



Crystallization of calcium carbonate mineral with hierarchical structures regulated by silk fibroin in microbial mineralization system

Tao Chen^{a,d,1}, Peiheng Shi^{a,1}, Yi Li^a, Jian Zhang^a, Tao Duan^{a,d}, Yang Yu^c, Jian Zhou^b, Wenkun Zhu^{a,d,*}

^a State Key Laboratory of Environmentally Friendly Energy Materials, Southwest University of Science and Technology, Mianyang, Sichuan 621010, PR China

^b Biomass Materials Ministry of Education Engineering Research Center, Southwest University of Science and Technology, Mianyang, Sichuan 621010, PR China

^c Mianyang People's Hospital, Mianyang, Sichuan 621010, PR China

^d Sichuan Co-Innovation Center for New Energetic Material, Southwest University of Science and Technology, Mianyang, Sichuan 621010, PR China

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ABSTRACT

In this work, we provide a novel idea to introduce silk fibroin (SF) into the microbial mineralization system and study the mechanism of mineralization of calcium carbonate by changing the mineralizing time and SF concentration. CaCO₃ superstructures with complex forms and hierarchical surface textures were obtained in the presence of SF as a crystal growth modifier in the microbial mineralization system. Moreover, with the prolonged reaction time, the shape of CaCO₃ in the additive-free mineralized system changes from a rough spherical to a smooth one, and then to a complex cube-like crystals. Surprisingly, superstructures CaCO₃ crystals with smooth hemispheres were obtained in low concentrations of SF. An emergent self-organization process and its combination with an aggregated mechanism have been proposed for the formation of the complex hemispherical superstructure with numerous fish-like flakes on the surface in high concentrations of SF. What's more, we found that high SF concentration is favorable for the formation of vaterite. The above results imply that the specific biomimetic synthesis strategy in the microbial mineralization system can provide a useful pathway to produce inorganic or inorganic-organic hybrid materials with a unique shape and specific textures.

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1. Introduction

Biom mineralization refers to the formation process of the ordered, functional, and function-specific organic-inorganic composites under the control of organic substrates in the body during metabolism in organisms [1–5]. Inspired by formation process of biom mineralization occurred in the nature, some artificial mineralization using bio-inspired techniques and mechanisms of formation has drawn considerable attention and remains to be a hot academic topic up to now.

Calcium carbonate is an industrially and scientifically important mineral because it resides in different organisms for protection or structural support, and also has been widely used in many industries for plastics, papermaking, cosmetics, paints, and recently pharmaceutical applications [1,6,7]. As a kind of biom mineralized minerals widely existed in the nature, calcium carbonate generally

includes three kinds of crystalline phases: calcite, aragonite and vaterite [8–10]. Aragonite and calcite have a better thermal stability than the other [8,11].

In the process of inorganic mineral calcium carbonate formation, not only are calcium ions and carbonate ions involved, but also a variety of inorganic small molecules [12–21] (such as phosphate ions, magnesium ions, etc.) or macromolecular macromolecules (such as hydroxyl group or carboxyl group Proteins) play an irreplaceable role [3,22–27], which affect the nucleation and growth of calcium carbonate crystals in a unique way and regulate their phase and shape. In the process of simulating biom mineralization, in order to better understand the biological mineralization mechanism, the vast majority of researchers have studied the effects of various additives on the mineralization of calcium carbonate [10,28–31].

As an organic macromolecule template and one of the most important substances in organisms, protein can influence the growth, spatial orientation, crystal form and shape of calcium carbonate crystals through its own pre-assembly in the process of biom mineralization [3,22,23,27]. Xue et al. studied the effects of three membrane materials on the shape, crystal structure and orientation of calcium carbonate mineralization with bovine serum

* Corresponding author at: State Key Laboratory of Environmentally Friendly Energy Materials, Southwest University of Science and Technology, Mianyang, Sichuan 621010, PR China.

E-mail address: zhuwenkun@swust.edu.cn (W. Zhu).

¹ These authors contributed equally.

albumin, pepsin and collagen as membrane materials [32]. Cheng et al. successfully prepared two typical calcium carbonate crystals using silk protein as an additive, one is a hollow rice grain (fusiform) calcite crystal, and the other is a solid spherical calcite and aragonite mixed crystal [22]. Zhang et al. successfully obtained dumbbell-shaped and rhombohedral calcium carbonate crystals by silk protein microspheres as an organic template [33]. Liu et al. successfully prepared rhombohedral crystals using casein, and at the same time they demonstrated that the stabilizing effect of casein resulted from the electrostatic attraction between the phosphate groups and the carbonate (especially the former) and the calcium ions, thus preventing the transition from unstable ACC to a more stable CaCO_3 crystalline phase [25].

Previous studies have focused on the control of the calcium carbonate crystal shape by adding proteins during the physicochemical synthesis of calcium carbonate crystals [12,24,34,35]. A few researchers used microbial mineralization to regulate the shape of calcium carbonate crystals. Hereupon, we introduced the microbial mineralization system into the field of crystal design. Taking Carbonate mineralization bacteria (*Bacillus pasteurise*) [36,37] as the research object, we have studied the shape and crystal phase of CaCO_3 by controlling the mineralization time and adding different concentrations of SF. This work can provide some theoretical basis for understanding the process of calcium carbonate mineralization in vivo.

