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The crystallization of Hydroxyapatite in the presence of sodium alginate

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The effect of sodium alginate on the crystal growth of hydroxyapatite (HAP) was investigated at sustained supersaturation by the constant composition technique. Sodium alginate was found to inhibit HAP crystal growth at low concentrations and reduced the crystal growth rates by 42–86% for inhibitor concentrations of 2.1×10^{-7}–12.6×10^{-7} mol/l. The inhibition effect on the crystal growth rate may be explained possibly through adsorption onto the active growth sites. A detailed kinetics analysis suggested a Langmuir-type adsorption of the alginate on HAP surface and a value of 1.63×10^{7} l/mol was obtained for the affinity constant of sodium alginate for the surface of HAP. The apparent order for the crystallization reaction was determined to be approximately 2, thus suggesting a surface diffusion controlled spiral growth mechanism.

1. Introduction

Alginic acid is a naturally occurring hydrophilic colloidal polysaccharide obtained from the various species of brown seaweed (Phaeophyceae). The principal source is the giant Kelp *Macrocystis Pyrifea* along the coast of North and South America, New Zealand, Australia and Africa. It is a linear copolymer consisting mainly of residues β-1,4 linked D-mannuronic acid and α-1,4 linked L-glucuronic acid. These monomers are often arranged in homopolymeric blocks separated by regions approximating and alternating sequence of two acid monomers. The molecular weight is about 120,000 (10,000–600,000 typical average). The polymer replaces pectin in the brown algae, from which is extracted by sodium hydroxide treatment. Alginates (usually as sodium, potassium and ammonium salts) are widely used in the food industry, in surgery as resorbable materials and in pharmaceutical and cosmetic in industries [1–5].

Hydroxyapatite [HAP, $Ca_{10}(PO_4)_6(OH)_2$] is thermodynamically the most stable calcium phosphate salt and is the inorganic component of hard tissues such as bones and teeth [6]. On the other hand, HAP formation occurs in several pathological cases such as undesirable biological calcification, atheromatic plaque formation, renal calculi and the formation of bladder and bile stones [7–9]. Thus, HAP has been considered as the model compound for the *in vitro* study of biomineralization processes.

In the present work, the effect of sodium alginate on the crystallization of HAP was investigated. For the kinetic study of HAP crystal growth, the constant composition method was employed [10–12].

2. Experimental procedure

All experiments were performed at 37.0 ± 0.1 °C in a thermostated double walled, water-jacketed Pyrex vessel, volume totalling to 0.250 dm^{-3}. Solid reagent-grade (Merck) calcium chloride, potassium dihydrogen phosphate, sodium chloride and triply distilled CO_2-free water were used in the preparation of the solutions (0.1 M $CaCl_2$, 0.05 M KH_2PO_4, 0.1 M KOH and 1 M NaCl). Potassium hydroxide solution was prepared from concentrated standards (Merck, Tritisol). The standardization of the stock solutions is described in detail in [10–12]. The supersaturated solution was prepared in the thermostated vessel by mixing the appropriate volumes of calcium chloride and potassium dihydrogen phosphate. The ionic strength of the solutions was adjusted to 0.15 mol dm^{-3} by the addition of sodium chloride. The pH of the solution was measured by a glass/saturated calomel pair of electrodes (Metrohm, 6.0101.100) and 6.0726.100, respectively) standardized before and after each experiment by NBS buffer solutions [13]. Following the adjustment of the pH by the addition of dilute potassium chloride (C_k, M), the crystal growth process was initiated by the addition of 20 mg of well-characterized HAP seed crystals prepared by a method described in [14].

The specific surface area of the seed crystals, as determined by a multiple-point BET method

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(Perkin-Elmer Sorptometer 212D), was found to be $34.6\,m^2\,g^{-1}$. The solid precipitates were analyzed by infrared spectroscopy (KBr pellet method, FT-IR Perkin-Elmer 16-PC) and by powder X-ray diffraction (Philips PW 1830/840) using aluminium as an internal standard and elemental analysis. The synthetic crystals displayed the characteristics powder X-ray diffraction pattern [15] and the infrared spectrum of stoichiometric HAP [13, 15] and the experimentally determined stoichiometric ration Ca:P was 1.67 ± 0.01.

