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Crystal growth of calcium carbonate in silk fibroin/sodium alginate hydrogel

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ABSTRACT

As known, silk fibroin-like protein plays a pivotal role during the formation of calcium carbonate ($CaCO_3$) crystals in the nacre sheets. Here, we have prepared silk fibroin/sodium alginate nanofiber hydrogels to serve as templates for calcium carbonate mineralization. In this experiment, we report an interesting finding of calcium carbonate crystal growth in the silk fibroin/sodium alginate nanofiber hydrogels by the vapor diffusion method. The experimental results indicate calcium carbonate crystals obtained from nanofiber hydrogels with different proportions of silk fibroin/sodium alginate are mixture of calcite and vaterite with unusual morphologies. Time-dependent growth study was carried out to investigate the crystallization process. It is believed that nanofiber hydrogels play an important role in the process of crystallization. This study would help in understanding the function of organic polymers in natural mineralization, and provide a novel pathway in the design and synthesis of new materials related unique morphology and structure.

1. Introduction

Calcium carbonate ($CaCO_3$) is an important mineral due to its significance as a biomineral and its various industrial applications such as a raw material for cement, paper coating, and medicine etc. [1,2]. $CaCO_3$ is also an important biomaterial for hard tissues in many organisms including mollusk shells, crustacean exoskeletons, and eggshells [3]. For example, the molluscan shell consists of more than 95% $CaCO_3$, which is present as calcite in the outer prismatic layer and as aragonite in the inner nacreous layer [4]. The unique properties of nacre seem to be due to the low amount of organic matrix (1–5% by weight), which is comprised of three major components: acidic proteins, β-chitin and silk fibroin-like protein [1,4,5,6]. Silk fibroin-like protein, rich in glycine and alanine, is a kind of insoluble protein with a secondary structure of anti-parallel β-sheet. It has been found that silk fibroin (SF) obtained from *Bombyx mori* is much more similar to silk fibroin-like protein in nacre in amino acid sequence and secondary structure [1,7] compared to the conventional synthesized acidic polymers. Therefore, SF has been used as a template to investigate the effect on $CaCO_3$ crystal growth in vitro study [8,9]. Shao et al. combined degummed silk fibers and regenerated silk fibroin (RSF) solution into a template/additive mineralization system to regulate the crystallization of $CaCO_3$ [10]. They found that $CaCO_3$ crystals could be nucleated and grown with uniform orientation along the longitudinal axis of the fiber. Wang et al. found $CaCO_3$ formation steps greatly depended on the presence of SF in mineralization process [11].

β-chitin occurs in many $CaCO_3$-based biomineral. However, in biomineral chitin occurs not only by itself but also constitutes insoluble two-dimensional or three-dimensional scaffolding to regulate and control crystallization in a confined space [12]. In mineralization process, it often requires the cooperation of other organic matrix, such as acidic protein [1], to control crystallization in a confined space. Comparing with β-chitin, sodium alginate (SA) is also one of polyanionic copolymers derived from brown sea algae and comprises linear chain of 1,4-linked β-D-mannuronic (M) and α-L-guluronic acid (G) residues in varying proportions [13]. SA is formed hydrogels in the presence of divalent cations (i.e., Ca^{2+}) through the ionic interaction between the carboxylic acid groups located on the polymer backbone and the chelating cation [14]. These chelating calcium ions provide the location of crystal growth and facilitate the crystal growth. Manoli et al. reported the effect of sodium alginate on the crystal growth of $CaCO_3$. Calcite crystals were grown in the presence of 16.7×10^{-7} M sodium alginate [15]. Xie et al. used alginate hydrogel network to control nanoscale calcium carbonate and hydroxyapatite. The result showed that calcite was the dominating polymorph in the calcium carbonate mineralized beads, while stoichiometric hydroxyapatite was formed in the calcium phosphate mineralized beads [16].

In recent years, $CaCO_3$ has been intensely studied with the aim of understanding how crystal polymorph and structural features can be controlled by organic additives. Some additives such as collagen [17], glutamic acid [18], chitosan [19], silk fiber [10] and

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silk-like proteins hydrogel [20–22] are used to control the crystallization of $CaCO_3$ in recent researches [23]. It demonstrates that SF protein is an excellent additive to control $CaCO_3$ morphology and crystal polymorph [24]. However, in several works, the mineral growth environment occurred in gel state [1]. The probable mechanism of $CaCO_3$ growth in gel state was not clearly elaborated. In our research, we used a silk fibroin/sodium alginate (SF/SA) nanofibers hydrogel system to regulate and control $CaCO_3$ crystal growth. SF protein and SA polysaccharides can be formed nanofiber hydrogel biopolymer system, which can better mimic the real mineralization system of nacre more than single protein system. The motivation for this research is to explore how the crystal growth of $CaCO_3$ is regulated and controlled in SF/SA nanofibers hydrogels. By studying the crystallization process in SF/SA hydrogel, a continuous change in the morphology and polymorphic nature of $CaCO_3$ crystals was observed during different stages of mineral process, which indicated that the nanostructure and spatial structure of hydrogels influenced the formation of structure and the crystallization of $CaCO_3$. Generally speaking, our work might not only offer an effective approach to design and synthesis of new materials, but also provide a new viewangle to investigation of the mechanism of biomimetic mineralization in hydrogel.

