Nucleation and Growth of Brushite in the Presence of Alginate

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ABSTRACT

Biomineral formation *in vivo* is a complex process regulated by functional molecules. Highlighting the mechanisms underlying biomineralization is necessary for a better understanding of *in vivo* processes and for enhanced *in vitro* model systems. Here, the effect of alginate and its well-defined oligomers with M- or G- block structure on brushite nucleation and growth is investigated by seeded and unseeded experiments. Growth kinetics were studied by seeded experiments and it was shown that molecular weight and functionality of alginate additives affect the crystal growth rates and the growth mechanisms. Growth retardation was most prominent when G-block additives were present. Growth proceeded by surface nucleation when alginate and G-block oligomers were added in the crystallization medium whereas in the presence of M-block oligomers parabolic rate laws were obtained. By decoupling the seeded and unseeded experiments, information was deduced on the effects of additives on brushite nucleation. In the presence of alginate and G-block oligomers nucleation was inhibited, however, M-blocks did not show a similar effect. Possible modes of interactions between the mineral and polymer additives are discussed by the evaluation of final crystal morphologies.

INTRODUCTION

The formation of inorganic minerals via a biological process (biomineralization) is a complex process involving the constructive interaction between organic and inorganic phases to result in composite tissues with intricate structures which give rise to unusual and often exceptional biological and physical properties.[1](#_ENREF_1) Such structures are difficult to reproduce synthetically in the laboratory, nevertheless, natural biomineralization processes may offer a great source of inspiration to design synthetic bottom-up formation processes to tailor biomaterials in an attempt to mimic or reproduce the structures and hence the properties of natural hard tissues.[2](#_ENREF_2) Both naturally and synthetically derived polymers, peptides and small molecules and ions may be used for this purpose either as mineralization templates or modifying agents. In the case of polymeric additives; concentration, molecular weight, functional groups and polymer block structure have been shown to impart significant influence over inorganic mineralization in terms of crystallinity, resulting crystal phase and crystal morphology, nucleation and growth rates.[3](#_ENREF_3),[4](#_ENREF_4) The role of macromolecules in controlling crystallization *in vivo* and *in vitro* model systems has been at the center of comprehensive research, and continues to be an advancing field.

Polysaccharides are recognized as a strongly influential group of polymers acting upon calcium phosphate and calcium carbonate mineralization that are important classes of minerals in vertebrate and marine hard tissues, respectively.[5-9](#_ENREF_5) Alginate is a linear polysaccharide copolymer, derived from brown sea algae and composed of 1-4 linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues with an alternating or block structure. The M- and G- units of alginate differ in the orientation of carboxyl groups that in turn highly affects their functionalty. The pKa values of M- and G- repeating units are 3.38 and 3.65, respectively and the pKa value of alginate polymer differs only slightly from its monomers. Calcium ions show specific affinity towards the outward carboxyl groups of the G- units of alginate and bind to G-blocks and alternating blocks of the polymer but not to M-blocks.[10](#_ENREF_10) Therefore, the physical properties of alginate and how it regulates mineralization are highly dependant on its chemical composition, sequential structure of the repeating units and molecular weight. Our group recently conducted detailed investigations on the nucleation and growth of calcium carbonate in the presence of alginate and well defined M- and G-block oligomers.[11](#_ENREF_11),[12](#_ENREF_12) G-block oligomers were shown to have prominent effects on nucleation characteristics, polymorph switching and resultant crystal morphology. Malkaj *et. al.* investigated the effects of alginate on hydroxyapatite crystallization. They showed the inhibitory effect of alginate on crystal growth rate, and attributed it to adsorption of polymer on the active growth sites.[13](#_ENREF_13) A reduction in hydroxyapatite growth rate in the presence of alginate was also observed by Coleman *et. al.* and further investigations in this study indicated varying effects of anionic polymers on crystal growth and morphology depending on chemical structure of polymer and subsequent changes in interaction mode with the crystal particles.[14](#_ENREF_14) To our knowledge, there are no similar studies in literature that have investigated the brushite - alginate system.

