In vitro crystallization of octacalcium phosphate on type I collagen: influence of serum albumin

C. COMBES, C. REY, M. FRECHE
INPT-ENSCT, Laboratoire des Matériaux, Equipe Physico-Chimie des Phosphates, UPRESA 5071, 38 rue des 36 Ponts, 31400 Toulouse, France

The heterogeneous crystallization of octacalcium phosphate (OCP, $Ca_8H_2(PO_4)_6 \cdot 5H_2O$) on demineralized Type I collagen has been studied from metastable supersaturated solutions, at 37 °C and pH = 6.50, using the constant composition crystal growth technique. The induction period, before OCP crystal growth, varied markedly with the degree of supersaturation of the solution. The data obtained allowed us to determine the apparent order for the precipitation and the growth mechanism of OCP on Type I collagen. Infrared spectroscopy analyses indicated the progressive mineralization of collagen and observations by scanning electron microscopy confirmed the development of OCP crystals on the collagen surface. The influence of bovine serum albumin on both the kinetics of OCP nucleation and growth has also been investigated. Because this protein was adsorbed on calcium phosphate nuclei, it exhibited two distinct effects as a function of its concentration in solution. We proposed a mechanism explaining the interaction between albumin and calcium phosphate nuclei or crystals and its incidence on the OCP crystallization kinetics. Observations by scanning electron microscopy revealed a modification of the size and the appearance of crystals grown on collagen due to the adsorption of albumin on the crystal surface.

1. Introduction

The calcification of implant materials has wide medical implications in orthopaedic, dental and cardiovascular surgery. In orthopaedic and dental surgery, it is generally recognized that apatite formation on implant surfaces is an essential requirement for osseointegration. In contrast, failure of bioprosthetic heart valves is often also associated with the formation of calcified deposits [1, 2]. In general, vascular deposits of calcium phosphate salts are chemically and crystallographically similar to bone mineral and their formation has been postulated to result from the hydrolysis of octacalcium phosphate (OCP, $Ca_8H_2(PO_4)_6 \cdot 5H_2O$) [3, 4].

There is no doubt that collagen plays a very important role in biological mineralization but many non-collagenous proteins, such as phosphoproteins, albumin, sialoproteins, etc., are also involved in this complex phenomenon and can either contribute to the initiation or, on the contrary, to the inhibition of calcification [5–8]. Because of its biological properties and availability, Type I collagen is included in some biomaterial formulations. Up to now, the real origin of normal or pathologic calcification has remained unknown but the implantation of collagen-based biomaterials requires the knowledge to control their ability to mineralize [9]. In order to understand the processes and the mechanisms involved in the precipitation of biominerals further, in the first part of this work, we studied the crystallization of OCP on a demineralized bovine Type I collagen at pH = 6.50 and temperature, *T*, equal to 37 °C. We used the constant composition crystal growth method with supersaturated solutions at various relative supersaturation ratios in order to elucidate the kinetics and the mechanism of OCP crystallization on the organic matrix. We chose to work at a slightly acidic pH to favour the precipitation of OCP that has been reported to be a precursor of biological apatite during bone and tooth mineralization [10–12], even though this compound has never been identified by X-ray diffraction (XRD) in bone fractions [13].

In the second part of this work, we present results concerning the influence of bovine serum albumin (BSA) at various concentrations on the kinetics of nucleation and growth of OCP. Albumin is a major component of blood – in human blood typical concentrations are about 50 g L^{-1} [14] – and one of the most important non-collagenous proteins of bone. Several authors have studied the adsorption of this protein on OCP and hydroxyapatite [15–17] and pointed out the high affinity of serum albumin for calcium phosphate surfaces. Furthermore, the inhibitory effect of adsorbed albumin on calcium phosphate crystallization has been demonstrated [18–20]. It may then be an essential factor in controlling the development of many biomineralization processes.

Scanning electron microscopy (SEM) allowed us to observe the evolution of calcium phosphate crystal morphology during the nucleation and growth stages in the presence or in the absence of BSA. Infrared (I.R.) spectroscopy analyses were performed to follow the mineralization.

2. Experimental procedure

2.1. Crystallization experiments

The constant composition crystal growth technique allows the investigation of nucleation and growth kinetics from metastable supersaturated solutions at a sustained thermodynamic driving force. With this method, developed by Tomson and Nancollas [21], we worked at supersaturations known with a precision impossible to achieve with the conventional free drift technique. The constant composition crystal growth technique included a 250 ml double-walled glass reactor thermostated by circulating water, an impulsomat, a pH meter, two electrically coupled burettes (Metrohm) and a recorder. The pH changes were detected by a combined glass electrode and the supersaturated solution was magnetically stirred. When collagen particles were added to the metastable supersaturated solution, they initiated the formation of the first nuclei of calcium phosphate on their surfaces. When nuclei reached the critical size, they grew, consuming ions of the solution and lowering the pH triggering the simultaneous addition of the two titrant solutions with concentrations such that their addition exactly compensated for the calcium and phosphate ions precipitated and maintained the concentrations in solution, the pH and the ionic strength constant. The one burette of titrant solution contained $CaCl_2 \cdot 2H_2O$ and KCl, and the other KH_2PO_4 and KOH. The burettes were connected to a recorder and from the plot of the volume of titrant added against time, the induction time, τ (min), and the initial growth rate, R_c (mol min^{-1} g^{-1}), were determined. The uncertainties on the τ and R_c determination were estimated to be 30 and 15%, respectively.

