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A correlative spatiotemporal microscale study of calcium phosphate formation and transformation within an alginate hydrogel matrix

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ABSTRACT

The modification of soft hydrogels with hard inorganic components is a method used to form composite materials with application in non-load-bearing bone tissue engineering. The inclusion of an inorganic component may provide mechanical enhancement, introduce osteoconductive or osteoinductive properties, or change other aspects of interactions between native or implanted cells and the material. A thorough understanding of the interactions between such components is needed to improve the rational design of such biomaterials. To achieve this goal, model systems which could allow study of the formation and transformation of mineral phases within a hydrogel network with a range of experimental methods and high spatial and time resolution are needed. Here, we report a detailed investigation of the formation and transformation process of calcium phosphate mineral within an alginate hydrogel matrix. A combination of optical microscopy, confocal Raman microspectroscopy and electron microscopy was used to investigate the spatial distribution, morphology and crystal phase of the calcium phosphate mineral, as well as to study transformation of the mineral phases during the hydrogel mineralization process and upon incubation in a simulated body fluid. It was found, that under the conditions used in this work, mineral initially formed as a metastable amorphous calcium phosphate phase (ACP). The ACP particles had a distinctive spherical morphology and transformed within minutes into brushite in the presence of brushite seed crystals or into octacalcium phosphate, when no seeds were present in the hydrogel matrix. Incubation of brushite–alginate composites in simulated body fluid resulted in formation of hydroxyapatite. The characterization strategy presented here allows for non-destructive, *in situ* observation of mineralization processes in optically transparent hydrogels with little to no sample preparation.

Statement of Significance

The precipitation and transformations of calcium phosphates (CaP) is a complex process, where both formation kinetics and the stability of different mineral phases control the outcome. This situation is even more complex if CaP is precipitated in a hydrogel matrix, where one can expect the organic matrix to modulate crystallization by introducing supersaturation gradients or changing the nucleation and growth kinetics of crystals. In this study we apply a range of characterization techniques to study the mineral formation and transformations of CaP within an alginate matrix with spatiotemporal resolution. It demonstrates how a detailed investigation of the mineral precipitation and transformations can aid in the future rational design of hydrogel-based materials for bone tissue engineering and studies of biomineralization processes.

1. Introduction

Hydrogels combined with inorganic materials are attractive candidates in the search for an injectable composite material for hard tissue regeneration. The hydrogel can be used as a carrier

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material for cells, drugs or other bioactive molecules and also act as a scaffold for tissue formation [1]. The inorganic phase provides nucleation sites and the necessary ions for *in vivo* bone formation and also modifies the mechanical properties of the resulting composite material [2,3]. In cases where calcium phosphate (CaP) has been used as the inorganic phase, hydroxyapatite (HAp) has long been the material of choice due to its similarity to the mineral found in bone [4–6]. However, HAp is thermodynamically stable under *in vivo* conditions, and therefore will not readily dissolve and provide ions for bone formation. Therefore, in recent years, less stable CaP phases such as Octacalcium phosphate (OCP) and brushite (the abbreviation DCPD has been used in sample names and figure legends to indicate brushite) have attracted increasing interest in this regard [7]. These acidic phases are often present in the early stages of precipitation *in vitro*, even at mildly alkaline conditions, as they tend to nucleate more easily than HAp [8]. In the more complex *in vivo* environment, evidence of such precursors has been elusive. Whether this stems from the influence of templating molecules or is due to dehydration or other artifacts during sample preparation is not entirely clear. Peptide motifs from dentin matrix proteins have been shown to accelerate the formation of crystalline HAp *in vitro*, which supports the first scenario [9]. On the other hand, using *in situ* characterization techniques or minimal sample preparation there have been reports of several non-apatitic precursor phases during early mineralization, including amorphous phosphate (ACP) and OCP [10]. More recently, ACP has been shown to act as a precursor to HAp during osteogenesis within a ceramic tissue engineering scaffold loaded with bone marrow mesenchymal stem cells and implanted in a murine model [11]. Also, cellularly derived ACP nanospheres have been shown to transform into crystalline platelets of HA upon contact with the collagen matrix of continuously mineralizing fin bones of zebrafish [12]. Similar mineralization pathways have also been suggested for other types of biominerals, such as calcium carbonate found in sea urchins and mollusks [13].

Our group focuses on the formation of alginate–CaP composite materials by counter-diffusion in which mineral is precipitated simultaneously with hydrogel crosslinking. This approach allows control over the resulting CaP phase and has recently been investigated in particular for the formation of HAp and brushite [14,15]. In order to produce phase pure alginate–brushite composites, seed crystals were used to initiate nucleation, since conditions which normally produce brushite when precipitated in solution, resulted in HAp inside the gel network, irrespective of the precursor concentrations and initial pH [15]. Further investigation into this phenomena revealed an inhibitory effect of alginate on the growth and nucleation of brushite in the presence of small amounts of alginate [16].