In the HAP growth experiments in the presence of sodium alginate (Sigma, from *Macrocystis pyrifea*), the alginic acid as dissolved in the phosphate solutions along with the HAP seed crystals. All this was carried out 30 min before the experiment initiated by the addition of calcium chloride. When following the above procedure, the adsorption phenomena did not interfere with the kinetic measurements. Throughout the course of the crystallization process water-saturated purified nitrogen was bubbled through the solution in order to preclude atmospheric carbon dioxide from dissolving into the solution.

During HAP formation, protons are released into the solution, thus offering a very sensitive means of monitoring its formation. A pH-meter (Metrohm 691) was used for measuring the pH. The pH-stat (Metrohm 614 Impulsomat with 654 Dosigraph) was modified so as to accommodate two burettes, mechanically coupled and mounted onto the shaft of the piston burette. The pH-meter was connected ti the pH-stat which allowed us, through the simultaneous addition of exactly equal volumes of reagents, to achieve invariability of all species in solution. The two mechanically coupled burettes were used with the following composition:

Burette 1: $(10Ca_t + 2Ca_t)$M $CaCl_2$ + {0.3 – $(20Ca_t + 30P_t + 10/5Ca_t\}$M NaCl

Burette 2: $(10P_t + 2P_t)$M KH_2PO_4 + $(20P_t + 10/5Ca_t + 2C_k)$M KOH

with molar ratios of Ca_t:P_t:OH = 5:3:1 (subscript t means total concentrations).

The monitoring of the crystal growth process and the constant supersaturation approach have been described in details in other publications [10, 12]. Therefore, at plethostatic conditions, the recorded motion of the burette piston can be easily translated into moles formed per unit time and area of the introduced seed crystals (R). However, owing to the reduction of the specific surface area during the crystallization process [16], the rates were taken at titrant additions corresponding to precipitated HAP ≈5%, with respect to the added HAP seed crystal concentration (20–45 min after the experiment started). Experiments with different amounts of seed crystals (10, 15 and 20 mg) showed the same initial rates normalized per unit surface area of the substrate. Also, changes in the stirring rate (between 60 and 300 rpm) had no effect on the initial rates (R). It may therefore be suggested that crystallization took place exclusively on the surface of the introduced seed crystals [17]. Higher amount of seed crystals led to erroneous results because of the high ionic strength, 0.15 M NaCl, that may cause aggregation. The reproducibility of the measured rates was better than 5%. During the crystallization process, samples were withdrawn (0.5, 1, 3, 6 and 12 h after the experiment was started) and filtered through membrane filters (Sartorius, 0.2 μm). The filtrants were analyzed for calcium by atomic absorption and for phosphate by spectrophotometric methods [18]. The constancy of calcium and phosphate was better than 3%.

3. Results and discussion

The experimental conditions were chosen in order to mimick those in nature namely pH 7.40, ionic strength adjusted to 0.15 M in NaCl and a working temperature of $37.0 \pm 0.1\,^{\circ}C$. In all cases, the crystallization material gave the characteristic X-ray diffraction patterns and infrared spectra of HAP. The morphology of the precipitated HAP is shown in Fig. 1. The kinetic results and thermodynamic data are summarized in Table I. As may be seen from this Table the rate of HAP crystal growth was reduced upon the addition of sodium alginate in the supersaturated solution. Furthermore, upon increasing the solution concentration of the sodium alginate further HAP crystallization inhibition was observed .