2. Materials and methods

2.1. Materials

Bombyx mori silk was bought from Zhejiang province, China. Sodium alginate was purchased from Sinopharm Chemical Reagent Co. Ltd. (Shanghai, China), and used without any further purification. All chemical reagents (lithium bromide, sodium carbonate, calcium chloride, ammonium carbonate, ethanol, etc.) were analytical grade, and also used without any further purification.

2.2. Preparation of regenerated SF solution

Bombyx mori silk fibroins were prepared according to our previously published procedures [25]. Raw *Bombyx mori* silk fibers were boiled in 0.05 wt% Na_2CO_3 solution for 30 min and then rinsed thoroughly with deionized water to extract the glue-like sericin proteins. Each step was repeated twice, and finally, the degummed silk fiber was dried at room temperature. The dried degummed SF was dissolved in a mixture of solvent composed of LiBr/Ethanol/H_2O (45/44/11, wt/wt/wt) at 70 °C for 4 h, yielding a 10 g dL^{-1} solution. This solution was dialyzed with deionized water for 4 days to remove the salt ions. Then, the solution was filtered and the final SF aqueous solution with desired concentrations was prepared.

2.3. Preparation of SF/SA nanofibers hydrogel

SA (2 g) was dissolved in deionized water at room temperature. The solution was mixed under constant stirring in a blender for 1 h, standing 24 h at 5 °C to obtain a uniformly 0.5 wt% SA solution. And then, the SF and SA aqueous solutions with various ratios were mixed by stirring, and the concentration of the mixture solution was controlled at 1.0 wt%. The various ratios of SF/SA solution were 100/0, 90/10, 80/20, 70/30, 60/40, and 50/50, respectively. All the solutions were stored overnight at 5 °C to achieve homogeneity and to avoid any premature precipitation of the protein, which occurred at room temperature. Finally, Hydrogels were prepared by adding 5 mL of blended solution in 24 well plates (Corning, USA). The solutions were allowed to gel in an incubator at 37 °C.

2.4. SF/SA hydrogel mineralization

The mineral process was used to grow $CaCO_3$ crystals in the SF/SA nanofibers hydrogels. First, SF/SA hydrogels were treated in 75% (v/v) ethanol aqueous solution for 30 min to prepare the water-insoluble hydrogels. The water-insoluble hydrogels were immersed directly in 0.1 M $CaCl_2$ solution for 1 h at room temperature and washed twice with deionized water to remove free ionic calcium. Then, the samples were placed in the desiccators containing ammonium carbonate. After being reacted for 6 h at 5 °C, the final samples were rinsed with distilled water and lyophilization for characterization.

2.5. Characterization

The morphology of SF/SA hydrogel and its $CaCO_3$ crystals growth was examined by SEM (S4800, Hitachi, Japan). Samples for the SEM experiment were observed with gold coating. At the same time, energy dispersive X-ray spectroscopy (EDS) was employed to determine the elemental composition. XRD experiments were carried out to investigate the secondary structure of hydrogel and the $CaCO_3$ crystal growth by using X Pert-Pro MPD (PANalytical, Netherlands) in transmittance mode. The incident beam wavelength was 0.154 nm. The intensity was finally corrected for changes in the incident beam intensity, sample absorption and background. To further confirmed the conformation structure of all samples, these samples were analyzed by FTIR on Nicolet5700 (Thermal Nicolet Company, USA) in absorbance mode. For each measurement, each spectrum was obtained by the performance of 32 scans with the wave number ranging from 400 to 4000 cm^{-1}.

3. Results and discussion

3.1. Preparation of SF/SA nanofiber hydrogels

SEM was used to examine the morphologies of the SF/SA hydrogels as well as the controls. Fig. 1 shows a series of SEM images acquired from different proportions of SF/SA hydrogels at 37 °C. The pure SF hydrogel shows homogeneous nanofibers morphology with an average 5 μm diameter (Fig. 1a). At the same time, Fig. 1a also depicts nanofibers morphologies for pure SF hydrogels with larger pore sizes ranging from 1 to 50 μm, which is different from typical interconnected macroporous morphology [26]. With a low SA content 10.0 wt% in the SF/SA hydrogels, nanofibers structures were also formed through self-assembly in gelation process (Fig. 1b). Many small nanofibers were assembled into larger nanofibers with small pores distributed inside. After adding 20.0 wt% SA in hydrogels, bundles of nanofibers was interconnected into macroporous morphology with many small pores (Fig. 1c). When the SA content increased to 40.0 wt%, the texture of hydrogels was an open nanofibers framework with a diameter of 1–5 μm (Fig. 1d and e). Bundles of nanofibers were gathered together. The most frequent contact between nanofibers was at nodes connecting the ends of other nanofibers. This kind of branching suggested that the nanofibers have been formed as a network, and the hydrogel was not the result of the random aggregation of independently formed nanofibers [27]. In addition, when the SA content was 50.0 wt%, Fig. 1f shows the morphology from nanofibers to flake-like in SF/SA hydrogels.

Structural changes in the SF/SA hydrogels were examined by XRD and FTIR (Fig. 2). Fig. 2A is the XRD results of SF/SA hydrogels with different SA content. Peaks at 9.0° and 20.5° appearing in the XRD spectra of pure SF hydrogel sample were attributable to the silk II (β-sheet) conformation; at the same time, peak at 24.3° was attributable to the silk I structure (Fig. 2A-a) [28,29]. Therefore, the XRD results indicated that the silk I and II crystalline structure