Alginate has been studied for many biomedical applications due to being nontoxic, biocompatible, biodegradable and its ability to form hydrogels. Alginate hydrogels can be prepared under mild conditions by ionic crosslinking and they show structural similarity to extracellular matrices of living tissues which in turn allows for many applications in bioactive agent delivery, wound healing and tissue engineering.[15](#_ENREF_15) A recent study conducted in our group showed alginate hydrogels mineralized with brushite were potential candidates for bone tissue engineering applications.[16](#_ENREF_16) Incorporation of bioceramics in alginate hydrogels enhances both the mechanical properties and the bioactivity of the polymer network. Brushite was chosen as the mineral phase due to its high solubility among calcium phosphates under physiological conditions, which enables for ready transformation and bone remodeling by providing necessary space and ion source for bone-like mineral formation *in vivo*.[17](#_ENREF_17) However in the complexity of a hydrogel environment control over mineral phase becomes more challenging due to varying effects of the polymer over the course of crystallization. When alginate solutions containing phosphate precursor were gelled and mineralized spontaneously in calcium containing gelling baths, multiple phases of calcium phosphates were precipitated, even at conditions which should favor formation of brushite. On the other hand, when brushite formation in the hydrogels was directed using small, crystalline seeds, mineral formation in the alginate network could be controlled to a much greater extent and the hydrogel was mineralized with pure brushite. Interestingly, growth of seed crystals, as well as nucleation of new brushite crystals was observed. These observations raised a number of interesting questions which are difficult to study in a gel system, and which need to be investigated under more controlled conditions, including polymer concentration, composition and a control of supersaturation.

In this study, the effect of alginate on brushite crystallization was investigated by both seeded and unseeded experiments. Alginates differing in molecular weight and block composition were used as additives and the effects of compositional variations of the polymer on nucleation and growth of brushite were examined. In the first part, brushite growth was studied by seeded desupersaturation experiments which best represents the model system of seeded alginate hydrogels. To our knowledge, no previous studies have reported brushite growth kinetics studied by this method. The constant composition method was also included for detailed investigation of brushite growth mechanism in the absence of additives. In the second part, unseeded batch experiments were conducted to show the effects of alginate additives on nucleation and growth stages. By evaluating the results of seeded and unseeded experiments together, information was deduced on the effects of additives on the nucleation stage. Finally crystal morphologies were also examined to discuss the possible modes of interactions between the mineral and polymer phases.

EXPERIMENTAL

Materials

All chemical reagents were purchased from Sigma-Aldrich unless stated otherwise. Deionized water (DIW) was used for all purposes. Alginate with molecular weight of 274 kDa and 68% G, isolated from *Laminaria hyperborea* stipe, was obtained from FMC Biopolymer. Two different oligomers of alginate, denoted as G- and M-blocks, consisted of 90% and 5% G, respectively, with the degree of polymerization ≈20 for both. The G-blocks were produced from alginate from *L. hyp* stipe by acid degradation and fractionation as described previously.[18](#_ENREF_18) The M-blocks were produced from alginate from *Ascophyllum nodosum* by acid degradation and calcium fractioning as described elsewhere.[19](#_ENREF_19)

Method

All experiments were carried out in a 1L double-walled glass reactor, stirred with Teflon coated 3-bladed impeller of propeller type controlled by a digital stirrer engine (IKA) and two baffels were attached to the lid. Temperature was controlled by a water bath at a temperature of 25°C for all experiments. Nitrogen, presaturated with water, was constantly bubbled into solutions throughout the precipitation reactions to prevent intrusion of atmospheric carbon dioxide. The chemical speciation and activity based supersaturation was determined by thermodynamic calculation program PHREEQC Interactive 3.1, using Minteq v4 database. The equilibrium constants used for solution speciation are given in supplementary information (Table S1). The pH was recorded constantly by means of a combined glass electrode with KCl reference electrolyte connected to Tiamo software (Metrohm) and calibrations were carried out weekly.

Calcium ion concentration in the vessel was monitored by offline titration (Mettler Toledo DL 53) of samples with 10 mM EDTA. For this purpose, 2 mL samples were withdrawn and filtered through a 0.22 µm syringe filter (Millipore) to remove solid crystals. Samples were kept overnight after addition of 3 mL of 0.1M HCl and dilution to 20 mL with water. Before analysis, samples were further diluted to 40 mL, 1 mL of 4M KCl and 1 mL of pH 10 NH3+/NH4 buffer were added.

