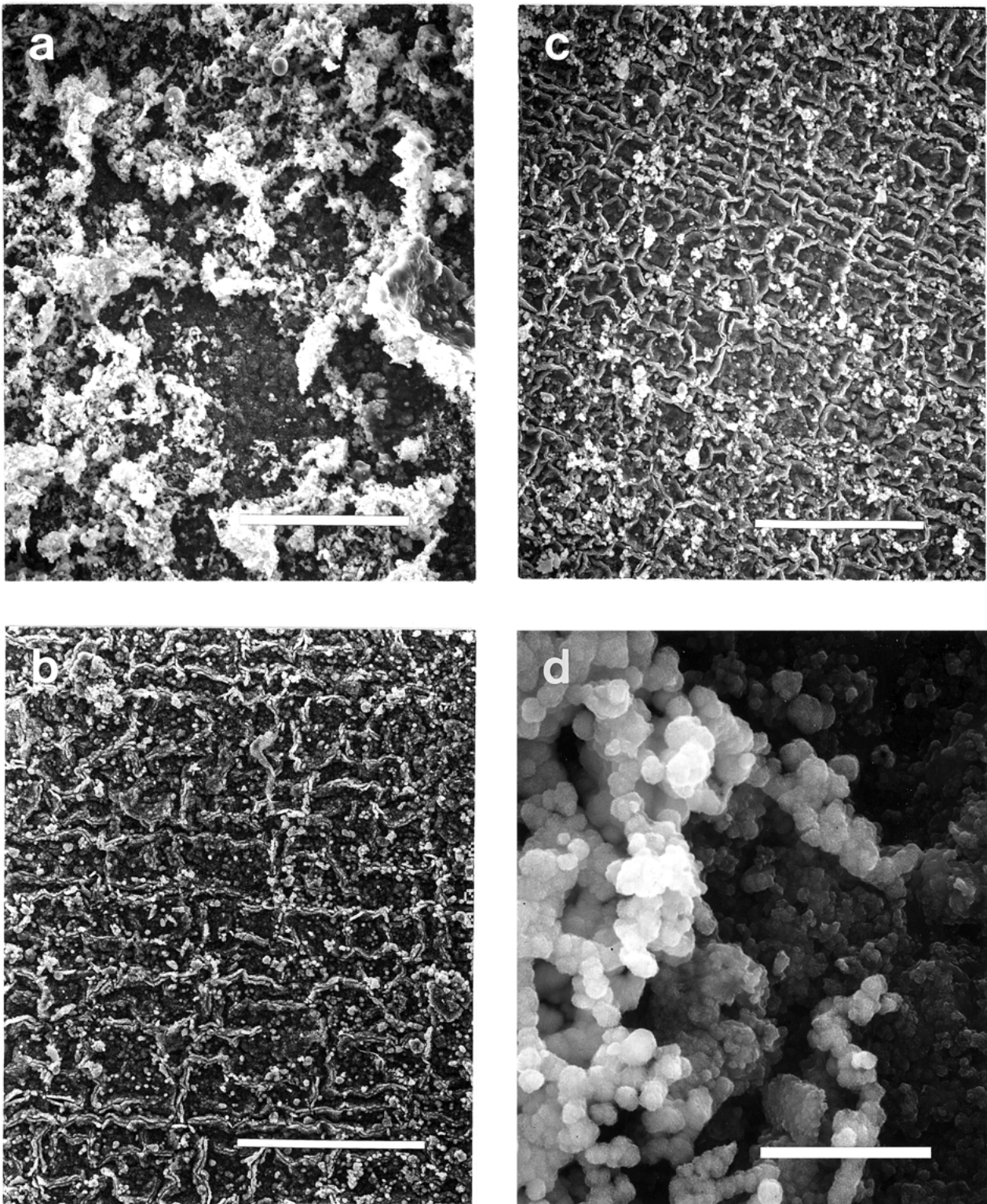
Figure 3 SEM micrographs of the calcium phosphates formed on regenerated cellulose hydrogels pre-incubated in calcium chloride and immersed in SBF for: (a) 4 days; (b) 7 days; (c) 15 days; (d) magnification of (a); (e) magnification of (b); and (f) magnification of (c). The bars in Figs. 3a to 3c correspond to $100\,\mu m$, and in Figs. 3d to 3f correspond to $10\,\mu m$.

