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Effect of chitosan on octacalcium phosphate crystal growth

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Chitosan has a strong effect on the crystallization of octacalcium phosphate. Octacalcium phosphate (OCP) is considered as one of the precursors in bony tissue formation. Chitosan is a polysaccharide prepared by chemical *N*-deacetylation of chitin. The interactions between the crystal and the polymer have been demonstrated by the growth of OCP crystals in the presence of soluble chitosan using the method of constant composition. These interactions correspond to a Langmuir adsorption type. The adsorption of chitosan on the OCP crystal surface is controlled by electrostatic interactions between the NH_3^+ functions of the polymer and negative groups on the crystal surface (HPO_4^{2-}). So, pH has a strong effect on the adsorption of chitosan. Copyright © 1996 Elsevier Science Limited.

INTRODUCTION

Chitosan is a linear polymer β-(1→4)-linked 2 amino 2-deoxy-D glucose residue (Fig. 1). It is prepared by chemical *N*-deacetylation of chitin, the natural polymer which forms the main organic part of crustacean shells. The presence of amino functions enables chitosan to exist in a soluble ($2.5 < pH < 6.2$) or solid form ($pH > 6.2$) (Rinaudo *et al.*, 1989). Chitosan presents numerous applications in various fields such as nutrition, metal recovery, (Domard, 1987; Shimizu *et al.*, 1995) and biomaterials (Muzzarelli *et al.*, 1993, 1994). With regard to the biomaterials applications, chitosan presents the aptitude to stimulate cell prolification and to organize the hystoarchitectural tissue structure. Recents works have shown that modified chitosan (particularly at the level of the nitrogen atoms) could play an active role in bone formation (Muzzarelli *et al.*, 1994; Biagini *et al.*, 1995).

Octacalcium phosphate, $Ca_8(HPO_4)_2(PO_4)_4.5H_2O$ (OCP), is considered as one of the calcium phosphates which participates in the early mineralization of the bony tissues (Legeros, 1994; Brown, 1962). The structure of OCP is formed by the intercalation of two layers; one apatitic and the other hydrated (Fowler *et al.*, 1993). Its chemical composition and its crystal structure are therefore close to calcium phosphate hydroxyapatite, the mineral of bones and teeth.

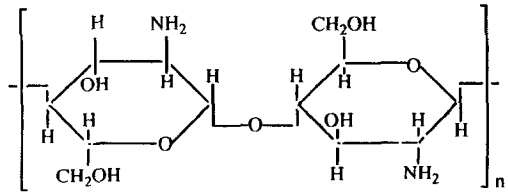

Fig. 1. Chitosan structure.

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The method of constant composition crystal growth has permitted the study of the crystallisation of OCP and of other calcium phosphates (Heughebaert *et al.*, 1984; Tomson *et al.*, 1978), and the growth mechanisms either in the absence or presence of ions (Salimi *et al.*, 1985) or organic molecules (Sharma *et al.*, 1992).

The studies reported in this paper were performed to determine the effects of chitosan on the crystal growth of the OCP at two pH values, using the constant composition crystal growth method.

EXPERIMENTAL

Crystallization experiments

The method of constant composition crystal growth consists of seeding metastable solutions, in which all the concentrations of the species in solution as well as the pH, the temperature and the ionic strength are kept constant (Koutzoukos *et al.*, 1980; Heughebaert *et al.*, 1984).

All the experiments reported herein were done at 37°C in a double-walled water jacketed Pyrex reactor thermostated to ±0.1°C. Supersaturated solutions (total volumes 100ml) were prepared in the reactor by mixing

equal volumes of calcium chloride and potassium dihydrogen phosphate solutions of the desired concentrations (Ca/P = 1.33). Next, the pH was adjusted by slowly adding small volumes of standard potassium hydroxide solution, followed by at least 2 h equilibration. The chitosan solution was introduced into the reactor during this period. Stability of the pH indicated that no precipitation took place: a process which is accompanied by proton release into the solution. The crystallization process was initiated by the addition of well characterized OCP seed crystals (15mg). A change in the solution pH as small as 0.005pH units, concomitant with the precipitation of calcium phosphate, triggered the addition of titrant solutions from two electrically coupled burettes (dosimat 665 Metrohm) of an automatic titrator (impulsomat 614 Metrohm). The two titrant solutions were prepared such that their addition exactly replaced the phosphate and calcium ions precipitated and maintained the concentrations, the pH and the ionic strength constant.