Characterization of solid phases were conducted via powder XRD (D8 Advance DaVinci, Bruker AXS GmBH) in the range of 5-75°with a step size of 0.013° and a step time of 0.67 s. SEM analyses (Hitachi S-3400N) were performed at an accelerating voltage of 5 kV, where samples were placed on carbon tape and sputter coated with gold.

Seed Preparation

Brushite seeds were prepared by mixing equimolar amounts of Ca(NO3)2.4H2O and KH2PO4 to a total volume of 1L under nitrogen environment and constant stirring at 800 rpm. Precipitates were aged in their saturated solution for at least 2h after completion of reaction and observation of stable pH. Crystals were then filtered, washed with DIW and ethanol prior to drying at room temperature. Phase purity of seeds was verified by XRD and SEM analyses. Specific surface area (SSA) was determined to be 0.646±0.023 m2/g by multiple point BET nitrogen adsorption method (Tristar II, Micromeritics) and volume distribution showed a mean diameter of 24.17 µm of an equivalent sphere with CV 40.3%, characterized by a Coulter Counter Multisizer (Beckman Coulter). For size determinations, an average of three measurements was used in which approximately 1x104 seeds were counted during each run. Seeds were stored for up to one month in their dry state prior to use.

Preparation of Solutions for Desupersaturation Experiments

Solutions of calcium and phosphate precursors were prepared separately from their stock solutions of CaCl2.2H2O and KH2PO4 (250 mmol/kg). An appropriate amount of KCl salt was added in calcium solution to ensure a final ionic strength of 0.15M whereas 1.0M KOH was added to phosphate solution for adjustment of desired pH. In the case of experiments incorporating alginate, corresponding amount of filtered polymer solution was added into the phosphate containing solution and stirred overnight prior to experiments. Alginate additives are fully deprotonated in the pH range of the growth experiments which was above the pKa values of the repeating units of alginate. Supersaturated solutions were prepared by the addition of calcium containing solution to phosphate containing solution while stirring at 300 rpm to give the final concentrations of calcium and phosphate equal to 15 mM. Final pH was carefully adjusted to 5.700±0.002 by dropwise addition of 0.1M KOH. Supersaturation with respect to brushite was calculated to be S=2.34 at initial conditions and solution was stable up to 15 min. Supersaturation calculations were repeated in the presence of additives assuming all G blocks present in solution bind to calcium ions. The low molar concentration of G blocks with respect to the total calcium ion concentration at any experimental condition did not cause any significant decrease in free ion concentration to affect the supersaturation.

Seeded Desupersaturation Growth Experiments

For brushite seeded experiments, 100 mg of seeds with well-known specific surface area (A) were added to the vessel as a suspension immediately after preparation of supersaturated solutions to initiate growth reactions. Samples were withdrawn for calcium analysis during the course of growth. At the end of each experiment, the vessel content was filtered, washed and dried for characterization by XRD and SEM.

Depletion in calcium ion concentration was measured by offline titration at arbitrary time points and fit to a simple exponential function later used to obtain an overall growth rate equation as a function of time (Equation 1). Also, using measured calcium concentration and pH, momentary supersaturation values were calculated by PHREEQC software. Changes in phosphate concentration were assumed equal to depletion in calcium since ion consumption proceeds only via brushite growth in solution. Spectroscopic methods could not be used efficiently for phosphate determination at these experimental conditions due to small changes in ion concentration with respect to high initial values. Combining the growth rate function with data points of momentary supersaturation, growth rate was represented as a function of relative supersaturation (σ) (Equation 2). The predicted models of growth rates showed good fit with the experimental data and are shown in supplementary information (Figure S1).

|  |  |
| --- | --- |
|  | (1) |
|  | (2) |

The relative supersaturation is defined as given in Equation 3 where IAP denotes ion activity product and Ksp is the solubility product of brushite.

|  |  |
| --- | --- |
|  | (3) |

Each experiment was repeated in multiple runs and the growth rate law was then used to obtain the growth rate constant, *k*, and the apparent order of growth, *g*.