All the experiments reported herein were performed with 100 ml of metastable supersaturated solution at $pH = 6.50 \pm 0.01$ and $T = 37 \pm 1\,^{\circ}C$ with an ionic strength of 0.1 mol l^{-1} and a weight of collagen seed of 20 mg. The stock solution contained $CaCl_2 \cdot 2H_2O$, KH_2PO_4 and KCl. The pH was carefully adjusted by the addition of standard potassium hydroxide solution (0.1 mol l^{-1}). The Ca/P ratio of the stock and the titrant solutions was 1.33. Blank experiments (no seeding with collagen) were performed to check the stability of the supersaturated solution.

Type I collagen was extracted from bovine bone and demineralized by treatment with ethylene-diaminetetracetic acid (EDTA) in the laboratory of Professor Glimcher in Boston [6]. The powder of demineralized Type I collagen obtained was preserved at $-20\,^{\circ}C$ between experiments.

For the experiments in the presence of albumin, the BSA (fraction V, 99%, Sigma) was dissolved in the metastable supersaturated solution before the addition of collagen.

It is important to note that all experiments were done with the same batch of collagen and with the same batch of BSA to ensure good experimental reproducibility.

2.2. Solids characterization

After each experiment, the formation of OCP was confirmed by I.R. spectroscopy (Perkin Elmer 1600 FTIR). The suspended collagen was withdrawn, filtered and dried at different stages during the precipitation of OCP in order to study the modifications occurring at the collagen surface. The morphology of the growing phase was observed by SEM (Jeol JSM 6400). The formation of OCP was checked by XRD analyses.

Samples of collagen withdrawn during the nucleation and the growth processes were, respectively, noted ''$t =$'' in minutes and ''$m_{OCP}/m_{colf} =$'' in per cent (as the weight of OCP precipitated compared to the initial weight of collagen powder).

The OCP reference sample, analysed by I.R. spectroscopy, was prepared using the constant composition crystal growth method with the introduction of well characterized OCP seeds (with Ca/P = 1.33) at pH = 6.50 and $T = 37\,^{\circ}C$.

3. Results

3.1. Kinetics of heterogeneous crystallization of OCP on Type I collagen

In the first series of experiments we studied the influence of the relative supersaturation, σ defined in Equation 1, on the nucleation and growth kinetics of OCP on collagen in order to determine the growth mechanism

$$\sigma = (IP/K_s^o)^{1/v} - 1 \qquad (1)$$

where IP is the ionic product and K_s^o the solubility product of OCP, respectively, at $37\,^{\circ}C$; v is the total number of ions in the chemical formula. For OCP, we considered the reduced formula, $Ca_4H(PO_4)_3 \cdot 2.5H_2O$, so $v = 8$.

The experimental results are reported in Table I. In all cases, an induction period preceded the onset of OCP precipitation. According to the classical theory, this period is inversely proportional to the nucleation rate and corresponds to the time needed to form stable nuclei on the collagen surface [22]. Fig. 1 illustrates the dependence of the induction time on the relative supersaturation ratio. When σ increased, τ decreased strongly. Because the critical radius of the nuclei, r_c, is inversely proportional to the supersaturation ratio [22] (Equation 2), upon increasing the relative supersaturation, the critical nuclei size become smaller.

TABLE I Induction time, τ, and growth rate, R_c, versus the relative supersaturation ratio of the solution, σ

Relative supersaturation ratio	Kinetic parameters	
	τ (min)	$R_c 10^6$ (mol min^{-1} g^{-1}
[Ca][a] = 2.80, [P][a] = 2.10 $\sigma = 1.15$	350	6.1
[Ca] = 3.00, [P] = 2.25 $\sigma = 1.27$	153	6.2
[Ca] = 3.20, [P] = 2.40 $\sigma = 1.40$	120	9.9
[Ca] = 3.60, [P] = 2.70 $\sigma = 1.63$	62	12.3
[Ca] = 4.00, [P] = 3.00 $\sigma = 1.87$	26	35.6

[a]Concentrations [Ca] and [P], are measured in millimoles.

Consequently the nucleation is favoured and the induction time is shorter

$$r_c = 2v_m\gamma/\Delta\mu = 2v_m\gamma/(kT\ln\Omega) \quad (2)$$

with $\Omega = IP/K_s^o$; where v_m is the growth unit volume: γ, the interfacial energy; $\Delta\mu$, the change in chemical potential for the transition process from supersaturated solution to equilibrium; k, the Boltzmann's constant ($1.38\ 10^{-23}$ J K^{-1}); T, the absolute temperature, and Ω, the supersaturation ratio.

Mineralization reactions controlled by surface processes, usually enable the growth rates to be expressed by the following simplified equation [22]

$$R_c = C \times s \times \sigma^n \quad (3)$$

where C is the kinetic constant for a given temperature, s is related to the number of active growth sites, and n the apparent order for the precipitation process.

The plots of $\ln R_c$ versus $\ln\sigma$, presented in Fig. 2, show a straight line with a slope that gives the global order to the reaction: n. The average value for n was 3.50 and the expression of the growth rate, R_c (in mol min^{-1} g^{-1}) is

$$R_c = 3.0610^{-6}\sigma^{3.50} \quad (4)$$

Several authors who studied the crystallization of various compounds showed that when $n > 2$, the growth mechanism was polynuclear [23]. Moreover, Heughebaert and Nancollas [24] showed that the growth of OCP on OCP seed occurs by a polynuclear mechanism with $n = 4$.

The suggestion that crystallization of OCP on Type I collagen proceeds by a polynuclear mechanism is satisfactory and in agreement with the SEM, observations (see Fig. 3). Indeed, we observed, at the beginning of the nucleation stage, many poorly crystallized nuclei of calcium phosphate on the collagen surface (see Fig. 3b). At the end of the induction period, these nuclei became more crystallized and the platelets jumbled together (see Fig. 3c). At the beginning of the growth process, the platelets were organized in the form of little ''sand roses'', which is the characteristic morphology of OCP (see Fig. 3d). At later stages, they spread over the collagen surface until they were joined together to cover the organic substrate entirely (see Fig. 3e).