The burettes were connected to a recorder, and from the plot of the volume of the titrants added, ΔV, against time, t, the rates of crystallization, R_c, were accurately measured:

$$R_c = (\Delta V.C)/(t.m.\mathrm{SSA})$$

where SSA is the specific surface area of the OCP seed crystals and C the "effective titrant concentration". Corrections of the rates for changes in the surface area were made using a factor $(m/m_0)^{2/3}$, assuming three-dimensional growth as simple spheres or cubes. m_0 and m are the masses of initial solid phase and solid phase at time t.

The concentration and the pH of the working solutions are given in Table 1. The chitosan concentration in the working solution was between 0 and 0.1 $g \cdot l^{-1}$.

At the end of the experiments, the solids were filtered and analyzed by X-ray powder diffraction (INEL CPS 120, Kα wavelength of cobalt), by infrared spectroscopy (Perkin Elmer 16000 FTIR), and by scanning electron microscopy (JEOL JSM 6400).

OCP preparation

Octacalcium phosphate was prepared by the method of constant composition crystal growth described above.

Table 1. Experimental conditions of the OCP growth

N°	pH	$T_{Ca} \cdot 10^3$ mol·l^{-1}	$T_P \cdot 10^3$ mol·l^{-1}	$T_{OH} \cdot 10^3$ mol·l^{-1}	$T_{KCl} \cdot 10^3$ mol·l^{-1}	σ
1	6.2	3.40	2.56	0.55	86.77	0.78
2	6.5	2.25	1.69	0.58	90.83	0.80
3	6.5	2.80	2.10	0.73	88.68	1.15

Relative supersaturation: $\sigma = (IP/K_{S0})^{1/u} - 1$, where IP = ionic product, K_{S0} = solubility product, and u = number of ions per formula unit (16 for OCP).

The concentrations of the supersaturated solution were: $[CaCl_2.2H_2O] = 3.40 \times 10^{-3}$ mol·l^{-1}, $[KH_2PO_4] = 2.56 \times 10^{-3}$ mol·l^{-1}, $[KCl] = 86.77 \times 10^{-3}$ mol·l^{-1}, $[KOH] = 0.55 \times 10^{-3}$ mol·l^{-1}, ionic strength = 0.1 mol·l^{-1}, pH = 6.2, and those of the titrations solutions: $[CaCl_2,2H_2O] = 86.80 \times 10^{-3}$ mol·l^{-1}, $[KH_2PO_4] = 65.12 \times 10^{-3}$ mol·l^{-1}, $[KCl] = 13.54 \times 10^{-3}$ mol·l^{-1}, $[KOH] = 101.1 \times 10^{-3}$ mol·l^{-1}. The seed (15mg) used to start the crystal growth process was previously obtained by dicalcium phosphate dihydrate hydrolysis (Brown *et al.*, 1957).

This method gives reproducible OCP batches with a specific surface area: SSA = 23 $m^2 \cdot g^{-1}$.

Chitosan solution preparation

Chitosan was obtained from ABER Technologie (France) (batch N° A39E23). Its degree of acetylation was equal to 20%, determined by infrared spectroscopy (Miya *et al.*, 1980) and its average molecular weight, determined by viscosity method, was 750000 g·mol^{-1}. The chitosan mother solution was prepared by dissolving 10 g of chitosan in 1000ml of 1% acetic acid. The solution was stirred for 12 h and filtered on a 60 micron sieve.