apatites. The Ca/P ratios obtained were 1.58 ± 0.04 after 4 days, 1.62 ± 0.07 after 7 days, and 1.63 ± 0.08 after 15 days immersion in SBF.

In order to further characterize the calcium phosphates formed after immersion in SBF, ATR-FTIR surface analysis was performed. Fig. 6 shows the spectra obtained for the layers formed on the different materials, compared to a standard hydroxyapatite sample. The most

significant evidence of their apatitic nature was the appearance of the peaks at near 557 and $597\,cm^{-1}$. These peaks, which are characteristic of the ν_4 vibration of PO_4 groups in apatitic structures precipitated from solution [9, 24, 44–47], were found in the control hydroxyapatite, as well as in every material immersed in SBF, but not on untreated cellulose. Cellulose hydrogels pre-incubated in $CaCl_2$ and immersed in
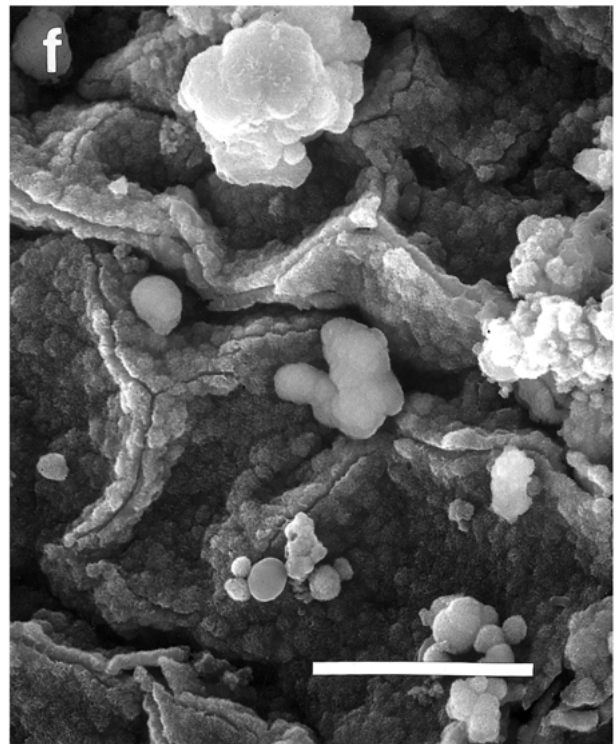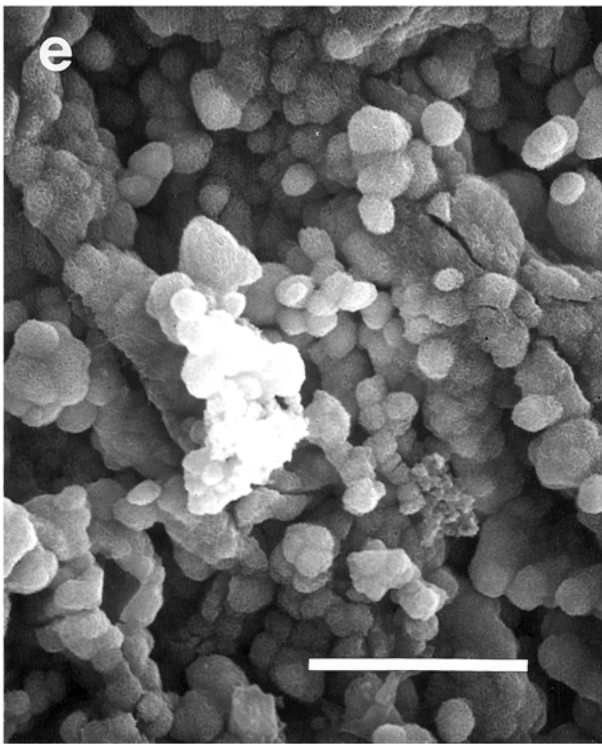
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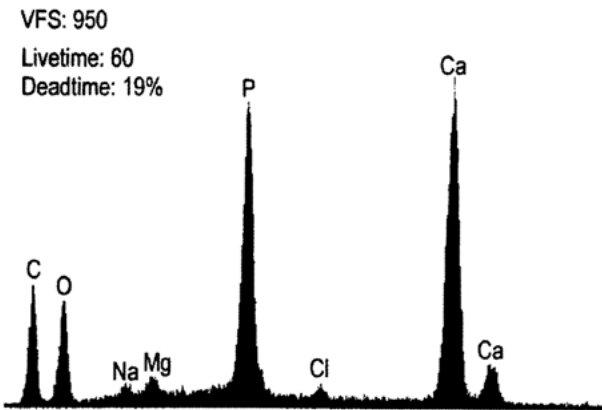*Figure 3* (Continued)