We estimated the interfacial energy of the OCP grown heterogeneously on titanium, from Equation 5, proposed by Mullin [22], and derived from the expression of the nucleation rate, which is inversely proportional to the induction period according to the classical nucleation theory

$$1/\tau = A \times \exp\{-(\beta \times \gamma^3 \times v_m^2)/[k^3 \times T^3 \times (\ln\Omega)^2]\} \quad (5)$$

where A is a constant and β a shape factor (assuming cubic shaped nuclei $\beta = 32$). Considering the reduced chemical formula of OCP ($Ca_4H(PO_4)_3 \cdot 2.5H_2O$: $v_m = 3.0310^{-28}$ m^3), we determined the interfacial energy from the slope of the straight line representing $\ln\ \tau$ versus $(\ln\Omega)^2$. The calculated value was $\gamma_{het} = 17.2$ mJ m^{-2} and is in excellent agreement with the interfacial energy $\gamma = 17$ mJ m^{-2}, calculated by Heughebaert *et al.* [25] from kinetic data of the heterogeneous nucleation of OCP on beta tricalcium phosphate.

Besides, as the presence of a foreign body or surface can induce nucleation at degrees of supersaturation lower than those required for homogeneous nucleation, the overall free energy change associated with the formation of a critical nucleus under heterogeneous conditions, ΔG_{het}, must be less than the corresponding free energy change associated with homogeneous nucleation, ΔG_{hom}, according to Equation 6

$$\Delta G_{het} = \phi\Delta G_{hom} \quad (6)$$

with

$$\Delta G = \beta \times \gamma^3 \times v_m^2/(kT\ln\Omega)^2$$

where the factor ϕ is less than unity and is related to the contact angle, θ, between the nucleus and the foreign substrate [22]

$$\phi = (2 + \cos\theta)(1 - \cos\theta)^2/4 \quad (7)$$

θ is determined by the different interfacial tensions between the crystal nucleus, the substrate and the

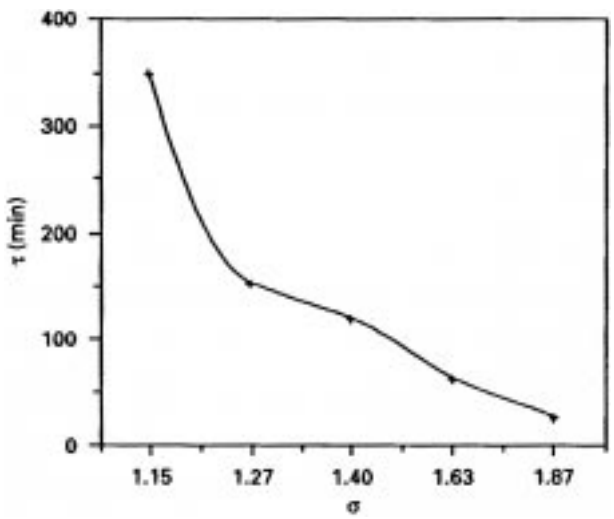

Figure 1 Induction time, τ, versus the relative supersaturation ratio, σ.

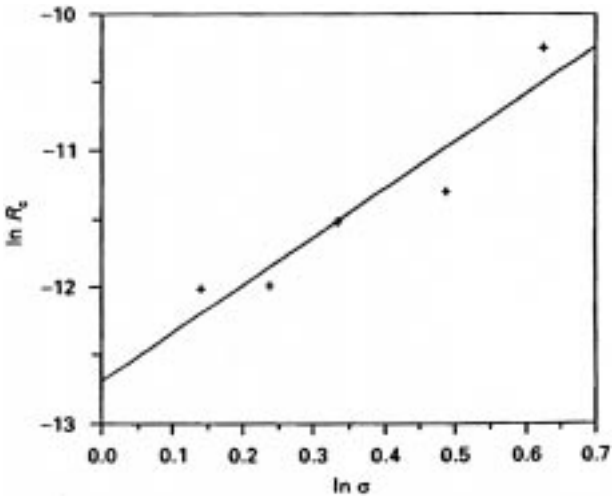

Figure 2 Determination of the apparent order of the reaction; plots of ln R_c versus ln σ.