TABLE I Crystallization of HAP on HAP seed crystals in the presence of sodium alginate at pH 7.40, 0.15 M NaCl, and Total Calcium (Ca_t): Total Phosphate (P_t) = 1.67

Ca_t (10^{-4} mol/l)	Sodium Alginate (10^{-7}mol/l)	ΔG_{HAP} (kJ/mol)	R_{HAP} (10^{-8} mol min^{-1} m^{-2})
5.0	2.1	−4.43	4.30
5.0	4.2	−4.43	2.60
5.0	6.3	−4.43	1.85
5.0	8.4	−4.43	1.62
5.0	8.4	−4.43	1.62
4.0	2.1	−3.94	2.37
3.5	2.1	−3.63	1.71
3.0	2.1	−3.29	1.35
2.5	2.1	−2.88	0.74
5.0	0	−4.43	9.71
4.0	0	−3.94	5.38
3.5	0	−3.63	3.85
3.5	0	−3.63	3.85
2.5	0	−2.88	1.68

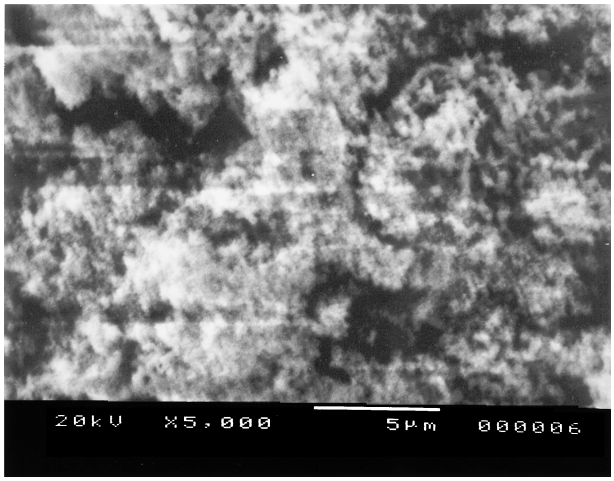

Figure 1 Scanning electron micrograph of HAP crystallization on HAP seed crystals in the presence of 12.6×10^{-7}mol/l sodium alginate.

Potentiometric titrations of the sodium alginate at the same experimental conditions employed in the kinetic studies (i.e. pH = 7.4, 0.15 M NaCl, 37 °C) in the presence and in the absence of total calcium 10×5^{-4} M did not show any appreciable complexation. It is therefore suggested that the observed inhibition effect is not due to a decrease of the solution supersaturation because of the Ca^{2+} sequestration by the sodium alginate. The suppression of the crystal growth rate, observed within the kinetic study, is attributable to extensive blocking of the active growth sites on the seed crystals by the adsorbed alginate. Fitting the kinetic results in a Langmuir-type isotherm may test this hypothesis.

Assuming that the additives adsorb onto the HAP seed crystals according to the simple Langmuir model and occupy a fraction θ, of the active growth sites ($0 < \theta < 1$), then the rates of crystal growth in the absence, R_0, and in the presence, R_i, of the inhibitor may be given from the equation [19]:

$$R_i = R_0(1 - \alpha_{\text{ads.}}\theta) \tag{1}$$

where $\alpha_{\text{ads.}}$ is a factor introduced in the adsorption isotherm equation in order to take into account deviations of the model. When $\alpha_{\text{ads.}} > 1$, then complete inhibition of the crystallization is expected even at low concentrations of the adsorbed molecules (irreversible and strong adsorption on the surface of the crystals) [19]. If $\alpha_{\text{ads.}} < 1$, then tha crystal growth may be remarkably reduced but it will not stop completely even at very high concentrations of the additive in the working solution [19]. According to the Langmuir model, at equilibrium the rates of adsorption and desorption of the additive compound of the surface are equal [20]:

$$k_a(1 - \alpha_{\text{ads.}}\theta)c_i = \alpha_{\text{ads.}}\theta k_d \tag{2}$$

where k_a and k_d are the adsorption and desorption rate constants of the adsorbate on the adsorbent, respectively and c_i is the concentration of the additive. Combination of Equations 1 and 2 gives the crystal growth rates in the presence and in the absence of the inhibitor as a function of its concentration in the supersaturated solution, c_i:

$$\frac{R_0}{R_0 - R_i} = \frac{1}{\alpha_{\text{ads.}}} + \frac{1}{\alpha_{\text{ads.}}k_{\text{aff.}}}\frac{1}{c_i} \tag{3}$$

where $k_{\text{aff.}}$ (equal to k_a/k_d), is the affinity constant and is a measure of the affinity of the adsorbent for the surface. The $k_{\text{aff.}}$ and $\alpha_{\text{ads.}}$ may be determined from the slope and the intercept, respectively, of the linear plots of $R_0/(R_0 + R_i)$ as a function of the $1/c_i$ according to Equation 3. This plot is shown in Fig. 2. From the straight line, a value of 1.63×10^7 l/mol was obtained for the affinity constant of sodium alginate for the surface of HAP and $\alpha_{\text{ads.}}$ was found to be 1. For comparative reasons, values of the affinity constants for other inhibitors of HAP formation are given in Table II. A high value of the affinity constant indicates strong adsorption of the inhibitor onto the surface.

TABLE II Affinity constants for various inhibitors of HAP crystal growth

Inhibitor	$k_{\text{aff.}} \times 10^4$ l/mol	Ref.
Phytic acid	8.4	[21]
1-hydroxyethane-1,1-diphosphonic acid	208	[21]
Sodium pyrophosphate	20	[22]
Amino tris (methylene phosphonic acid)	62	[22]
1-hydroxyethylidene-1,1-diphosphonic acid	130	[22]
Melitic acid	160	[22]
Citric acid	1.5	[22]
Glucose	10.2	[23]
Bis (sulfonamides)	3.5	[24]
1,2-hydroxy-1,2-bis (dihydroxyphosphonyl) ethane	216	[25]
Titanocenes $[Cp_2Ti(H_2O)_2]^{2+}$	68.9	[26]
Zirconocenes $[Cp_2Zr(H_2O)_2]^{2+}$	57.8	[27]
Vanadocenes $[Cp_2V(H_2O)_2]^{2+}$	11.9	[28]
Hafnocenes $[Cp_2Hf(H_2O)_2]^{2+}$	10.0	[29]
Homo-Aza-Steroids	213	[30]
Sodium Alginate	163	This work

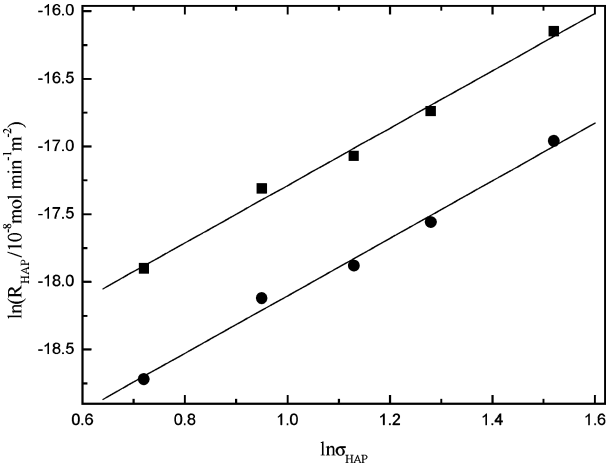

Figure 2 Kinetics of HAP crystal growth in the presence of various concentrations of sodium alginate, according to a Langmuir-type kinetic model; 37 °C, pH 7.40, $Ca_t = 10 \times 5^{-4}$ mol/l.

The driving force for a crystal growth process may be expressed as the change in Gibbs free energy, ΔG_{HAP} for the transfer from a supersaturated solution to an equilibrium state:

$$\Delta G_{\text{HAP}} = -\frac{R_g T}{9}\ln \Omega_{\text{HAP}} = -\frac{R_g T}{9}\ln \frac{IP}{k^0_{s,\text{HAP}}} \tag{4}$$