VFS: 950
Livetime: 60
Deadtime: 19%

Figure 4 EDS spectra of the calcium phosphate formed on regenerated cellulose hydrogels pre-incubated in calcium chloride and immersed in SBF for 7 days.
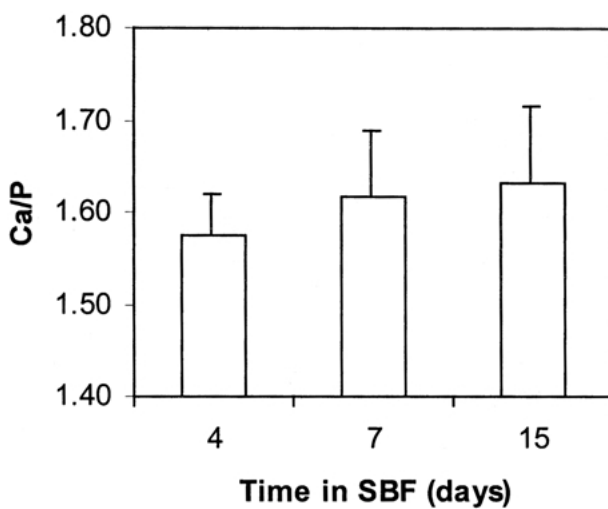

Figure 5 Ca/P ratio of the calcium phosphates formed on regenerated cellulose hydrogels pre-incubated in calcium chloride and immersed in SBF, at varying immersion times, as determined by EDS.

SBF for 15 days showed the sharpest peaks. There is also evidence of CO_3^{2-} bands, at 867 and 1407 cm^{-1}, which are typical of carbonated and biological apatites [1, 46–52].

3.2. Regenerated cellulose sponges

Regenerated cellulose sponges, pre-incubated in calcium acetate solution, mineralized after immersion in SBF for 4 days (Fig. 7). Calcium phosphates covered the whole available structure, including the inside of the pores.
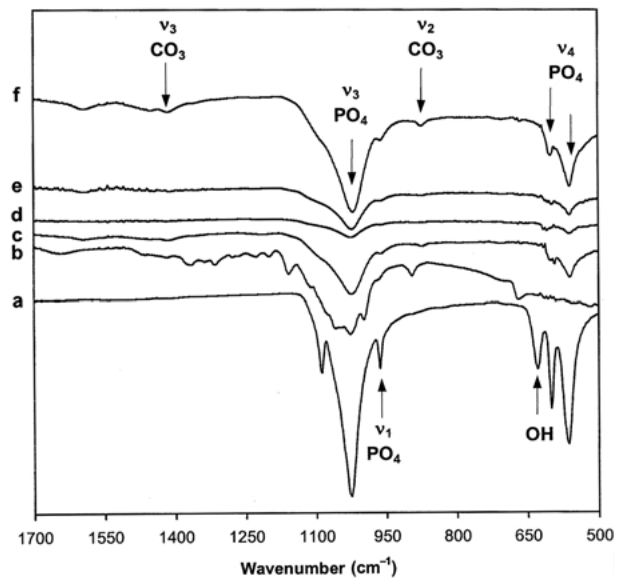