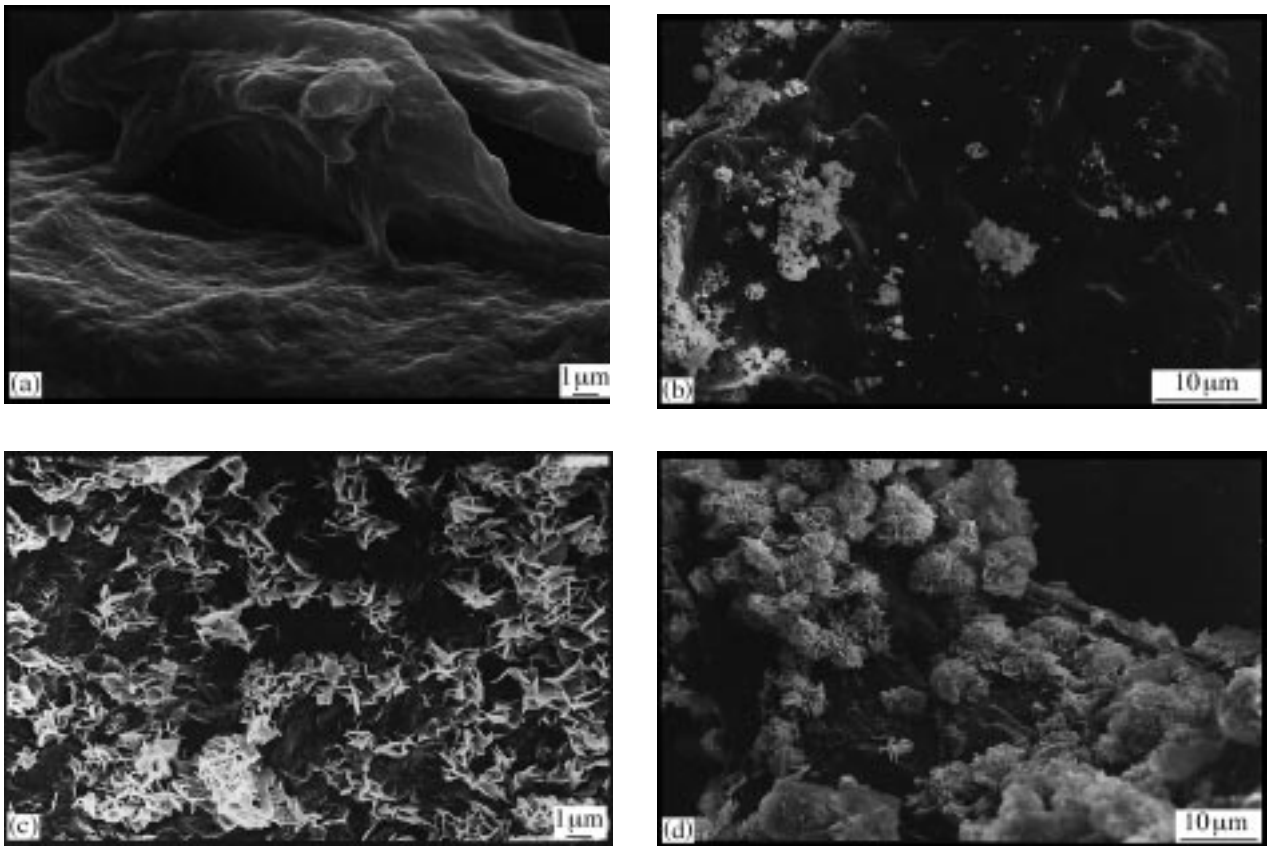

Figure 3 Scanning electron micrographs of samples of collagen withdrawn at different stages during the crystallization of OCP. (a) osseous demineralized collagen, (b) at the beginning of the nucleation stage, (c) at the end of the nucleation stage, (d) at the beginning of the growth stage, and (e) at the end of the growth stage.

solution and depends on the affinity between the precipitating phase and the foreign substrate. θ varied between 0 and 180° and the better the contact, the lower the values of θ and ϕ and the greater the nucleation rate. We estimated θ from the calculated interfacial energy for the heterogeneous nucleation of OCP on demineralized Type I collagen ($\gamma_{het} = 17.2$ mJ m^{-2}) and from the interfacial energy determined from the OCP homogeneous crystallization data of Boistelle and Lopez-Valero [26] ($\gamma_{hom} = 60$ mJ m^{-2}); $\theta = 35°$. This relatively low value of θ indicates that collagen is a good substrate for the nucleation of OCP.

It should be noted that the estimated interfacial energy and contact angle are useful parameters to compare different systems with each other but we cannot consider them as intrinsic characteristics of a system.

Fig. 4 gathers the i.r. absorbance spectra, in the range 4000–400 cm^{-1}, of collagen samples withdrawn at different stages during mineralization. We can note a decrease of C–H and amide vibration bands as mineralization progresses and, for a growth of 93%, they represented a very weak absorbance. Besides, when the percentage of growth reached 93%, the spectrum presented all the bands characteristic of the phosphate groups in OCP. We can particularly see the band specific of OCP, at 917 cm^{-1}, corresponding to the stretching of P–OH in HPO_4 groups of OCP. Between 1250 and 400 cm^{-1}, the spectrum of the OCP grown on collagen (Fig. 4d) and the OCP reference spectrum (Fig. 4e) were quite superimposable. Nevertheless, we noticed that the bands for the OCP precipitated on collagen were broader indicating a less crystallized product.

3.2. Influence of albumin

The results concerning the experiments, for a given relative supersaturation ratio ($\sigma = 1.63$), in the presence of BSA at various concentrations, are reported in Table II. Fig. 5 shows the effect of albumin concentration on the induction time. We see that the induction period increases with the concentration of albumin. For the typical human serum albumin concentration (50 g l^{-1}) [14], τ was ten times higher than in the absence of albumin.

The effect of the concentration of BSA on the growth stage is represented in Fig. 6. R_c and R_{c_o} are the growth rates measured in the presence and in the absence of BSA in the crystallizing system, respectively. Even though the shape of the curve is complex, it can be decomposed in two parts.

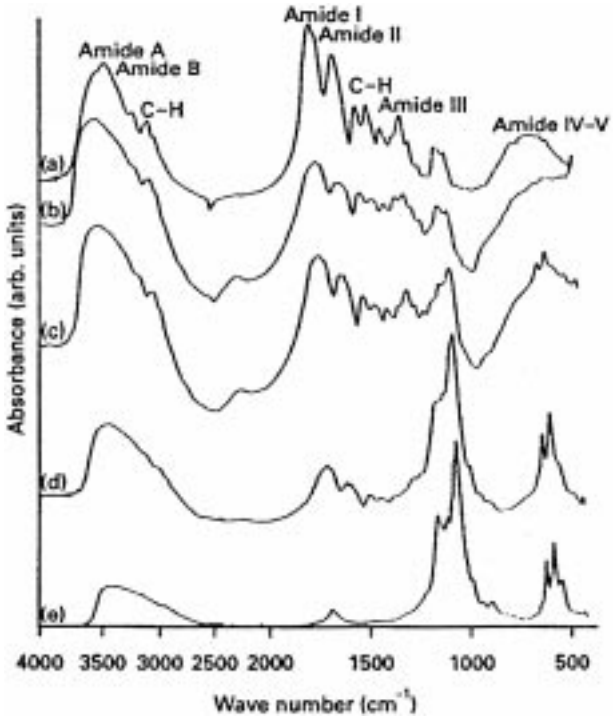

Figure 4 I.r. absorbance spectra, in the range 4000–400 cm^{-1}, of samples of collagen withdrawn at different stages during the crystallization of OCP. (a) demineralized Type I collagen, (b) $t = 90$ min, (c) $m_{OCP}/m_{coll} = 16\%$, (d) $m_{OCP}/m_{coll} = 93\%$, and (e) OCP reference sample.