Seeded Constant Composition Growth Experiments

Desupersaturation experiments are difficult to conduct at low supersaturation regions where crystal growth proceeds slowly and ion consumptions are too low to collect sufficiently reliable data. In order to overcome this drawback constant composition experiments were performed in the relative supersaturation range of 0.20-0.55. Initially stable supersaturated solutions at pH 5.70 were prepared and 50 mg of seeds in suspension were added in the vessel to initiate growth, as described above. A decrease in pH of more than 0.002 units associated with brushite growth triggered the addition of titrant solutions from two mechanically coupled titrant burettes (902 Titrando, Metrohm) to maintain a constant supersaturation.

Compositions of titrant solutions were adjusted for each working solution to maintain a constant thermodynamic driving force during the brushite growth by compensating for ion consumption, changing ionic strength and alkalinity in the reaction media. Detailed preparation of titrant solutions has been previously given for calcium carbonate by our group.[20](#_ENREF_20) In this study, the same methodology was used with the modifications for brushite growth. For this purpose, first the effect of the extent of brushite crystallization on total alkalinity and ion consumption were computed in moles (*n*) according to the equations given below:

|  |  |
| --- | --- |
|  | (4) |
|  | (5) |
|  | (6) |
|  | (7) |
|  | (8) |

In order to compensate for the calcium ion consumption by crystallization, an arbitrary concentration (*c*) was determined for the calcium to be added to the reaction medium from burette 1. The calcium concentration in burette 1 should be higher than the concentration in the working solution and should be adjusted according to the growth kinetics. Next, the composition of titrant solution 2 was calculated, in accordance with the titrant solution 1 and the total volume of titrant solutions to be added, to fulfil Equations 9-14. The adjustment for the ionic strength was also ensured with burette 2 in order to keep constant ionic activity coefficients used in the calculation of activity based supersaturation.

|  |  |
| --- | --- |
|  | (9) |
|  | (10) |
|  | (11) |
|  | (12) |
|  | (13) |
|  | (14) |

Note that the calculated composition in burette 2 is only valid for the chosen initial working solution and the determined calcium concentration in burette 1.

By the addition of titrant solutions, pH was controlled at a constant value with an overshoot to a maximum of 0.004 units only at the initial growth period. Constancy of supersaturation was confirmed with calcium analyses at arbitrary time points that showed less than 1% deviation in concentration during titrant addition.

The growth rate of brushite was calculated at each supersaturation value by using titrant addition curves (Equation 15), where the effective concentration (*Ceff*) denotes number of moles precipitated per liter of titrant addition and *dV/dt* is the titrant curve gradient. Titrant addition curves were normalized to an increasing surface area of the seeds with proceeding growth. For this purpose, the mass change of brushite was calculated from the volume of titrant added and related to the change in surface area by a factor of [*(wi/w) 2/3*], where *wi* is the weight of seed crystals added initially and *w* is the weight of crystals present at time *t*, assuming three dimensional growth with a constant shape factor.[21](#_ENREF_21),[22](#_ENREF_22) Obtained results were then used to calculate the growth rate constant, *k*, and the apparent order of growth, *g* (Equation 2).

|  |  |
| --- | --- |
|  | (15) |

Unseeded Batch Experiments

Supersaturated solutions were prepared as described above for desupersaturation experiments and spontaneous precipitation was allowed to occur at constant stirring speed and temperature, under nitrogen environment. Solution pH was recorded continuously and samples were withdrawn at arbitrary time points for calcium analysis after the first detectable drop in pH. Reactions were continued until S≈1.0 for the additive free system and S≈1.5 for all others. At the end of each experiment, precipitates were filtered, washed and air dried for characterization by XRD and SEM.

RESULTS AND DISCUSSION

Seeded Growth Experiments

Seeded batch experiments were conducted in order to investigate and quantify the effects of different alginates on brushite growth. Initial experimental conditions were determined in accordance with preliminary experiments to give solutions supersaturated with respect to brushite and stable for up to 15 min. Brushite seeds were well characterized prior to usage in terms of phase purity and morphology (Figure 1). The surface area of seeds was determined by multiple point BET as 0.646±0.023 m2/g.

