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Biological control of apatite growth in simulated body fluid and human blood serum

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Abstract The surface transformation reactions of bioactive ceramics were studied in vitro in standard K9-SBF solution and in human blood serum (HBS)—containing simulated body fluid (SBF). The calcium phosphate ceramics used for this study were stoichiometric hydroxyapatite (HA), β-tricalcium phosphate (β-TCP) and brushite. Immersion of each calcium phosphate tested in this study, in simulated body fluid, led to immediate surface precipitation of apatite. The use of HBS resulted in a delay in the onset of precipitation and a significant inhibition of the dissolution reaction normally observed for brushite in solution. However, apatite formation still occurred. The use of HBS and SBF in this investigation, which has shown the ability to induce similar crystal growth as that observed in vivo, suggests that there is scope for the use of serum proteins in simulated body fluid in order to create a protein-rich surface coating on biomedical substrates.

1 Introduction

The use of simulated body fluid (SBF), a solution with ion concentrations and pH similar to that of human blood plasma, for the precipitation of bioactive calcium phosphate (Ca-P) has attracted extensive research interest since its first use by Kokubo et al. [1] in 1990. The apatite which forms in such a solution is similar to biological mineral, hence its suitability in in vitro work. However, the presence of proteins in blood are considered to be important in establishing the acceptance or rejection of an implant when placed in vivo [2]. When an implant is placed into the body, proteins immediately become adsorbed on the surface of the material [3], which gives an indication of the clinical success of an implant in the body. Simulated body fluid mimics the ion levels found in human blood, but does not include proteins (Table 1). Various groups have attempted to alter the ionic composition of SBF to create a solution with a higher carbonate content more similar to the levels in human blood serum, and to observe their effect on calcium phosphate crystallization [4]. Oyane et al. [5] produced a solution known as I-SBF, which simulated the influence of proteins by including the free ions which are not bound to the proteins. However, to give a more accurate view of the effect of proteins on dissolution and crystallisation processes, these compounds would need to be present in the testing solution.

Brushite, tricalcium phosphate and hydroxyapatite are currently among the calcium phosphates used in vivo for bone replacement. Observing the effect of SBF and HBS on bone-like apatite formation in vitro will allow for a better understanding of the surface modifications that can take place during in vivo apatite formation. Human blood serum contains many proteins, the most abundant of which are fibronectin and albumin. These are the first proteins that come into contact with an implant placed in the body before the attachment and effect of more specific extracellular proteins involved in bone apatite formation, such as osteonectin, osteocalcin, bone sialoprotein and osteopontin. In nature, these extracellular proteins are necessary for the subsequent growth, orientation and organisation of

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Table 1 Ion concentrations of different types of simulated body fluid

Ion	Concentration/mM			
	Blood plasma [4, 5]	S-SBF [1]	R-SBF [4, 5]	I-SBF [5]
Na^+	142.0	142.0	142.0	142.0
K^+	5.0	5.0	5.0	5.0
Mg^{2+}	1.5	1.5	1.5	1.0
Ca^{2+}	2.5	2.5	2.5	1.6
Cl^-	103.0	187.8	103.0	103.0
HCO^{3-}	27.0	4.2	27.0	27.0
HPO_4^{2-}	1.0	1.0	1.0	1.0
SO_4^{2-}	0.5	0.5	0.5	0.5
Buffer	–	6.063[a]	11.928[b]	11.928[b]

[a] 50 mM trishydroxymethylaminomethane ($NH_2C(CH_2OH)_3$) (g/L)

[b] HEPES 2-(4-hydroxyethyl)-1-piperazinyl)ethane surfonic acid (g/L)

these mineral crystals [6]. These adsorbed proteins can also encourage cell adhesion [7] and therefore control the interactions that take place during bone formation.

The aim of this article is to study the influence of proteins present in human blood serum on calcium phosphate crystallisation. Brushite, tricalcium phosphate and hydroxyapatite have been used in this investigation in order to use a range of calcium phosphates with different Ca/P ratios. Simulated body fluid has been used as the protein-free environment in vitro and human blood serum as the natural solution that simulated body fluid is mimicking, enabling the effect of proteins to be observed.