TABLE II Induction time, τ, and growth rate of OCP, R_c, and R_{rmc}/R_{c_o} ratio, versus the concentration of BSA for $\sigma = 1.63$ ([Ca] = 3.6 m mol l^{-1} and [P] = 2.7 m mol l^{-1})

[BSA] gl^{-1}	τ (min)	$R_c 10^5$ (mol min^{-1} g^{-1})	R_c/R_{c_o} (%)
0	42	1.14[a]	100
1	66	2.30	202
5	106	2.15	189
10	145	0.91	80
20	215	0.51	45
40	333	0.65	57
60	463	0.22	19

[a]This value is for R_{c_o}.

For low BSA concentrations ([BSA] < 10 gl^{-1}), the BSA favoured the growth of OCP. In this range of concentrations, the growth rate was strongly dependent on the BSA concentration. R_c in the presence of about 1 gl^{-1} of BSA was twice as high as R_{c_o}.

For high BSA concentrations ([BSA] ≥ 10 gl^{-1}), the BSA inhibited the growth of OCP. For the typical human serum albumin concentration (50 gl^{-1}), we observed a reduction of R_c of about 65%. The growth rate slightly decreased as the BSA concentration increased.

It can be noticed that the presence of albumin in the crystallizing system did not seem to modify the growth mechanism of the OCP crystals; it was still a polynucleation mechanism. However, characterization by i.r. spectroscopy and observations by SEM revealed some differences between samples of collagen mineralized in the absence or in the presence of BSA. Fig. 7 gathered the i.r. absorbance spectra of samples of collagen mineralized in the absence and in the presence (40 gl^{-1}) of BSA. The phosphate vibration bands, in the

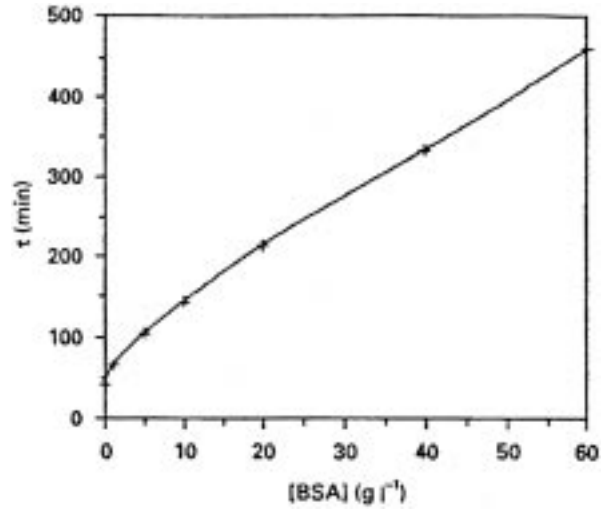

Figure 5 Effect of the concentration of BSA on the induction time, τ.

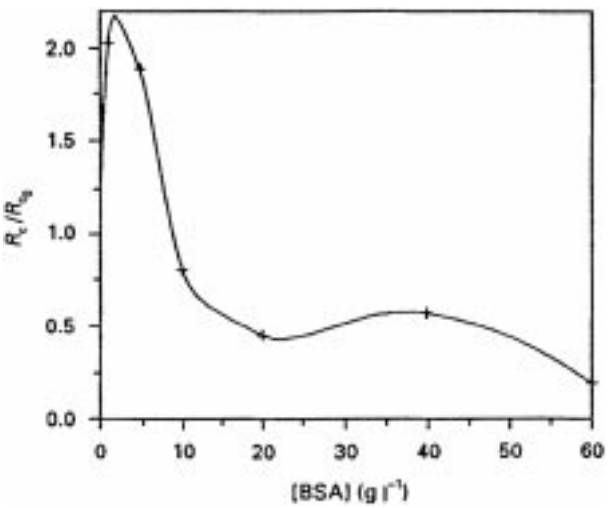

Figure 6 Effect of the concentration of BSA on the growth rate, R_c/R_{c_o}.

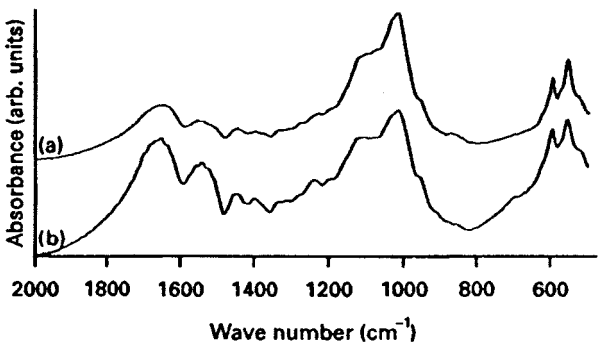

Figure 7 I.r. absorbance spectra, in the range 2000–500 cm^{-1}, of samples of collagen mineralized (a) in the absence of BSA, and (b) in the presence of BSA ([BSA] = 40 gl^{-1}).

ranges 650–500 and 1250–900 cm^{-1}, were quite superimposable. But, the amide bands were more intense, between 1800 and 1300 cm^{-1}, for collagen samples mineralized in the presence of BSA compared with those mineralized in the absence of BSA, showing that the protein is associated with the growing OCP crystals.