**Figure 1.** SEM image and XRD spectrum of brushite seeds.

Upon addition of seeds into the reaction medium, the pH started to drop immediately which was accompanied by a simultaneous decrease in calcium ion concentration. To ensure ions were only consumed by the growing seeds, coulter counter measurements were taken during growth experiments of brushite in the absence of alginate. Analysis of samples withdrawn during the course of the reaction showed steady numbers of crystals with increasing average size. In experiments including alginate additives, reliable data could not be obtained from size measurements due to aggregation of the crystals. However, SEM images of end products indicated that a non-nucleating environment was achieved as no new particles smaller than the original seeds were observed (Figure 2).

**Figure 2.** SEM images of samples collected at the end of seeded experiments where S≤1.5 in the presence of 10 ppm M-blocks at different magnifications.

There was a significant change both in calcium consumption (Figure 3) and pH drop rates (not shown) when different types of alginate were added in the reaction vessel. By using the obtained calcium and pH depletion curves growth rate constants (*k*) were calculated to quantify the effects of additives on the overall growth rate (Table 1). In the absence of alginate, a fast growth process was observed and the reaction was almost complete within 50 min. After that point, calcium depletion curves and pH showed constant values that indicated the end of the reactions. Calculations of supersaturation with the momentary inputs of pH and ion concentrations at that time points resulted S≈1.00 that supports the accuracy of thermodynamic calculations used. The growth rate constant was calculated to be 1.063 mmol/min.m2 in the absence of any additive from the desupersaturation experiments. Addition of high molecular weight alginate resulted in a decrease in the rate of calcium consumption and pH drop starting from the initial stages of growth and continuing throughout the reaction. This retarding effect was observed to be highly dependent on additive concentration. When G-block oligomers of alginate were used at the same concentration as the alginate polymer, the concentration effect was less pronounced between 10 and 25 ppm additive concentrations and growth rate constants obtained at those two G-block concentrations were very similar. Among all types of alginate additives, G-blocks resulted in the strongest inhibition on the overall course of growth. Calcium ion depletion slowed down even further with decreasing supersaturation in the vessel and was inhibited almost completely after consumption of 50% of available calcium (Figure 3B). In the presence of M-block oligomers a reduced rate of calcium consumption was observed when compared to additive free systems, however, growth proceeded further when compared to both alginate and G-block counterparts (Figure 3C).

**Figure 3.** Calcium consumption with respect to time in the presence of different additives with 10 ppm and 25 ppm concentrations (A,B,C). Comparison of additives at equal concentration is given separately for 10 ppm addition (D). The additive free system is included in all graphs. Note the different time scales.

Crystal growth inhibition in the presence of polymeric additives has mostly been attributed to the adsorption of additives on active growth sites on the crystal surfaces. Previously, ourselves and others have reported reduced growth rates for calcium carbonate and hydroxyapatite with addition of alginate, where the growth retarding effect was related to the high affinity of carboxyl groups in alginate towards calcium and subsequent blocking of growth sites.[11](#_ENREF_11),[13](#_ENREF_13) Similarly, polyelectrolytes other than alginate with carboxyl and hydroxyl functionality showed an inhibitory effect on hydroxyapatite and calcium fluoride growth and the adsorption hypothesis was confirmed by fitting of experimental results to Langmuir type adsorption curves.[23](#_ENREF_23),[24](#_ENREF_24) In the present study, alginate and its oligomers are fully deprotonated in the pH range of growth experiments that in turn allows for ionic interactions with calcium ions. Therefore, by reference to the previous studies on calcium phosphate and calcium carbonate systems with alginate and other polyelectrolytes and the similar chemistry in solution, the inhibitory effect of alginate on brushite growth can be related to molecular adsorption mechanisms. Further inhibition by increasing polymer concentration and effective G units in parallel, supports the hypothesis that high affinity functional groups can result in more effective adsorption to active growth sites and thus lower the growth rate. The magnitude of inhibition is increasing with additive concentration but can reach a maximum beyond which growth inhibition becomes less significant.[25](#_ENREF_25). Comparable growth rates observed at two different concentrations of G-block oligomers indicate saturation of the active sites after which the extent of growth inhibition becomes less dependent on the additive concentration. A G-block concentration of 10 ppm can maximally yield ~13 molecules per nm2 of brushite seeds which is high enough for an efficient surface coverage that inhibits growth. An additional experiment performed with 1 ppm G-block oligomers showed that inhibition follows a concentration dependent manner before reaching saturation. (Figure 3B).