RESULTS

Chitosan is soluble in acidic medium and precipitates at a pH close to 6.2 (Domard, 1987; Rinaudo *et al.*, 1989); OCP crystal growth studies are generally performed in a pH range 6–7. The first part of this work consisted of defining the pH of chitosan precipitation in a solution with the composition of the working solution. This study was performed by pHmetry (Domard, 1987) using KOH (0.1 mol·l^{-1}) as titrant. The characteristics of the supersaturated solution used were: volume = 50ml, ionic strength = 0.10 mol·l^{-1}, $[CaCl_2 \cdot 2H_2O] = 3.4 \times 10^{-3}$ mol·l^{-1}, $[KH_2PO_4] = 2.56 \times 10^{-3}$ mol·l^{-1}, $[KCl] = 86.77 \times 10^{-3}$ mol·l^{-1}, the initial pH was adjusted to 5.70 with KOH. The chitosan concentration was equal to 0.1 g·l^{-1}, the highest concentration used during crystal growth studies. After the KOH addition, the pH was measured immediately (time $t = 0$) and after 6 h (time $t = 6$, which corresponds with the longest duration for a crystal growth assay) in order to check for a possible very slow chitosan precipitation. The pH values are plotted in Fig. 2.

The two curves ($t = 0$ and $t = 6$) are perfectly identical. The inflexion point, at pH = 6.65 (determined by annulation of the second derivative), corresponds to the beginning of chitosan precipitation. In these conditions, especially in the presence of calcium and phosphate ions, chitosan precipites at a pH over 6.65. From these results, the studies of the chitosan influence on OCP crystal growth were performed in a pH range where the polymer remains soluble, pH = 6.2 (solution 1) and 6.5

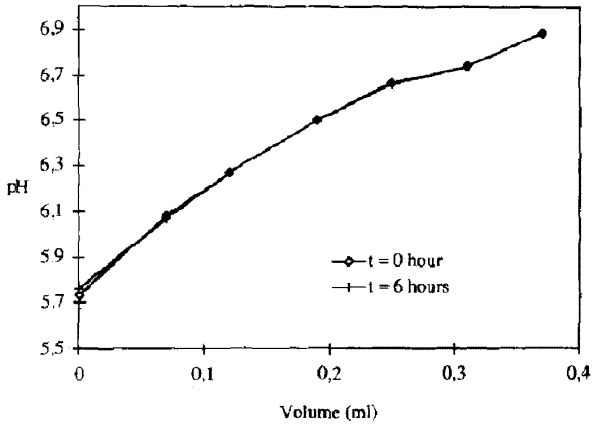

Fig. 2. pHmetry curves: pH of the chitosan solution ($0.1\,g\cdot l^{-1}$) versus the volume of KOH ($0.1\,mol\cdot l^{-1}$).

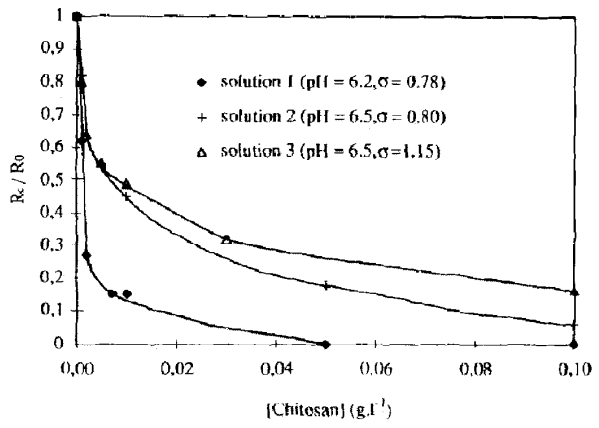

Fig. 3. R_c/R_0 versus chitosan concentration at two pHs. R_0 = growth rate of pure OCP. R_c = growth rate with inhibitor.

(solutions 2 and 3). The influence of supersaturation on the chitosan–OCP interactions was also considered for just one pH value: pH = 6.5. The chitosan concentration was between 0 and $0.1\,g\cdot l^{-1}$. The results are presented in Table 2 and Fig. 3.

Whatever the initial conditions, X-ray diffraction, infrared spectroscopy (Fig. 4) and scanning electron microscopy analysis showed that the solid samples obtained after crystal growth are octacalcium phosphate; their morphology was not modified by the presence of chitosan in the solution.