Figure 6 ATR-FTIR spectra of samples analyzed, in the 500–1700 cm^{-1} window: (a) control hydroxyapatite; (b) regenerated cellulose hydrogel; (c) regenerated cellulose immersed in SBF for 15 days; regenerated cellulose pre-incubated in calcium chloride and immersed in SBF for (d) 4 days, (e) 7 days, and (f) 15 days.

Figure 8 ATR-FTIR spectra of samples analyzed, in the 500–1700 cm^{-1} window: (a) regenerated cellulose sponge; (b) cellulose sponge pre-incubated in calcium acetate and immersed in SBF for 4 days.
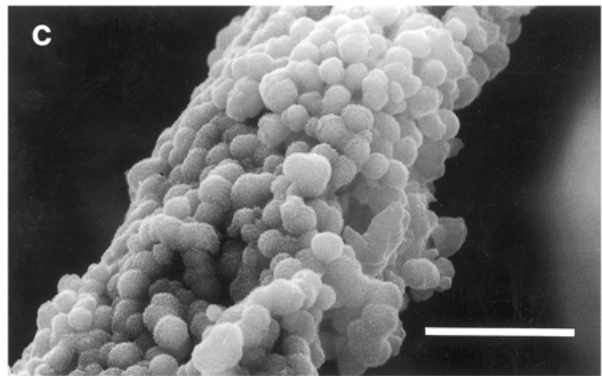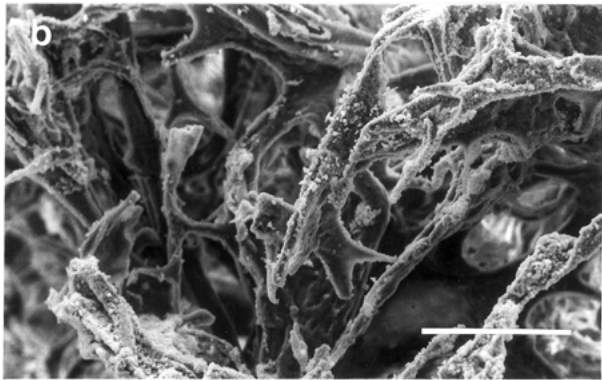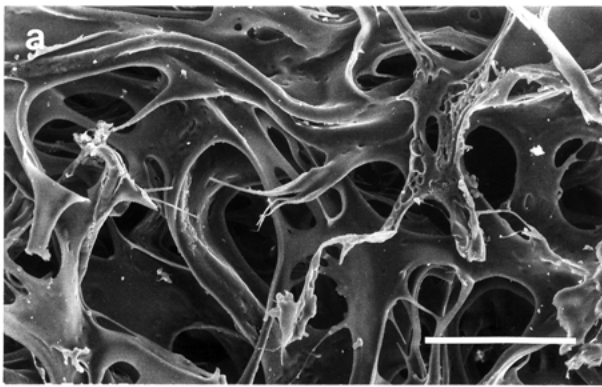
Figure 7 SEM micrographs of the mineralization of cellulose sponges. (a) original unmodified regenerated cellulose sponges; (b) cellulose sponges pre-incubated in calcium acetate and immersed in SBF for 4 days; (c) magnification of (b). The bars in Figs. 7a and 7b correspond to 100 μm, and in Fig. 7c to 10 μm.

EDS microanalyses indicated an average Ca/P ratio of 1.72.