2 Experimental

Ceramic discs of brushite, β-tricalcium phosphate (β-TCP) and hydroxyapatite (HA) were produced. Brushite was prepared by the dropwise addition of 1.0 M calcium nitrate ($Ca(NO_3)_2{\cdot}4H_2O$) to 0.375 M diammonium hydrogen orthophosphate ($(NH_4)_2HPO_4$) at a Ca:P molar ratio of 1.0. Tricalcium phosphate (TCP) was prepared via an aqueous precipitation reaction [8] by the addition of phosphoric acid (H_3PO_4) to calcium hydroxide ($Ca(OH)_2$). HA was also prepared by an aqueous precipitation reaction but the orthophosphoric acid, H_3PO_4, was added dropwise to an agitated suspension of calcium hydroxide, $Ca(OH)_2$ in water, as described by Akao et al. [9] and Jarcho et al. [10].

Calcium phosphate discs of 5 mm diameter and 1 mm thickness were pressed and then sintered at 1,000 and 1,200 °C for tricalcium phosphate and hydroxyapatite, respectively. Brushite samples were compacted but did not require sintering for SBF testing. Samples were soaked in 2 mL of SBF [1] and human blood serum (HBS) at 36.5 °C. The HBS was supplied by Sigma–Aldrich and was type male, rhesus positive, AO blood. The SBF was prepared by dissolving reagent-grade NaCl, $NaHCO_3$, $K_2PO_4 \cdot 2H_2O$, $MgCl_2 \cdot 6H_2O$, and $CaCl_2$ in ion-exchanged water in a polyethylene bottle and buffered at pH 7.4 with 50 mM trishydroxymethyl aminomethane ($(CH_2OH)_3CNH_2$) and 45 mM HCl. All solutions were filtered through 0.22 µm Millipore filters before use. After soaking, the samples were removed from the solutions, washed with distilled water to remove excess solution, and then allowed to dry in air.

The microstructures and crystal phases of the surfaces of the calcium phosphate substrates were analysed using scanning electron microscopy (SEM;Philips) and X-ray diffractometry (XRD) (Siemens D-5000, θ-2θ). Samples were coated for 3 min with carbon and during analysis, an accelerating voltage of 10 kV and a working distance of 10 mm were used. During XRD analysis a step size of 0.02° was used, taking 5 min at each 2θ angle of 3–42°. FTIR spectra were also obtained at 8 cm^{-1} resolution, operating from 650 to 4,000 cm^{-1}, averaging 128 scans on a Digilab FT7000 Spectrometer.

3 Results

The XRD patterns of the brushite, TCP and HA compacts are shown in Fig. 1. The phase composition of all three were found to be pure (compared to ICDD (JCPDS) standard patterns) with no secondary phase formation such as TCP or CaO in the HA trace.

The results in Fig. 2 show the effect of placing brushite in SBF. With increasing immersion time, there was a transformation of brushite to octacalcium phosphate on the surface of the brushite samples. After only 1 day in SBF, the 100% relative intensity peak for hydroxyapatite was also apparent in the XRD trace.

Soaking brushite samples in HBS for periods of up to 28 days showed a less dramatic transformation into OCP at the surface (Fig. 3). However, there seemed to be a faster

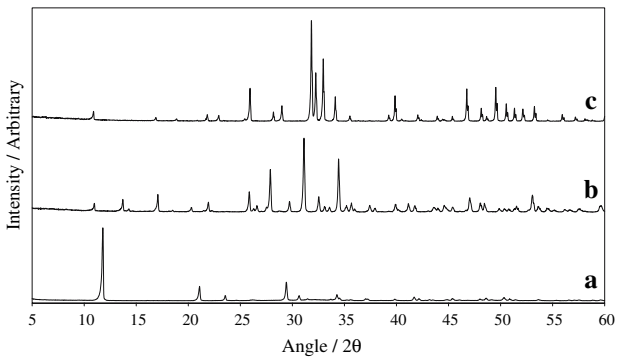

Fig. 1 X-ray diffraction patterns for brushite (**a**), TCP (**b**) and HA (**c**)