The apparent order of growth (*g*) was determined from the rate expression (Equation 2) and used to discuss possible mechanistic models of crystal growth. Although it is not possible to directly relate changes in solution chemistry to microscopic growth processes occurring at crystal surface or distinguish between individual faces without support from direct methods such as AFM, general deductions can be made regarding overall growth mechanisms from indirect bulk measurements.[26](#_ENREF_26)

The growth mechanism of a crystal depends highly upon supersaturation and it is possible to obtain different and multiple mechanisms dominating at different regions of the supersaturation range.[27](#_ENREF_27) In the case of brushite growth without additives seeded desupersaturation experiments resulted in an average *g* value close to 1, which indicates a diffusion controlled growth mechanism. High initial supersaturation (S=2.34) used in the batch experiments can promote high surface nucleation rates resulting in fast surface integration kinetics and hence a diffusion controlled process with a linear dependence on supersaturation. In order to gain more insight into the growth mechanism at low supersaturation, constant composition experiments were conducted and the value of apparent growth order was calculated as 2.40 at the determined range from the slope of the logarithmic *R* vs *σ* curve (Figure 4). The values of the apparent growth orders demonstrated varying growth mechanisms for brushite under two different conditions that correlates with the dependence of the dominating growth mechanism on supersaturation. In desupersaturation experiments most of the growth took place at high supersaturation range where a diffusion controlled process becomes rate determining. The constant composition experiments were conducted at lower values of supersaturation. Spiral growth has been stated as the most dominant growth mechanism for sparingly soluble salts at low to moderate supersaturation range and previous studies of brushite growth showed parabolic rate laws (*g* ≈ 2).[21](#_ENREF_21),[28-30](#_ENREF_28) Surface nucleation might accompany growth when supersaturation is sufficiently high to create new step edges. Therefore, the value of 2.40 can imply a combined mechanism of spiral growth (*g* ≈ 2) and surface nucleation (*g* > 2) which has previously been observed for brushite growth.[21](#_ENREF_21)

**Figure 4.** The plot of rate vs relative supersaturation obtained from constant composition experiments.

Experiments with alginate and G-block oligomers resulted in even higher apparent growth orders indicating a surface nucleation mechanism. In the presence of additives which cause an effective reduction in active growth sites by adsorption, growth may proceed via surface nucleation to generate new growth sites when the driving force is high enough.[31](#_ENREF_31) Thus, the transition in growth mechanism might result from specific interactions between the additive and active sites on the crystal surface. When M-block oligomers were used as additives, *g* values were close to 2 which might be interpreted as growth is still proceeding via spiral growth and mechanism is not changed. Carboxyl groups of M-blocks do not exhibit a specific affinity towards calcium ions, however, nonspecific interactions with the crystal surface are likely to occur via ionic interactions or H-bonding.[10](#_ENREF_10) Oligomer chains might be non-specifically adsorbed onto the crystal surface without significant blockage of active sites and therefore growth mechanism is not altered. However, their presence is effective in decreasing the rate of incorporation as seen evidently from the growth rates. Thus, it can be concluded that the presence of M-blocks affects the accessibility of the active sites in a concentration dependent manner and retards the growth.

**Table 1.** Values of growth rate constants (*k*) and apparent growth orders (*g*)

|  |  |  |
| --- | --- | --- |
|  | *k* (mmol/min.m2) | *g* |
| no alginate | 1.063±0.128 | 1.091±0.086 |
| no alginate\* | 0.962±0.155 | 2.402±0.148 |
| 10 ppm alginate | 0.743±0.016 | 2.779±0.030 |
| 25 ppm alginate | 0.193±0.022 | 3.964±0.441 |
| 10 ppm G-block | 0.116±0.006 | 3.996±0.083 |
| 25 ppm G-block | 0.115±0.003 | 3.395±0.008 |
| 10 ppm M-block | 0.210±0.024 | 2.126±0.095 |
| 25 ppm M-block | 0.096±0.005 | 2.134±0.079 |

\* Values given are calculated from constant composition experiments.