Moreover, the presence of albumin in the crystallizing system induced a change of the crystals appearance as observed by SEM (Fig. 8). Fig. 8a,c show the attachment of the calcium phosphate nuclei to the collagen surface. In the presence of BSA, the OCP crystallites seemed to

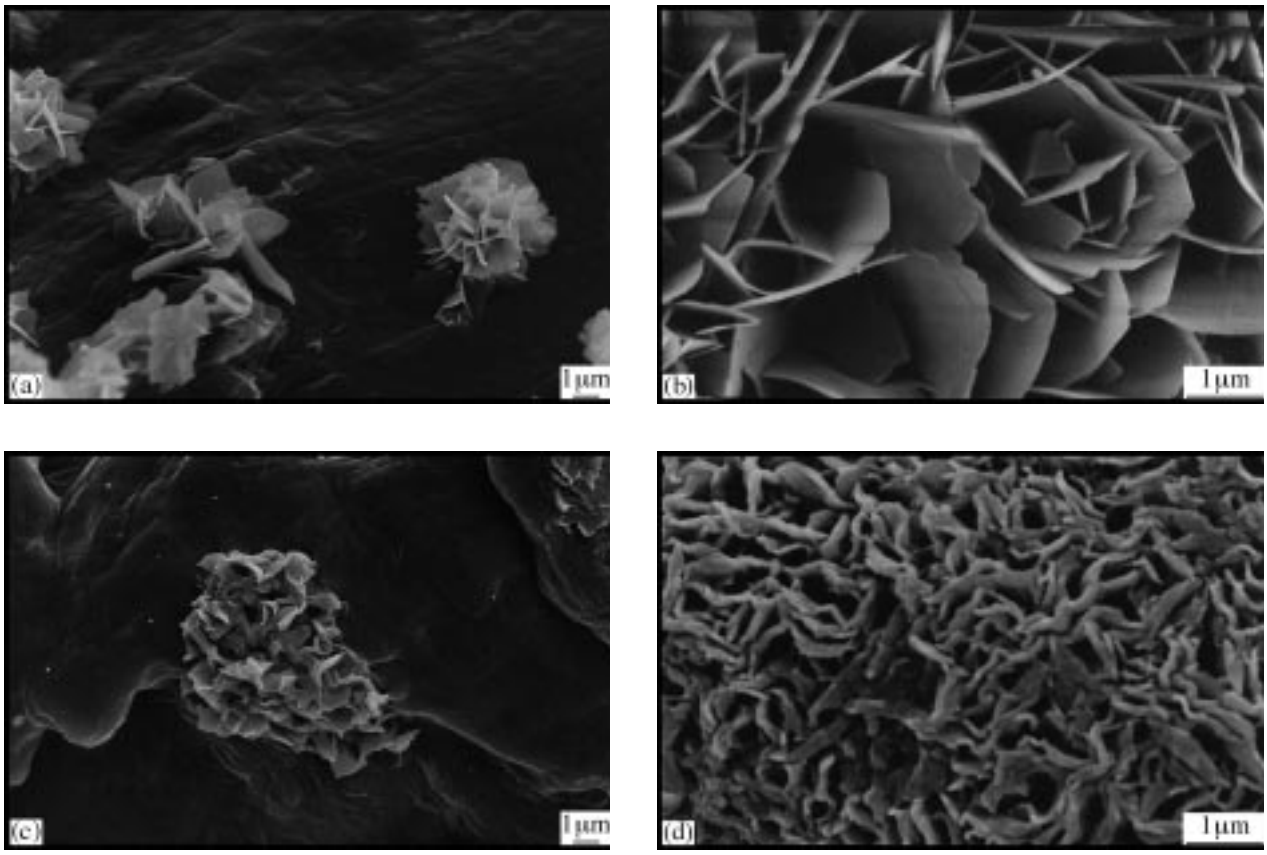

Figure 8 Scanning electron micrographs of samples of collagen withdrawn during the nucleation stage (a,c) and (b,d) during the growth stage. (a,b) in the absence of BSA, and (c,d) in the presence of BSA.

be less well crystallized and smaller (Fig. 8c). The micrographs of collagen samples withdrawn during the growth process were taken at the same magnification and are presented in Fig. 8b,d. In the presence of BSA, the OCP crystals appeared plate-like, as in the absence of protein, but they were smaller and showed smooth edges unlike the crystals obtained without albumin. Besides, the association of OCP crystals, grown in the presence of BSA, seems denser. These observations point out the ability of albumin to interact with OCP crystals.

4. Discussion

Our kinetic data are in good agreement with the work of Koutsoukos and Nancollas [9] on the crystallization of OCP on bovine achilles tendon collagen at pH = 7.40 and 37 °C. Their experiments, at high supersaturation, pointed out the formation of OCP, following well defined induction periods. OCP subsequently hydrolysed to hydroxyapatite, which is the most stable phase in these experimental conditions.

With regards to the results presented in this paper, it is interesting to note that BSA has two opposite effects on the kinetics of growth of OCP on Type I collagen, depending on its concentration. Because albumin is associated with numerous biomineralization processes, the kinetic study might give a clue to the role played by this protein in the control of normal and pathological calcification.

We noticed an inhibiting effect of albumin both on the nucleation and the growth stages. This could be attributed to the capacity of albumin, firstly to complex calcium ions by its carboxylate groups (COO^-) and, secondly to interact and to be adsorbed on organic and inorganic compounds and especially calcium phosphates.