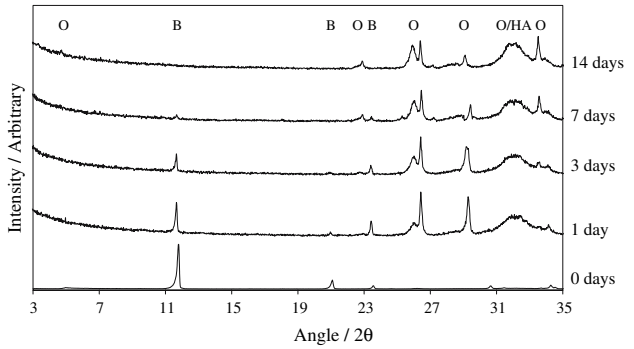

Fig. 2 X-ray diffraction patterns for brushite immersed in SBF for 0, 1, 3, 7, and 14 days. The presence of brushite, octacalcium phosphate and hydroxyapatite peaks are indicated by B, O, and HA, respectively

transformation to a hydroxyapatite structure. The immersion of TCP in both SBF and HBS indicated the presence of HA peaks in the XRD data after 7 days immersion (data not shown). However, the SBF immersed samples resulted in more pronounced apatite peaks which were produced more rapidly. When HA was placed in SBF and HBS, no significant change was observed in the XRD traces with increasing immersion time (data not shown).

Comparing the FTIR spectra of brushite before and after soaking in SBF and HBS (Fig. 4) demonstrated the presence of carbonate groups, indicating the formation of a carbonated apatite at the surface. In both solutions, there appeared to be an increase in the v_4 stretching mode of phosphate at the surface.

FTIR analysis of the TCP samples showed the formation of carbonate bands for the samples soaked in the SBF. This was not seen for the samples soaked in HBS. However, there was an increase in the peaks between 900 and 1,200 cm^{-1}, indicating an increase in intensity of the phosphate groups (data not shown).

Figure 5 shows the FTIR spectra of HA immersed in SBF and HBS. The time points shown are an indication of when the bands for carbonate became apparent in each solution. The characteristic sharp peaks attributed to the

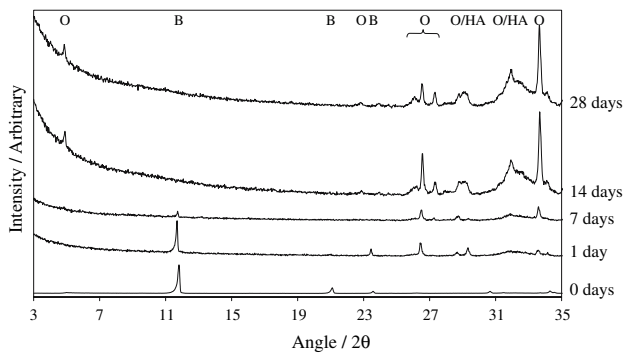

Fig. 3 X-ray diffraction patterns for brushite immersed in HBS for 0, 1, 7, 14 and 28 days. The presence of brushite, octacalcium phosphate and hydroxyapatite peaks are indicated by B, O, and HA, respectively