The changes in *g* values and proposed mechanisms for brushite growth in the presence of additives also shed light on the differences in growth behavior at low supersaturation range. In the presence of alginate and G-block additives almost complete inhibition of growth was observed at low supersaturation because certain driving force is needed to continue growth via surface nucleation. However, in the presence of M-blocks crystal growth continued even at low supersaturation range with the spiral growth mechanism.

Unseeded Batch Experiments

Unseeded experiments were conducted to evaluate the effects of alginate based additives on spontaneous crystallization of brushite. Although other calcium phosphate phases were observed within the gel matrix in our model system, all experiments in solution yielded phase pure brushite as the final product, but differences were observed in the progression of the reactions and morphology of the final particles.

Calcium depletion curves for unseeded experiments are given in Figure 5. In the absence of any additive, calcium consumption was fast and the precipitation reaction was complete after approximately 100 min. When high molecular weight alginate was added in the reaction vessel, calcium depletion slowed down in proportion to the polymer concentration. In the presence of oligomers the retardation effect was most prominent. Addition of 10 ppm M-blocks in the reaction medium resulted in a prolonged time interval where calcium concentration and pH changes were very small, and this time lapse was followed by a faster consumption region at a rate comparable to high molecular weight alginate at the same concentration. In the case of G-blocks, the course of precipitation slowed down substantially compared to any other additives (Figure 5 inset).

**Figure 5.** Calcium consumption curves of unseeded experiments with no additive, with alginate and M-blocks at indicated concentrations. Inset shows the calcium consumption curve in the presence of 10 ppm G-block. Note different time scales.

Samples were collected at initial and final reaction time points and at comparable levels of supersaturation from each batch for morphological evaluation by SEM. Appropriate time points were determined by calculations of supersaturation that is dominantly depending on pH. Since all experiments were conducted under nitrogen atmosphere pH values are sufficient input data in order to calculate momentary supersaturation values. Early samples were collected at S≈2.00 revealing the effects of the various additives on initial stage of the precipitation reactions whereas final samples were collected at S≈1.0 for additive free reactions and S≤1.50 for all others, by which point most of the available calcium had been consumed. In the early stages brushite crystals were observed to precipitate with a well-defined, platelet like morphology in additive free experiments (Figure 6A). Crystals precipitated in the presence of 10 ppm alginate were larger initially (Figure 6B) and more prominent size differences were observed for the final products of the reactions (Figure 7B). Since seeded experiments showed that the growth rate was reduced with the addition of alginate, large sized particles observed at same levels of supersaturation illustrates that the nucleation rate was reduced and resulted in a lower number of particles consuming supersaturation for growth. This effect was even more pronounced at the final stages of the reactions. The brushite crystals precipitated in the presence of alginate were much larger than additive free counterparts despite the lower growth rates and higher supersaturation at the end point of reactions. M-blocks did not show a retardation effect on nucleation (Figure 6C). As seen in the calcium and pH depletion curves (Figure 5), high supersaturation levels were maintained for prolonged times in the presence of oligomers which can result in upholding high nucleation rates. Simultaneously reduced growth rates in the presence of M-block oligomers consequently led to precipitation of small size brushite particles (Figure 7C). In precipitation experiments including G-block oligomers, with the same upholding effect on supersaturation for much longer time and lower growth rates, even smaller particles would be expected but the opposite was observed as in the case of alginate (Figure 6D, 7D). These results suggest a retardation effect on nucleation by the G-blocks that are present in both additives, however further explanations can not be offered within the experimental data of this study.

**Figure 6.** SEM images of samples collected at S≈2.0 during unseeded batch experiments with no additive (A), 10 ppm alginate (B), 10 ppm M-block (C) and 10 ppm G-block (D).

**Figure 7.** SEM images of final precipitation products from unseeded batch experiments with no additive (A), 10 ppm alginate (B), 10 ppm M-block (C), and 10 ppm G-block (D). Note the different scale bars.

The morphology of a crystal is determined by the relative growth rates of its individual faces. When additives are adsorbed preferentially to a certain face, its relative growth rate decreases and that face is expressed more in the final morphology. Therefore, observation of the final morphologies may reveal the interactions of alginate based additives with brushite.

