Acta Biomaterialia 6 (2010) 3665–3675

Contents lists available at ScienceDirect

Acta Biomaterialia

journal homepage: www.elsevier.com/locate/actabiomat

Alginate-controlled formation of nanoscale calcium carbonate and hydroxyapatite mineral phase within hydrogel networks

Minli Xie [a,1], Magnus Ø. Olderøy [a,1], Jens-Petter Andreassen [b], Sverre Magnus Selbach [c], Berit L. Strand [d], Pawel Sikorski [a,*]

[a] Department of Physics, Norwegian University of Science and Technology, Trondheim NO-7491, Norway
[b] Department of Chemical Engineering, Norwegian University of Science and Technology, Trondheim NO-7491, Norway
[c] Department of Materials Science and Engineering, Norwegian University of Science and Technology, Trondheim NO-7491, Norway
[d] Department of Biotechnology, Norwegian University of Science and Technology, Trondheim NO-7491, Norway

ARTICLE INFO

Article history:
Received 13 January 2010
Received in revised form 22 March 2010
Accepted 23 March 2010
Available online 30 March 2010

Keywords:
Biocomposite
Alginate
Hydroxyapatite
Calcium carbonate
Nanostructure

ABSTRACT

A one-step method was used to make nanostructured composites from alginate and calcium carbonate or calcium phosphate. Nanometer-scale mineral phase was successfully formed within the gel network of alginate gel beads, and the composites were characterized. It was found that calcite was the dominating polymorph in the calcium carbonate mineralized beads, while stoichiometric hydroxyapatite was formed in the calcium phosphate mineralized beads. A combination of electron microscopy, Fourier-transform infrared spectroscopy, thermogravimetric analysis and powder X-ray diffraction showed that alginate played an active role in controlling mineral size, morphology and polymorphy. For the calcium phosphate mineralized beads, alginate was shown to modulate stoichiometric hydroxyapatite with low crystallinity at room temperature, which may have important applications in tissue engineering. The results presented in this work demonstrate important aspects of alginate-controlled crystallization, which contributes to the understanding of composite material design.

1. Introduction

In tissue engineering and regenerative medicine, there is a great need for biomaterials which can be used as structural support, as scaffolds for specific cell types or as fillers. These materials must be biocompatible, and biodegradability might be an advantage [1] in providing optimal functionality to the tissue which is to be regenerated. It is also a great advantage for such biomaterials to be lightweight. This is a good incentive to look at biomimetic fabrication strategies, where the structure is highly controlled and optimized at several length scales. In bone-tissue engineering, the mechanical properties of the biomaterial are vital, and may be satisfied by structurally clever designs inspired by fabrication strategies found in nature.

There are numerous examples of organic/inorganic composite materials in nature, where the arrangement of different phases is tightly controlled at several length scales. A good example is bone, in which collagen fibres are mineralized with hydroxyapatite (HA). The HA crystallization is controlled in such a way that the high compression strength of HA and the elasticity of collagen is combined to yield a material with both these properties [2]. The key to understand the structure/function relationship in bone is to study the collagen/HA interplay and structural arrangement from the nanometer to the micrometer scale. HA particle size and alignment in the collagen fibres are vital to the mechanical properties, and a well-defined structure on higher length scales provides macroscopically consistent mechanical properties [3]. Recently, the nanostructure of mineralized collagen was mimicked with the aid of poly-L-aspartic acid [4], which was shown to play an important role in the mineralization process.

It is important to realize that well-defined structures at the nanometer scale in natural materials are a result of interactions between the organic and inorganic phases. For example, Metzler et al. [5] showed, by X-ray adsorption near edge spectromicroscopy, that calcite-interacting peptides and proteins (mollusk nacre-associated polypeptides and sea urchin spicule matrix protein) disrupt C—O bonds in calcite. Ordering of the amino acid side chains as a consequence of polypeptide association with the mineral phase and carboxylate binding was also observed [5]. Metzler et al.'s paper clearly showed that carboxyl groups in aspartate and glutamate participate in polypeptide–mineral associations. Therefore, the biomimetic fabrication approach should utilize such interactions and not depend on preformed mineral particles. A one-step method, where the inorganic phase is formed within an organic matrix to

* Corresponding author. Tel.: +47 73598393.
E-mail address: pawel.sikorski@phys.ntnu.no (P. Sikorski).
[1] These authors contributed equally to this work.

1742-7061/$ - see front matter © 2010 Acta Materialia Inc. Published by Elsevier Ltd. All rights reserved.
doi:10.1016/j.actbio.2010.03.034

produce structural composite materials, would thus be the most versatile and flexible way to mimic the structures found in nature.

Several mineralized biomaterials have been reported in the literature, including calcium phosphate mineralization of alginate gels [6], polyesters [7], poly(ethylene imine) [8], collagen [9,10] and collagen–chitosan scaffolds [11], but little attention has been given to the nanometer-scale structure and the interplay between the inorganic and organic phases in the mineralization process. To take full advantage of the fabrication strategies found in nature, these considerations, which are of vital importance, must be given attention. Developing straightforward fabrication methods which allow for making composite materials with well-defined mineral content, mineral distribution, crystal size and crystal polymorphy is therefore of major importance. Such methods could include one-step preparation of scaffolding and the mineral phase (described here), as well as other strategies to produce mineralized composites, e.g. enzymatic mineralization (where mineral precursors are produced by an enzymatic reaction).