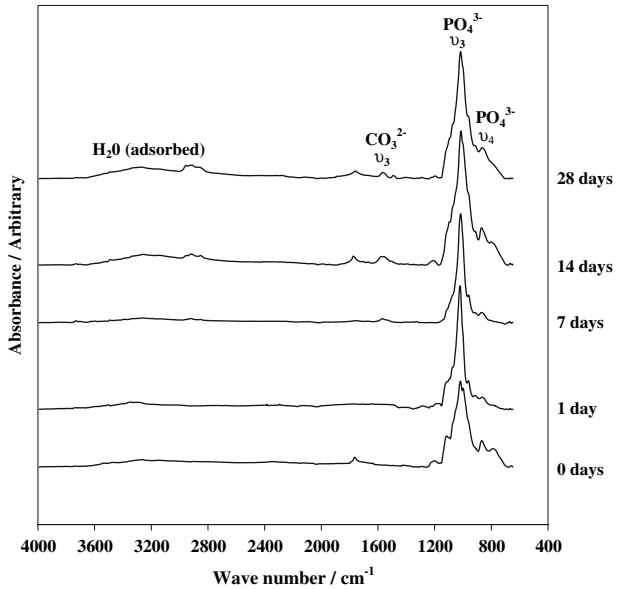

Fig. 4 FTIR data for brushite immersed in HBS for 0, 1, 7 14 and 28 days

vibration of phosphate were seen in all traces increasing with increasing immersion time. Peaks for OH^- groups and adsorbed water at the surface were also noted.

Scanning electron microscopy (SEM) allowed observation of the deposits on the surfaces of the calcium phosphates in this investigation. The phase transformation of brushite into OCP and HA was confirmed by the presence of these precipitated structures on the surface of brushite (Fig. 6). In SBF, the OCP structure (plate-like structure) was more pronounced even up to 14 days immersion. Whereas in HBS, small apatitic nuclei had formed which were characteristic of hydroxyapatite (carbonated in this case as shown by FTIR).