In additive free experiments, brushite was precipitated with a typical platelet like morphology with prominent (010) and lateral (*h0l*) faces (Figure 8A). In aqueous solution, it is assumed that the dominant (010) face of brushite crystal is composed of bilayers of structural water molecules and lateral faces express a mixed ionic nature with exposed calcium and phosphate ions.[32](#_ENREF_32) When alginate was added in the reaction medium, large crystals with the same platelet shape were obtained (Figure 8B). In that case, it can be assumed that sterically hindered macromolecules of alginate mostly adsorb on the dominant faces. In contrast, addition of small M-block oligomers with functional groups resulted in particles with a noticeable increase in thickness, which can be interpreted as they were preferentially adsorbed on the lateral faces of brushite crystals primarily due to electrostatic interactions (Figure 8C). In their study of additive interactions with brushite, Sikiric *et. al.* presented similar findings where small molecules with sufficient charge density through functional groups preferentially interacted with lateral faces whereas macromolecules and molecules with hindered structures specifically adsorbed on the dominant (010) face.[33](#_ENREF_33) The most prominent morphological changes were observed in the presence of G-block oligomers where smooth (010) faces were no longer retained. In the presence of G-block oligomers, the specific affinity of the additives towards calcium ions might be more operational than size and charge effects (Figure 8D). This drastic change in morphology was also reflected in the XRD data. The most intense peaks of brushite are found at 2θ= 11.68°, 20.93°, 29.26°, 30.50° and 34.15° that correspond to X-ray diffractions from {020}, {021}, {041}, {-221} and {220} planes, respectively. The plate like structure and dominant (010) face of brushite leads to preferential orientation of the crystals and affects the diffraction pattern by enhancing the intensity of {020} reflection at 11.68°.[34](#_ENREF_34) Although this effect was eliminated to some extent by grinding the samples, it could not be completely removed. Figure 9 shows the comparison of diffraction patterns of the precipitates with ideal isotropic XRD data of brushite at the five highest intensity peaks. As the preferential orientation due to the plate like structure of brushite was disturbed with the presence of G-block oligomers, relative peak intensities showed a closer fit to the ideal case.

**Figure 8.** SEM images of final crystals from unseeded batch experiments with no additive (A), 10 ppm alginate (B), 10 ppm M-block (C), and 10 ppm G-block (D). Note the different scale bars.

**Figure 9.** Relative intensities of XRD peaks of brushite precipitates.

CONCLUSION

Investigations of brushite crystallization in the presence of alginate additives revealed significant effects of compositional variations of alginate on the nucleation and growth processes. Growth kinetics in the presence of additives was studied by seeded desupersaturation experiments and this method was shown to be applicable for brushite growth under specified experimental conditions. Although the constant composition method provides better accuracy and control particularly over the kinetic studies of slow growing minerals at low supersaturation range, for a fast growing mineral like brushite, growth kinetics can be efficiently studied at desupersaturation conditions and a relatively high level of supersaturation. Growth experiments showed decreased growth rates in the presence of all additives, but the extent of reduction and proposed growth mechanisms varied between the different types of alginates used. In the presence of alginate and G-block oligomers specific affinity of G-blocks towards calcium ions resulted in a growth mechanism more dominated by surface nucleation possibly by blocking the active growth sites on the crystal surface. On the other hand, non-specific interactions of M-block oligomers caused a reduction in growth rates, and a parabolic dependence of growth on relative supersaturation which is indicative of a spiral growth mechanism. Differences in growth mechanisms led to changing growth profiles at the low supersaturation region where spiral growth could proceed further, but growth via surface nucleation was almost completely inhibited with decreasing driving force. Unseeded batch experiments showed the effects of additives on the initial stages of precipitation. By decoupling the results of seeded and unseeded experiments, information was deduced regarding the nucleation stage where comparing particle sizes with the knowledge of relative growth rates suggested a retardation effect on brushite nucleation by additives with G- units.

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Nucleation and Growth of Brushite in the Presence of Alginate

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Synopsis

The effects of alginate and oligomers of well-defined M- and G-blocks have been investigated on the nucleation and growth of brushite mineral in terms of possible modes of interaction. Desupersaturation batch experiments have been efficiently used for the growth rate determinations.