A thorough understanding of CaP formation and transformation processes is essential for both fundamental studies of biomineralization and for the development of synthetic hard tissue engineering scaffold biomaterials. CaP mineralization, although dependent on reaction conditions such as pH and ionic strength, is often dictated by kinetics rather than thermodynamics. In addition, the crystallization process may be influenced by both (bio) organic molecules and spatial confinement [17–25]. This represents a particular scientific challenge, since it is difficult to precisely monitor mineralization processes *in situ*. We have recently presented a new approach that enables the correlative application of a range of characterization techniques to closely monitor crystallization processes within hydrogels [26]. Here we apply this toolbox to study the formation and transformation of CaP–mineral within an alginate matrix at low pH (approx. pH 5) and the influence of brushite seeds dispersed in the matrix under otherwise identical conditions. The non-destructive characterization techniques were also used to monitor the transformation behavior of minerals within alginate hydrogels during incubation in simulated body fluid (SBF), providing a means to measure the same samples over several time points. This resulted in a thorough spatiotemporal description of the gel and mineral formation, maturation and transformation pathways at the microscale in unprecedented detail.

2. Experimental

2.1. Flow cell samples

De-ionized water (DIW, with a resistivity of 10–15 MΩcm) was used in all of the experiments. Alginate solutions were prepared with 1.8 mass% alginate (LF200S, $M_w = 2.74 \times 10^5$ g mol^{-1}, $F_G = 0.68$, FMC Biopolymer, Sandvika, Norway), 0.9 mass% NaCl (27810.295, VWR, Philadelphia, PA, USA) and a mixture of $Na_2HPO_4{\cdot}7H_2O$ (206515000, Thermo Fisher Scientific, Oslo, Norway) and $NaH_2PO_4{\cdot}2H_2O$ (04269, Sigma Aldrich, Oslo, Norway) to a phosphate concentration of 100 mM or 300 mM at pH 7. A 1.5 μL droplet of alginate solution was placed between two glass slides separated by 140 μm in order to produce a disc. A 1 M $CaCl_2$ (C8106, Sigma Aldrich, Oslo, Norway) solution buffered either at pH 5 with sodium acetate (NaAc) (A6283, Sigma Aldrich, Oslo, Norway) or at pH 7 with tris(hydroxymethyl) aminomethane (TRIS) (252859, Sigma Aldrich, Oslo, Norway) was introduced into the flow cell initiating the gelling and mineralization process as the calcium diffused into the disc. The reaction occurs in a large excess of calcium ions to ensure proper gelation of the alginate. SBF was made according to Kokubo *et al.* [27]. Samples were placed in 50 mL of SBF and the solution was replenished with fresh SBF every 24 h.

2.2. Preparation of crystals for seeding and Raman analysis

Brushite seed crystals were made by mixing 500 mL of 0.4 M $Ca(NO_3)_2{\cdot}4H_2O$ (31218, Sigma Aldrich, Oslo, Norway) and 500 mL of 0.4 M KH_2PO_4 (P3786, Sigma Aldrich, Oslo, Norway) and 26 mM KOH (221473, Sigma Aldrich, Oslo, Norway). The resulting precipitate was aged for 2 h before they were washed and filtered with DIW and ethanol. The size of the crystals was measured using a Coulter Counter Multisizer 3 (Beckman Coulter, CA, USA). The seed crystals were ground using an agate pestle and mortar in order to disrupt any aggregation and 0.2 mass% were added to alginate solutions under stirring. The solutions were left stirring for 1 h to ensure uniform distribution of the seed crystals.

OCP and HAp were made according to methods described by Elliott [28]. Briefly, OCP was made by hydrolysis of brushite crystals in 0.5 M NaAc (pH > 9) at 37 °C for 1 week. The solution was replenished daily. HAp was made by slowly dripping a solution with 640 mM $Ca(NO_3)_2$ into an equal volume of 250 mM $(NH_4)_2HPO_4$ (215996, Sigma Aldrich, Oslo, Norway) under rapid stirring. Both solutions had an initial pH above 10 and NH_4OH (221228 Sigma Aldrich, Oslo, Norway) was used to maintain pH above 10. The resulting precipitate was aged over night.

The resulting crystals were in all cases washed and filtered using DIW and ethanol and crystalline phase purity was measured using powder XRD (D8 Advance DaVinci, Bruker AXS GmBH, Karlsruhe, Germany) prior to Raman measurements, see Figs. S1–S3 in the Supplementary Information.

2.3. Characterization

Dark-field and phase contrast images of alginate samples with varying phosphate content, see Table 1, were recorded using an optical microscope (Eclipse TS100, Nikon Instruments Europe BV, Amsterdam, Netherlands) through a 4× lens at 4 FPS for the first