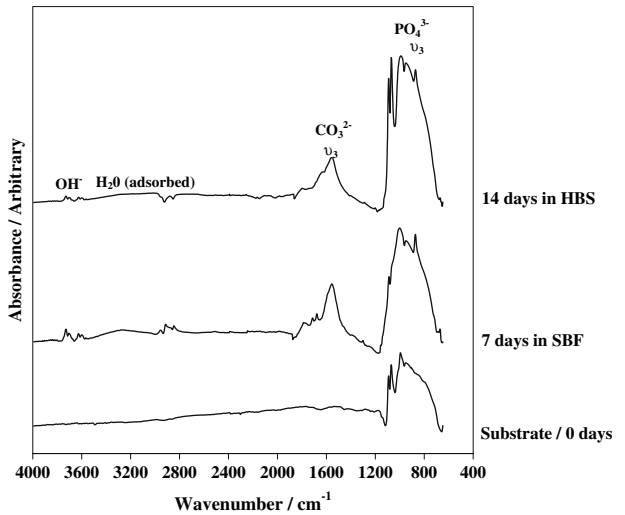

Fig. 5 FTIR data for HA in SBF and HBS for 7 and 14 days, respectively

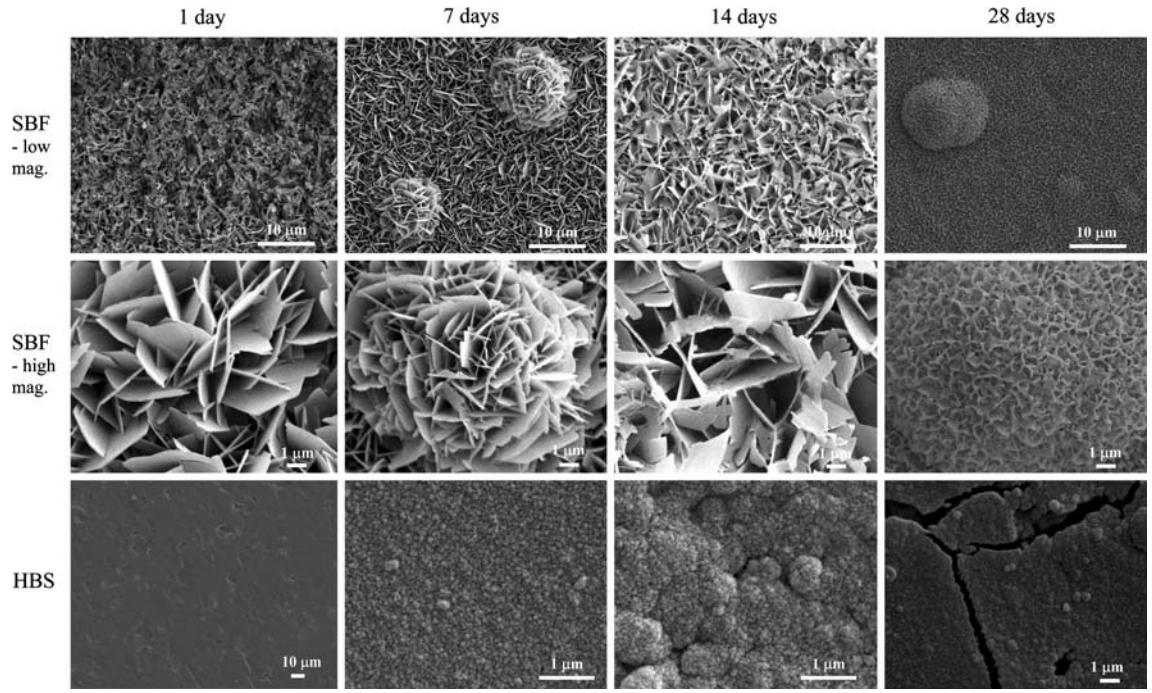

Fig. 6 Scanning electron microscopy images of apatite formation on brushite samples in both SBF and HBS (high and low magnification images) after 1, 7, 14 and 28 days immersion

When TCP was placed in SBF, there was slight dissolution at the surface and edges of individual grains of TCP, as well as the formation of small apatite crystals (Fig. 7). Most of the apatite crystals remained sub-micron sized, whereas others grew into larger apatite clusters. In HBS, TCP did not undergo such a dissolution process, and no sub-micron sized apatite crystals were observed. However, apatitic clusters did occur as in SBF. Figure 7 also shows the structural changes which took place on the surface of HA samples.

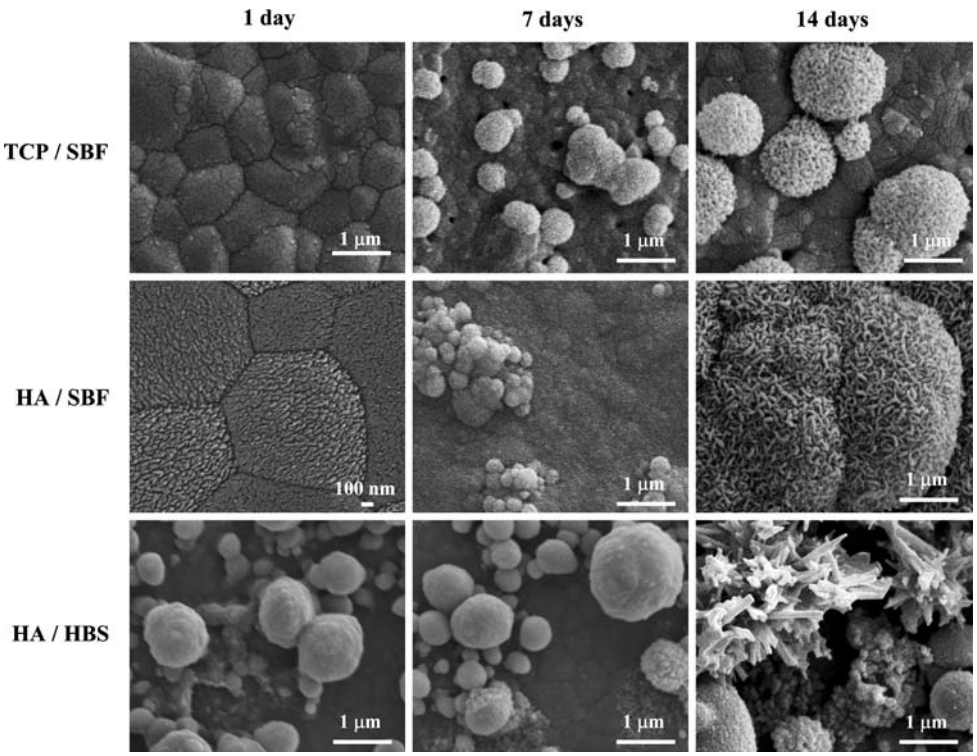

Fig. 7 Scanning electron microscopy images of apatite growth on the surface of TCP when soaked in SBF and HA when soaked in SBF and HBS for 1, 7 and 14 days