The ability of albumin to bind calcium was tested by immersing a dialysis bag, containing albumin, in the supersaturated calcium phosphate solution at 37 °C for one day. Considering that the pKa of the amino acids is about 3.6 [27], we calculated the ratio $[COO^-]/[COOH]$ at pH = 6.50. Even though COO^- groups were largely predominant ($[COO^-]/[COOH] = 794$), in our experimental conditions, we did not detect any appreciable calcium uptake by albumin during the dialysis experiments. Thus, the complexation of calcium did not seem to be a determining phenomenon.

The isoelectric points of albumin, collagen and OCP are 4.5,9 and 7.5, respectively [28–30]. Consequently, at pH = 6.50, collagen and OCP were positively charged and albumin was negatively charged, so we can assume electrostatic interactions between OCP-albumin and albumin-collagen.

The experimental results emphasized the strong dependence of the induction time on the albumin concentration. This phenomenon can be explained either by considering the effect of albumin on the collagen substrate or on the OCP nuclei.

The adsorption of albumin at the nucleation sites on the collagen surface, could reduce the nucleation capabilities of the substrate and delay the appearance of the first nuclei. It should be noticed, however, that albumin is very easily extracted from bone after demineralization and, therefore, does not bind strongly to collagen. On the contrary, the adsorption of albumin on calcium phosphate surfaces is well documented.

Indeed, Addadi *et al*. [31] showed that acidic proteins like albumin preferentially adsorbed on the (100) face of OCP crystals. Besides, the adsorption of BSA onto OCP and its hydrolysates affects the precipitation process of OCP and its hydrolysis into hydroxyapatite [32]. Taking all these considerations into account, the modifications of the OCP heterogeneous crystallization kinetics seem mostly due to the adsorption of BSA on OCP nuclei. Thus, the attachment of albumin molecules to the forming mineral surface would slow down the nucleation process as developing nuclei surfaces could be at least partly inactivated by the protein coating. For a given relative supersaturation ratio, this phenomenon might be limited in solutions with low albumin concentrations, by depletion of free albumin molecules in solution as more and more nuclei are covered by the protein with time.

The influence of albumin on the growth rate is more difficult to interpret as two opposing effects have been brought to light: an increase of the growth rate for low albumin concentrations and a decrease of the growth rate for high albumin concentrations. This dual effect of the additive appears intriguing and indicative of the complex role a protein may have on crystal growth. This observation is not isolated and Ebrahimpour *et al*. [33] reported the same phenomenon in the case of nucleation and growth of calcium oxalate at hydroxyapatite surfaces in the presence of human serum albumin.

At high albumin concentrations, the slowing down of the crystal growth rate can be attributed to its adsorption on stable nuclei or on growing crystals as demonstrated by others [18, 19, 29]. Indeed, in the presence of a large amount of BSA, albumin molecules are available, in large quantities, to block the active growth sites on the growing OCP crystal surface. However, even at very high concentrations crystal growth never stops, and the platelet crystal morphology is not fundamentally altered, indicating that the adsorbed albumin does not constitute a strong barrier and that ionic species may still have access to the mineral surface. This behaviour could be related to dynamic processes at the liquid-solid interface [16].

The increase of the crystal growth rate at low albumin concentrations means that the number of stable nuclei on the collagen surface in a solution containing large amounts of BSA is higher than in an albumin-free solution. Several explanations can be proposed: albumin attached to the collagen surface may act as a nucleating agent for OCP, increasing the number of nucleation sites. This type of interaction has been reported for different proteins in various systems [34, 35]. Nevertheless, albumin does not seem to interact strongly with collagen, and despite many studies *in vitro* and *in vivo*, adsorbed albumin has never been suggested as a potential nucleator of OCP or apatite at the moderate supersaturations we used in these experiments. Another explanation could be that the adsorption of albumin decreases the interfacial energy of the calcium phosphate nuclei and therefore stabilizes nuclei with smaller radii than in albumin free solution. The formation of such small nuclei is easier and their number can thus increase rapidly. Because they are covered by albumin, however, they cannot be very active for crystal growth, but they may grow very slowly during the induction period. At low albumin concentrations, the adsorption process is not fast and not efficient enough to inhibit all potential sites. The new mineral surfaces generated by both slow nucleus development and nucleus multiplication processes increases rapidly. Consequently, as the number of stable nuclei is larger, the crystal growth rate is higher than in the absence of albumin. With larger amounts of albumin the protein concentration is so high that its adsorption is favoured over mineral ion uptake.

This mechanism is supported by the observation of smaller but more numerous crystals on the collagen fibers mineralized in the presence of BSA.

The findings illustrate the complex role of proteins in crystal growth, and adsorbing protein may have positive effect on crystal development by stabilizing nuclei as they form and favouring their multiplication and thus, the emergence of smaller but more numerous crystals. This phenomenon should be general and not limited only to albumin. Concerning albumin, it should be remembered that this protein, at human serum concentrations, is a strong inhibitor of crystal growth. Although most nucleation experiments to test the ability of biomaterials to induce apatite formation in body fluids use simulated body fluids (SBF), the data reported here show clearly that proteins, especially albumin, which is the major protein of blood, may strongly affect nucleation ability and crystal growth. Thus, we wonder if, in addition to its mineral composition, SBF should not also contain albumin to mimic biomineralization processes more closely [36].

As the crystallization of OCP occurred rapidly on the collagen surface, we can assume that the mineralization did not take place within the collagen fibrils. In order to confirm this assumption, we will have to complete this work by transmission electron microscopy observations. In order to gain further understanding of biomineralization, it would also be interesting to work close to physiological conditions.