Calcium phosphates formed on cellulose sponges pre-incubated in calcium acetate, after immersion in SBF, were also analyzed by ATR-FTIR. Fig. 8 shows spectra similar to those previously described using cellulose hydrogels. The peaks at 557 and 597 cm^{-1}, characteristic of the ν_4 vibration of PO$_4$ groups in apatitic structures, were also found in materials immersed in SBF, but not on untreated cellulose. There is also evidence of carbonate bands, at 867 and 1407 cm^{-1}.
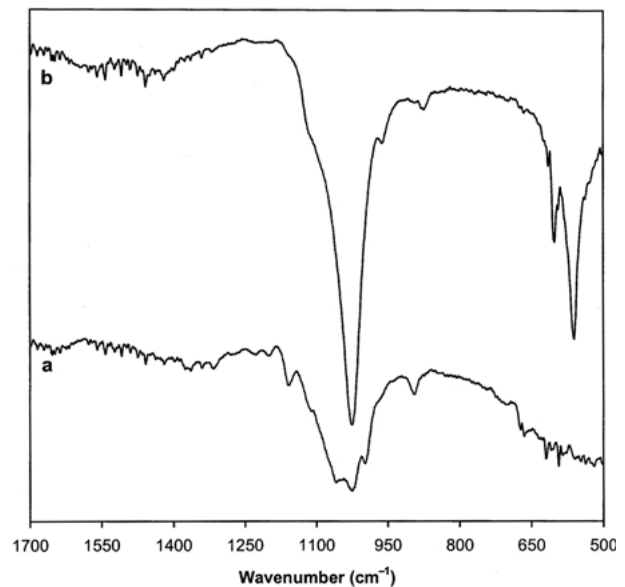
4. Discussion

Pre-incubation of regenerated cellulose hydrogels in a Ca-containing solution promoted their mineralization. Calcium may have been incorporated in the structure of cellulose hydrogels by two different mechanisms: (a) absorption – physical entrapment, when the polymer has swollen in CaCl$_2$ solution; and (b) adsorption – ionic interaction with the negatively charged OH groups, largely available on cellulose. Incorporated calcium ions can then bind phosphate ions, thus becoming nuclei for the formation of the calcium phosphate mineral.

In the case of cellulose samples not previously incubated in CaCl$_2$, some round shaped calcium phosphate particles were formed. They were randomly distributed and did not cover the surface.

The process of formation of the calcium phosphate formed on materials pre-incubated in CaCl$_2$ was accelerated. This was probably due to the existence of large amounts of calcium ions on the surface, made available by their entrapment during swelling of cellulose in CaCl$_2$. This fact seems to be responsible for the homogeneous lateral growth of the apatitic film. Mineral propagation over the substrate seemed to be hindered by adjacent mineral growing fronts, thus growing vertically upon confrontation, and forming the characteristic nodules reported above.

The Ca/P ratio of the minerals formed increased with time, indicating the presence of a precursor phase. It should be taken into consideration that values obtained by EDS are only semi-quantitative [53]. However, in conjunction with ATR-FTIR results, values obtained suggest that octacalcium phosphate was the precursor phase of hydroxyapatite [47, 48, 50, 54, 55].

ATR-FTIR analyses carried out on films formed on the different materials demonstrated their apatitic nature. A standard hydroxyapatite was used for comparison, where its main bands can be observed, namely: the ν_1 PO$_4$ peak, at 962 cm^{-1}, in the form of a very sharp band; the

ν_2 PO$_4$ peak, as a weak band at 470 cm^{-1}; the intense ν_3 PO$_4$ peaks centered at 1023 and 1086 cm^{-1}; the ν_4 PO$_4$ peaks centered at 557 and 597 cm^{-1}; and the OH peak at 626 cm^{-1}. Untreated cellulose gives a main band 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 and sponges swell considerably in solution, and consequently the surface becomes irregular upon drying.