In a first approach, the three curves presented a similar shape: the OCP growth rate decreased and tended to zero when the chitosan concentration increased. Nevertheless, the three curves can be decomposed into two main domains:

(1) 0–$0.01\,g\cdot l^{-1}$ of chitosan, the crystal growth rate R_c drastically decreases with the increase in chitosan concentration.
(2) 0.01–$0.1\,g\cdot l^{-1}$ of chitosan, the growth rates decrease more slowly and tend to zero.

For the experiments at the same pH (6.5), a difference was observed: when the chitosan concentration was above $0.01\,g\cdot l^{-1}$, the rate diminution for solution 2 ($\sigma = 0.80$) appeared to be greater than for solution 3 ($\sigma = 1.15$).

For the experiments at the same supersaturation (solution 1 and 2), the R_c/R_0 ratio decreased quicker at pH6.2 than at pH6.5, especially at low chitosan concentrations (lower than $0.01\,g\cdot l^{-1}$). Beyond this concentration, the growth rates tended to zero whatever the supersaturation was: this value was reached for solution 1 when the chitosan concentration was $0.05\,g\cdot l^{-1}$. On the other hand, for solution 2, the rate was still equal to 6% of R_0 when the chitosan concentration reached $0.1\,g\cdot l^{-1}$.

DISCUSSION

Two main reasons can be proposed for the decrease of the growth rate of OCP when the chitosan concentration increases: (i) the formation of a soluble complex of calcium and/or phosphate with chitosan; this can contribute to the decrease in the amount of free calcium or phosphate in the solution and consequently to the decrease of the supersaturation; (ii) the effect of the chitosan molecule on the OCP crystal growth phenomena.

Table 2. Crystal growth rate of OCP on OCP seed crystals in the presence of chitosan

[Chitosan] $g\cdot l^{-1}$	Solution 1 pH = 6.2; $\sigma = 0.78$ $R_c\cdot 10^7$ ($mol\cdot min^{-1}\cdot m^{-2}$)	Solution 2 pH = 6.5; $\sigma = 0.80$ $R_c\cdot 10^7$ ($mol\cdot min^{-1}\cdot m^{-2}$)	Solution 3 pH = 6.5; $\sigma = 1.15$ $R_c\cdot 10^7$ ($mol\cdot min^{-1}\cdot m^{-2}$)
0	3.08	5.74	13.48
0.001	1.90	4.69	10.87
0.002	0.83	—	8.65
0.005	—	3.18	7.48
0.007	0.47	—	—
0.01	0.46	2.60	6.65
0.03	—	—	4.36
0.05	0	1.06	—
0.1	0	0.36	2.16

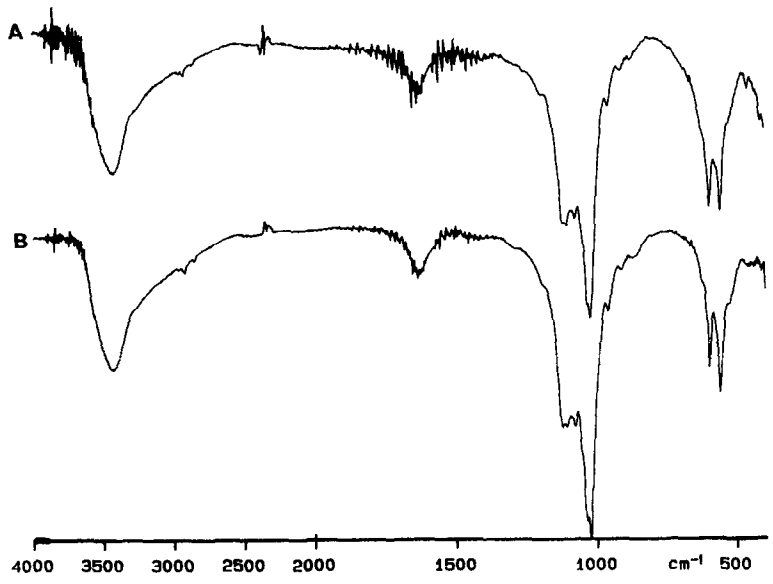

Fig. 4. Infrared transmittance spectra of OCP after crystal growth. (A) $0\,g\cdot l^{-1}$ of chitosan. (B) $0.1\,g\cdot l^{-1}$ of chitosan.