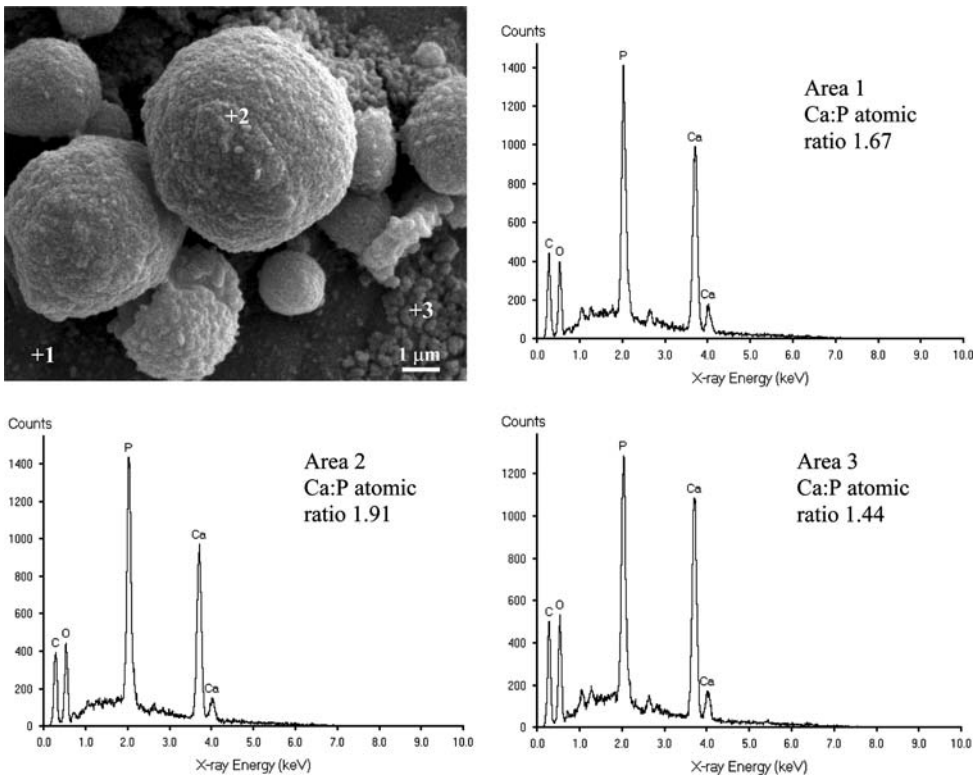

Fig. 8 Scanning electron microscopy image and EDX traces of HA soaked in HBS for 1 day

In SBF, dissolution of the grains of HA was observed (high resolution image at 1 day), and then the formation of a 'standard' apatitic structure which gradually covered the entire surface. In HBS, as well as sub-micron sized apatite crystallisation, hollow spheres of what appeared to be apatite, were observed. By 14 days, needle-like structures were also observed. Figure 8 demonstrates that the hollow spheres were indeed apatitic, with a high Ca:P atomic ratio of 1.91. EDX analysis showed the needle-like structures to also be rich in calcium and phosphate ions, with an equally high Ca:P atomic ratio. The sub-micron sized structures had a lower phosphate content, resulting in a lower Ca:P atomic ratio.

4 Discussion

Soaking brushite in SBF and HBS caused the precipitation of both octacalcium phosphate ($Ca_8(HPO_4)_2(PO_4)_4 \cdot 5H_2O$, OCP) and a hydroxyapatite-like phase ($Ca_{10}(OH)_2(PO_4)_6$, HA). The formation of these calcium phosphates was more pronounced in SBF than HBS. It is proposed that the reduction in precipitation rate was due to the presence of serum proteins which blocked dissolution sites at the surface, and which may interfere with the integration process [11].