Every material pre-incubated in a calcium-containing solution and immersed in SBF showed their ability to induce the formation of calcium phosphates. ATR-FTIR analyses, in conjunction with previous evidence from SEM/EDS, showed the difference in the structures obtained when materials were pre-incubated in CaCl$_2$. Figs. 6c and 6e show the spectra of untreated cellulose immersed in SBF for 15 days and cellulose pre-incubated in CaCl$_2$ and immersed in SBF for 7 days, respectively. In both spectra, weak peaks of the ν_1 PO$_4$ can be observed, as well as main peaks resembling the ν_3 PO$_4$ vibration and peaks centered at 555 cm^{-1}, typical of the ν_4 PO$_4$ vibration. However, in Fig. 6f, the spectrum of cellulose preincubated in calcium and immersed in SBF for 15 days showed significant differences: the ν_1 PO$_4$ vibration was better defined; the ν_3 PO$_4$ vibration was more pronounced; and the ν_4 PO$_4$ vibration showed a doublet at 557 and 597 cm^{-1}. Comparison of Figs. 6c and 6f clearly shows that, for the same immersion time in SBF (15 days), cellulose pre-incubated in calcium promoted a much better resolved spectrum. Furthermore, peaks at 866 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 [1, 46–52]. A closer inspection of the previous spectra allowed to identify those carbonate peaks also. The ν_1 PO$_4$ peak at 962 cm^{-1}, appeared in the form of a shoulder on the ν_3 PO$_4$, instead of the very sharp band found in hydroxyapatite, which seems to indicate the presence of a poorly crystalline apatite, resembling bone mineral [50]. During the immersion period in SBF (from 4 to 15 days) there was a gradual intensification of absorption in the ν_1, ν_3 and 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 since the early stages (Fig. 6d), indicate that the precursor phase of the calcium phosphate formed was octacalcium phosphate (OCP) and not amorphous calcium phosphate (ACP) [47, 48, 50, 54, 55]. Minerals formed were poorly crystalline but not amorphous, due to the presence of band splitting, whereas in ACP these bands are broad singlets [47, 48, 50, 54, 55]. ATR-FTIR results allowed to identify the calcium phosphates formed as apatitic structures, similar to hydroxyapatite [1, 9, 24, 44–52, 54, 56–60]. These results also showed the relevance of the pre-incubation in calcium in order to achieve the formation of calcium phosphates from simulated plasma solutions.

Regenerated cellulose sponges are promising materials for biomedical applications [40, 41]. Their structural similarity with the hydrogels described here allowed us to confirm the use of the pre-incubation in calcium procedure for inducing the mineralization of a porous material. After 4 days incubation in SBF, cellulose sponges pre-incubated in calcium acetate were homogeneously mineralized (Fig. 7), even inside the pores. Calcium acetate was used in order to check if another calcium-containing solution would promote similar results. It was demonstrated that, independently of the calcium solution used, pre-incubation of high swelling materials in a Ca-containing solution is effective in promoting their mineralization, in a relatively accelerated and homogeneous way.

5. Conclusions

Regenerated cellulose hydrogels have some promising properties for use in orthopedic applications but they lack osteoinduction. This disadvantage can be partly overcome by a simple pre-incubation treatment in a Ca-containing solution, since materials with limited or no tendency to mineralize can be transformed into mineralizing ones. In addition to the fixation of calcium ions by ionic interactions with the OH groups from cellulose, the high swelling ability of these hydrogels allowed the anchoring of calcium ions by physical entrapment. Calcium ions can then bind phosphate ions, which then nucleate a calcium phosphate mineral. The minerals formed had an apatitic carbonated and poorly crystalline structure, resembling carbonated hydroxyapatite found in bone mineral. There was some evidence that octacalcium phosphate occurred as the precursor phase of hydroxyapatite. The apatitic layer formed showed a relatively accelerated growth using this technique, exhibiting nodules in their macroscopic structure, which seem to indicate lateral growth. The porous structure of regenerated cellulose sponges was also homogeneously mineralized using this technique.

It is possible to envisage the application of this simple treatment to other high-swelling non-bioactive candidates for orthopedic applications, as the means to promote their mineralization.

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