References

1. W. J. PAULE, S. BERNICK, B. STRATES and M. E. NIMMI, *J. Biomed. Mater. Res.* **26** (1992) 1169.
2. T. CHANDY, M. MOHANTY, A. JOHN, S. BHASKARA RAO, R. SIVAKUMAR, C. P. SHARMA and M. S. VALIATHAN, *Biomaterials* **17** (1996) 577.
3. B. B. TOMAZIC, W. E. BROWN, L. A. QUERAL and M. SADOVNIK, *Astherosclerosis* **69** (1988) 5.
4. B. B. TOMAZIC, E. S. ETZ and W. E. BROWN, *Scann. Microsc.* **1** (1987) 95.
5. M. J. GLIMCHER, in ''Calcium in biological systems'', edited by R. P. Rubin, G. B. Weiss and J. W. Putney (Plenum, 1985) p. 607.
6. A. ENDO and M. J. GLIMCHER, *Connect. Tissue Res.* **21** (1989) 179.
7. J. D. TERMINE, H. K. KLEINMAN, S. W. WHITSON, K. M. CONN, M. L. MCGARVEY and G. R. MARTIN, *Cell* **26** (1981) 99.
8. J. T. TRIFFITT, in ''Fundamental and clinical bone physiology'', edited by M. R. Urist (J. B. Lippincott, Philadelphia, PA, 1980) p. 45.
9. P. G. KOUTSOUKOS and G. H. NANCOLLAS, *Colloids Surfaces* **28** (1987) 95.
10. W. E. BROWN, J. R. LEHR, J. P. SMITH and A. W. FRAZIER, *J. Amer. Chem. Soc.* **79** (1957) 5318.
11. R. Z. LEGEROS, in ''Hydroxyapatite and related materials'', edited by P. W. Brown and P. Constantz (CRC Press, 1994) p. 3.

12. D. J. WHITE and E. R. COX, A.C.S. Symposium Series, ''Surface Reactive Peptides and Polymers: Discovery Commer'', **444** (1991) p. 177.
13. M. J. GLIMCHER, L. C. BONAR, M. D. GRYNPAS, W. J. LANDIS and A. H. ROUFOSSE, *J. Cryst. Growth* **53** (1981) 100.
14. D. C. CARTER and J. X. HO, in ''Advances in protein chemistry-lipoproteins apolipoproteins and lipases'', Vol. 45, edited by C. B. Anfinsen, J. T. Edsall, F. M. Richards, D. S. Eisenberg and V. N. Schumaker (Academic Press, 1994) p. 153.
15. V. HLADY and H. FUREDI-MILHOFER, *J. Colloid Interface Sci.* **69** (1979) 460.
16. M. J. MURA, S. BEHR, E. F. BRES and J. C. VOEGEL, in ''Interfaces in biomaterials sciences'', Vol. 13, edited by D. Muster and G. Hastings (Elsevier, North Holland, 1989) p. 189.
17. K. KANDORI, S. SAWAI, Y. YAMAMOTO, H. SAITO and T. ISHIKAWA, *Colloids Surfaces* **68** (1992) 283.
18. J. GARNETT and P. DIEPPE, *Biochem. J.* **266** (1990) 863.
19. K. O. A. CHIN, M. JOHNSSON, E. J. BERGEY, M. J. LEVINE and G. H. NANCOLLAS, *Colloids Surfaces A Physicochem. Engng Aspects* **78** (1993) 229.
20. W. M. MULLINS and C. R. ELSASS, *B. J. Lett.* (1992) 326.
21. M. B. TOMSON and G. H. NANCOLLAS, *Science* **200** (1978) 1059.
22. J. W. MULLIN, in ''Crystallization'', 2nd Edn (Butterworths, London, 1972) 189.
23. A. E. NIELSEN, in ''Kinetics of precipitation'', (Pergamon, New York, 1964) 1–21.
24. J. C. HEUGHEBAERT and G. H. NANCOLLAS, *J. Phys. Chem.* **88** (1984) 2478.
25. J. C. HEUGHEBAERT, S. J. ZAWACKI and G. H. NANCOLLAS, *J. Cryst. Growth* **63** (1983) 83.
26. R. BOISTELLE and I. LOPEZ-VALERO, *ibid.* **102** (1990) 609.
27. M. N. TARAVEL, PhD thesis, University Claude Bernard, Lyon **1** (1994).
28. M. IIJIMA, Y. MORAWAKI and Y. KUBOKI, *J. Cryst. Growth* **137** (1994) 553.
29. S. A. SMESKO, R. P. SINGH, A. C. LANZALACO and G. H. NANCOLLAS, *Colloids Surfaces* **30** (1988) 361.
30. M. H. SALIMI, PhD thesis, Sunny, Buffalo (1985).
31. L. ADDADI, J. MORADIAN-OLDAK, H. FUREDI-MILHOFER, S. WEINER and A. VEIS, in ''Chemistry and biology of mineralized tissues'', edited by H. Slavkin and P. Price (Excerpta Medica: Elsevier Science Publishers BV, 1992) p. 153.
32. O. SUZUKI, H. YAGISHITA, M. YAMAZAKI and T. AOBA, *Cells Mater.* **5** (1995) 45.
33. A. EBRAHIMPOUR, L. PEREZ and G. H. NANCOLLAS, *Langmuir* **7** (1991) 577.
34. A. LINDE, A. LUSSI and M. A. CRENSHAW, *Calcif. Tissue Int.* **44** (1989) 286.
35. S. MANN, *Nature* **332** (1988) 119.
36. T. KOKUBO, K. HATA, T. NAKAMURA and T. YAMAMURO, in ''Bioceramics'', Vol. 4, edited by W. Bonfield, G. W. Hastings and K. E. Tanner (Butterworth-Heinemann, London, 1991) p. 113.

Received 23 December 1997
and accepted 26 January 1998