Kokubo's research group demonstrated that the surface precipitation of a HCA layer also occurred on dense stoichiometric HA [12] as seen in the present investigation, although their study was predominantly applied to the development of glass-ceramic apatite-wollastonite. However, the mechanism by which this HCA layer formed on the surface of HA is not clearly understood. One hypothesis is the partial dissolution of calcium and phosphate ions from the apatite, which combine with ions from the SBF solution. This is believed to lead to the creation of local areas of supersaturation that can promote the nucleation and precipitation of the HCA crystals [13] and which has been observed in the present results.

HA, compared to other calcium phosphates, is considered to be the most thermodynamically stable in the physiological environment. In vivo, brushite is a precursor of HA as is OCP, hence the mechanism of bone mineral formation progresses via the transformation from the least kinetically stable form [14, 15]. Other groups have reported the formation of HA in SBF, however, as has been observed in this study, OCP formation in similar environments has also been reported [16]. It is thought that certain apatite forms in other published works are also composed of OCP and not only HA [17]. There is a higher thermodynamic driving force for HA precipitation in SBF as compared with OCP or brushite. However, OCP precipitation is kinetically favourable in SBF, hence the increased likelihood of its formation in other studies with this solution. When all three types of calcium phosphate were placed into the protein-rich HBS, the formation of apatite occurred, but resulted in precipitates with different

morphologies, due to the presence of organic and biological compounds. Parts of these compounds are in solution, and others, in the solid state, but most have an influence on calcium phosphate crystallisation [18, 19]. Certain compounds, if dissolved, will act as crystal growth inhibitors. If these same compounds are immobilised at the surface, they can act as crystal nucleators [20–22]. For crystallisation, this dual effect may be caused by the influence of the biological compounds on the crystal nucleation process. Homogeneous nucleation from a solution, of calcium phosphates is inhibited by the Ca-chelating properties of the biological compounds, which cause a decrease in the supersaturation state of the solution, hence preventing crystal formation. However, heterogeneous nucleation, which occurs at the surface, may be enhanced by the adsorbed biological molecules which can either change the surface charge on the substrate, or create necessary nucleation sites on the surface, allowing crystals to form. The presence of charged groups results in characteristics which make them potentially good mediators of interactions with the charged crystal surfaces [23]. Hence, the surface charge of the substrate prior to the adsorption of molecules will affect how these biological compounds change the surface of the material. This explains the difference in behaviour of the three calcium phosphates in HBS, all of which have very different crystal structures prior to exposure to the solutions.

The presence of proteins slowed down the transformation of the structures on the calcium phosphates examined in this investigation. However, as EDX and XRD analyses demonstrated, the formation of an intermediate phase such as OCP, occurred in both SBF and HBS, which is normal in solutions that are supersaturated with respect to this calcium phosphate phase [24]. The FTIR results indicated that as OCP formed and as it transformed into carbonated HA, the peaks ascribed to the PO_4^{3-} ions increased resulting in an increase in the Ca:P atomic ratio. Therefore, FTIR analysis confirmed that in both solutions OCP gradually transformed into carbonated hydroxyapatite with extended immersion time. Hence, with increased immersion time, the eventual transformation into a kinetically more stable structure would take place. This also occurred when the hollow, spherulitic structures formed on the surface of HA when placed in HBS (Fig. 8). It is thought that air bubbles trapped in the serum caused preferential apatite growth, most probably induced by the compounds in the serum, around these structures.

Protein layers at the surface are known to enhance cell adhesion, which would aid the mineralisation process [25]. The growth layer prepared in vitro in the presence of HBS has a close resemblance to natural bone mineral, i.e. is composed of carbonated apatite and OCP, and has low crystallinity, as demonstrated by the XRD data, than the SBF samples. Further investigations are needed to observe whether HBS could be added to SBF in order to mimic the reaction observed in HBS and in vivo and potentially, create a protein-coated material.

5 Conclusions

HBS caused a delay in carbonated apatite formation, reducing the amount of dissolution that could take place at the surface of brushite, TCP and HA. The use of HBS does however, mimic the crystallisation process that takes place in vivo. Hence, by using SBF with additions of HBS, the influence of proteins can be assessed and provide a more accurate in vitro model.

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