Considering the first hypothesis, analysis of free calcium and phosphate ions after dialysis (Spectra Por 1: 6000–8000 $g\cdot mol^{-1}$) of solutions containing $0.1\,g\cdot l^{-1}$ of chitosan shows no change in the concentration of these ions. Consequently, no soluble complexes between chitosan and calcium and/or phosphorus are formed and the supersaturation of the working solutions remains constant.

It is possible to compare the results obtained at a similar supersaturation level (solution 1, pH = 6.2 and solution 2, pH = 6.5), with an empirical model deduced to the Langmuir isotherms (Christoffersen *et al.*, 1981, 1984). This model allows calculation of the adsorption constants from kinetic values by using the following equation:

$$R_0/(R_0 - R_c) = 1 + 1/(K_e \cdot C_l)$$

where C_l is the equilibrum concentration of the inhibitor in the working solution, K_e an empirical affinity constant characteristic of the inhibitor affinity for the crystal. When adsorption is checked by the Langmuir law the variation of the ratio $R_0/(R_c - R_0)$ versus $1/C_l$ is linear. The curves plotted for the two assays (Fig. 5) are linear (the linear regression coefficients were 0.999 for solution 1 and 0.992 for solution 2) and so a mechanism of adsorption in agreement with the Langmuir isotherm can be proposed. The affinity constants K_e are deduced from the slopes.

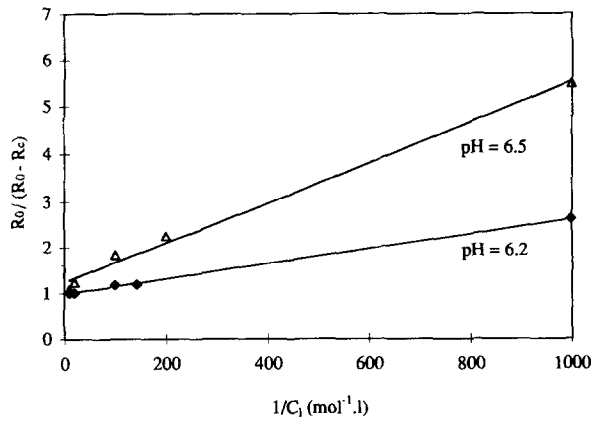

Fig. 5. Langmuir-type kinetic plots: $R_0/(R_0 - R_c)$ against chitosan inverse concentration.

The values obtained at pH = 6.2 and pH = 6.5 were respectively equal to 612 and $283\,g^{-1}\cdot l$. Chitosan adsorption on the OCP crystal is largely dependent on the pH of the solutions.

Considering the experiments at the same pH and two different supersaturations $\sigma = 0.80$ and $\sigma = 1.15$ (Table 2), the growth rate, in the absence of chitosan was about 2 times greater at higher rather than lower supersaturation: this can be attributed to the well known effect of the supersaturation. In the presence of a large amount of chitosan the growth rate is then 6 times greater: this can be attributed to the simultaneous effect of supersaturation and chitosan. It can be shown that the effect of chitosan appears to be greater when the influence of supersaturation is at its weakest (solution 2).

The OCP crystal growth is governed, under conditions of supersaturation and pH in this work, by polynucleation mechanisms on the crystal surface (Heughebaert *et al.*, 1984; Salimi, 1985). In aqueous solutions, the negative charges on the OCP surface are due to orthophosphate ions especially HPO_4^{2-} groups whose number increases with the pH. Concerning the chitosan, it changes from a polycationic form to a free amino form at pH = 6.65 (Fig. 1); therefore the number of NH_3^+ functions decreases when the pH increases from 6.2 to 6.5. So, it can be considered that the lower

the pH, the greater the number of NH_3^+ from the chitosan molecules and the number of HPO_4^{2-} groups on the OCP surface. This can be related to the fact that the chemical interactions between NH_3^+ and HPO_4^{2-} control the adsorption of chitosan on the OCP crystal surface. A similar role for NH_3^+ was proposed for the binding of protein to the surface of apatite crystals (Gorbunff, 1984).

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