In Equation 4, IP is the ionic product of the precipitating HAP, $k^0_{s,\text{HAP}} = 2.35 \times 10^{-59}$ [31] is the thermodynamic solubility product at 37 °C, R_g the gas constant, T the absolute temperature and Ω_{HAP} the supersaturated ratio. The ionic products of the supersaturated solutions were calculated taking into account all equilibria. The computation of the solution speciation was made using expressions for mass balance for total calcium and total phosphate, electroneutrality, and ion association equilibrium constants after successive approximations for the ionic strength [10]. For this reason a computer program was developed [12]. Solution phase ionic concentrations were calculated from the dissociation constants

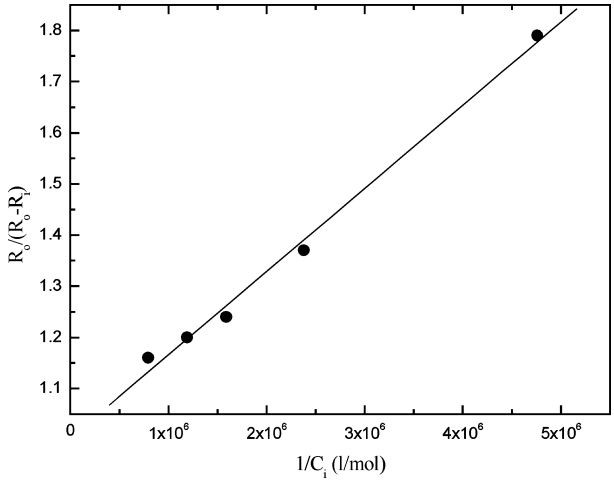

Figure 3 Kinetics of HAP crystal growth on HAP seed crystals (pH 7.4, 37 °C). Dependence of the rates of HAP crystallization on the relative solution supersaturation in the absence (■) and in the presence (●) of 2.1×10^{-7}mol/l sodium alginate.

of phosphoric acid and the ion-pair formation equilibrium constants of calcium, potassium and sodium, which are present in the solution, with the phosphate species.

Crystal growth rates were found to be proportional to the relative solution supersaturation, σ_{HAP}, defined as [32]:

$$\sigma_{HAP} = \Omega^{1/9} - 1 \quad (5)$$

$$R_{HAP} = K\sigma_{HAP}^{n} \quad (6)$$

where R_{HAP} is the crystal growth rate, K the rate constant (also a function of the number of the active growth sites on the crystal surface) and n the apparent order of the crystallization reaction. Logarithmic plots according to Equation 6, yielded straight lines in the presence and in the absence of sodium alginate (Fig. 3). From the slope of the linear plot a value of 2.11 ± 0.11 was obtained in the case of HAP crystallization in the absence of any additive while in the presence of sodium alginate the corresponding value is 2.13 ± 0.12. These values suggests a surface diffusion controlled spiral growth mechanism [33].

As may be seen from Table I and Fig. 3, when sodium alginate was present in the supersaturated solution the rate of HAP crystal growth decreased significantly. The presence of a foreign compound in the supersaturated solution (sodium alginate in this case) in which the crystal growth process is taking place, very often results in the interaction of the solute species with the surface of the precipitating solid. When the solute species have functional groups (C=O in the case of sodium alginate) they may adsorb on the crystal surface. Thus the crystal growth centers are blocked and the adsorbed molecules (inhibitor) prevent the crystal lattice species from incorporation into the crystal.

4. Conclusions

In the present work the effect of sodium alginate on HAP crystallization was investigated in supersaturated solutions with respect to HAP by the constant composition technique. Sodium alginate was found to inhibit HAP crystal growth at low concentrations and reduced the crystal growth rates by 42–86% for inhibitor concentrations of 2.1×10^{-7}–12.6×10^{-7} mol/l. Adsorption and further blocking of the active growth sites may explain the inhibitory effect. The adsorption assumption was justified through the satisfactory fit of the experimental results to a kinetic Langmuir-type model. Finally the advantage of using physiological substances such as sodium alginate to prevent pathological formation of HAP over the commercial synthetic drugs (which have been accused of being responsible for several side effects) is that the concentration excess of the inhibitor on the tissue can be regulated via physiological pathways and therefore the liver function is not charged.

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