Here we focus on the mineralization of calcium carbonate and calcium phosphate, as these are the two most abundant minerals found in living organisms. Calcium carbonate is usually found in organisms living in water and calcium phosphate in organisms on land. The calcium carbonate system consists of three different anhydrous polymorphs: vaterite, aragonite and calcite, in increasing order of stability. Usually, these are found as polycrystalline spherulites, needle-like particles and single-crystalline cubes, respectively. In nature, however, the morphology is tightly controlled by organic molecules and the mineral is designed to meet the structural needs of the organism [12]. The calcium phosphate system is more complex, consisting of several crystalline compounds, including brushite, octacalcium phosphate, β-tricalcium phosphate (β-TCP) and HA, in increasing order of thermodynamic stability. HA is an important constituent of dental enamel, dentine and bone. In natural materials it is also common to find calcium-deficient, carbonate- or fluoride-substituted HA, these chemical modifications being crucial to the unique mechanical properties of natural mineralized materials [2,13].

Alginates are natural polysaccharides found in seaweed. Their production does not represent a risk of contamination by allo- or xeno-proteins or viruses, unlike, for example, the production of collagen. They are linear copolymers of 1-4-linked β-D-mannuronic acid (M) and α-L-guluronic acid (G). The monomers are arranged in a blockwise pattern along the chain with homopolymeric regions of M and G, termed M- and G-blocks, respectively, interspaced with regions of alternating structure (MG-blocks) [14]. The ratio and order of G and M units are of great importance for the physicochemical properties of the polymer, such as affinity to cations, gelling properties and chain stiffness. Alginates form gels with divalent cations, such as Ca^{2+}, that bind preferentially to the G-blocks in the alginate in a highly cooperative manner [15,16]. Thus, the length of the G-blocks is the main structural feature contributing to gel formation [17,18]. Recently, the alternating sequences of M and G have also been shown to form cross-links with Ca^{2+} [19–21]. Because it is gentle and cell-friendly, the mechanism of gelation has been used extensively in medicine, including cell therapy and tissue engineering applications [22]. In recent years, various alginate gels and composites have been proposed for use in bone engineering [23–25]. Alginate has been shown to effectively reduce the growth rate, but not the nucleation rate, of calcium carbonate vaterite crystals [26] and may therefore be used to control the particle size of the mineral phase in biocomposites. Alginate/HA composites have also been investigated as drug and growth factor delivery systems, where bone-specific glycosaminoglycans were encapsulated in alginate/HA composite beads [27]. It was shown in that study that inclusion of HA increased the encapsulation efficiency of the glycosaminoglycans.

In this work, we used a one-step method to make alginate gel beads mineralized with calcium carbonate or calcium phosphate, and characterized the materials with a wide range of techniques to address structural properties from the nanometer to the micrometer scale. Microbeads were chosen as an appropriate model material in this work to provide simple geometric considerations in the mineralization process and because the spherical shape is easy to reproduce with a narrow size distribution. It was also important to choose a method that offers an easy one-step procedure, which also has the potential to encapsulate cells. Our goal was to produce small (100 nm or less) mineral particles evenly distributed within the beads, which is expected to modify the mechanical properties of the gel network. The motivation to make such materials is the need for well-characterized nanostructured materials with applications in tissue engineering, regenerative medicine, medical implants and three-dimensional cell cultivation.

2. Materials and methods

2.1. Materials

Sodium alginate (FMC Biopolymer) was from *Laminaria hyperborea* stipe, with a molecular weight $M_W = 2.2 \times 10^5$ g mol^{-1}, fraction of G units $F_G = 0.67$, fraction of GG diades $F_{GG} = 0.56$, fraction of alternating M and G $F_{MG} = 0.11$ and intrinsic viscosity of $[\eta] = 1018$ ml g^{-1}. Characterization of the alginate was done by ^{1}H nuclear magnetic resonance spectroscopy [28] (sequence characterization) and by size-exclusion chromatography with online multiangle laser light scattering and viscometry [29] (molecular weight and intrinsic viscosity). $CaCl_2 \cdot 2H_2O$, Na_2CO_3, Na_2HPO_4 and the surfactant Tween-20 were purchased from Sigma Aldrich. All reagents and solvents were of analytical grade and used as received.

A custom-built electrostatic bead generator [30] was used to make the alginate gel beads. The generator basically consists of a power supply of 7 kV, a switch for fine tuning of the applied voltage, an autoclavable holder for 1–10 needles and a safety cage with an electric safety switch. The needles used were made of metal and had an outer diameter of 0.4 mm. The alginate solution was fed by a syringe pump (see Fig. 1).

2.2. Preparation of Ca-mineralized alginate composite beads

Sodium alginate was dissolved in deionized water to a final concentration of 1.8% (w/v) containing 0.9% NaCl with pH 7.2–7.4. For gel beads mineralized with calcium carbonate or calcium phosphate, mineral precursor (Na_2CO_3 or Na_2HPO_4) was added to the alginate solution, according to Table 1. Mineralized alginate beads were prepared by dripping the Na-alginate solution containing mineral precursor into the calcium containing gelling bath (500 mM or 1 M $CaCl_2 \cdot 2H_2O$, 0.9% NaCl, 0.5 ml l^{-1} Tween-20, pH 7.2–7.4) under constant stirring at room temperature. The droplets were left for 1 h in the gelling bath before being removed and rinsed in distilled water. In the following text, the mineralized and non-mineralized alginate beads are represented by abbreviations according to Table 1.

Non-mineralized alginate beads (NMB) were prepared in a similar procedure except that no mineral precursor was added to the alginate solution prior to droplet formation.

Calcium phosphate precipitates were made using MQ-grade water containing 300 mM Na_2HPO_4 instead of alginate solution. The solution was added dropwise into the calcium-containing gelling bath according to the same procedure as making beads.

All synthesized beads were dried in a critical point dryer (CPD; Emitech K850) before characterization with scanning electron