2. Materials and methods

2.1. Materials

All chemicals are of analytical grade. Glucose, Anhydrous ethanol, Peptone, Beef paste, NaCl, LiBr, $\text{Ca}(\text{NO}_3)_2$ and Na_2CO_3 were purchased from Chengdu Cologne Chemical Co., Ltd., and Bombyx mori silk from local farmers. And water was deionized.

2.2. Methods

2.2.1. Strain culture

Bacillus pasteurise was inoculated into beef extract-peptone agar medium (beef extract 10 g/L, sodium chloride 5 g/L, peptone 5 g/L, agar 15 g/L, H_2O 1 L, pH value 8.0). *Bacillus pasteurii* was inoculated into a liquid medium (glucose 20 g/L, sodium chloride 10 g/L, peptone 10 g/L, H_2O 1 L, pH 8.0). The bacteria were cultured in a biochemical incubator at 30 °C for 24 h.

2.2.2. Preparation of SF solution

The degumming and dissolving process of *Bombyx mori* silk were followed by established procedures [38]. Silk fibers were boiled for 30 min in an aqueous solution of 0.02 M Na_2CO_3 , and then rinsed thoroughly with distilled water to extract the sericin fibroins. Then the extracted SF was dissolved in 9 M LiBr solution at 80 °C for 4 h, yielding a 20 w/v% solution. The solution was dialyzed against distilled water using a dialysis tube for 2 days for removing salt. The dialyzed solution was centrifuged at 5000 rpm for 10 min for removing silk aggregates formed during the process. The final concentration of aqueous SF solution was about 5 wt%, determined by weighing the remaining solid after drying.

2.2.3. Preparation of calcium carbonate by microbial mineralization in the presence of SF

1 L of liquid medium was prepared with deionized water, glucose 20 g, peptone 10 g, and NaCl 5 g and placed in a sterilizer at 120 °C for 20 min, then taken out on a sterile operation table for natural cooling. And 0.05 mol/L of $\text{Ca}(\text{NO}_3)_2/\text{Urea}$ was added to the medium by the microporous membrane filter sterilization

method. Then 0 ml, 2 ml, 4 ml, 6 ml, 8 ml SF solution were separately pipetted into the above liquid medium, respectively. Finally, 5 ml of the bacterial liquid was inoculated into the above liquid medium. The liquid medium was incubated at 30 °C for 6, 12 and 18 h, respectively. The mineralized sample obtained by static and suction filtration was washed three times with distilled water and absolute ethanol, and then dried at 45 °C for 24 h.

2.2.4. Preparation of calcium carbonate by chemical method in the presence of SF

In a typical procedure, the solutions of calcium nitrate (0.05 mol/L, 30 ml) and SF solution (4 ml) were mixed with mechanic stirring for 5 min to obtain a mixed solution. Then, an anhydrous sodium carbonate solution (0.05 mol/L, 30 ml) was added rapidly to the mixed solution with stirring under ambient condition. The mineralized sample was obtained by static and suction filtration, and the mineralized sample was washed three times with distilled water and absolute ethanol, and then dried at 35 °C for 12 h. For additional details, please see the materials in the supporting part.

2.3. Characterization

The samples amplified 1000–20000 times were observed by the SEM. XPS analyses were carried out with a Kratos Axis Ultra photoelectron spectrometer. In the temperature range of 40–900 °C, samples were characterized by TG at the heating rate of 20 °C/min. The crystal forms were analyzed by X-ray diffraction analyzer, Cu K α radiation, wavelength 1.54056 Å, tube voltage 40.0 kV, tube current 100 mA, scan range 3–80°, and scan step 0.02°. FT-IR spectra of samples were taken by Nicolet Avatar 370 of FT-IR spectrometer at wavenumbers from 4000 to 400 cm^{-1} . The samples mixed with KBr were compressed into tablet and analyzed by infrared spectroscopy, resolving power 4 cm^{-1} , scanning 20 times, scanning range of 4000–400 cm^{-1} .

3. Results and discussion

3.1. Effects of mineralization time on shape of calcium carbonate in the presence of SF

In this study, it has been found that the shape of calcium carbonate can be effectively modified by adding SF in the process of preparing calcium carbonate physically or chemically. Fritz Vollrath et al. [22] successfully prepared rice grains calcium carbonate by controlling the molecular weight of SF. It stimulates great curiosity about whether SF has a certain influence on the shape of calcium carbonate in the microbial mineralization system or not. So we have conducted experiments to verify the effects of SF on microbial mineralization of calcium carbonate. Representative SEM images of the mineralized samples collected in the presence of SF after 6 h of reaction are shown in Fig. 1. Fig. 1a shows spherical CaCO_3 particles obtained in the mineralization time of 6 h. Spherical calcium carbonate crystals gradually grow with the increase of mineralization time. The spherical particles were partially converted into hemispherical calcite with superstructure after 12 h (Fig. 1b). It can be seen from Fig. 1(c, d) that the spherical particles were all converted into hemispherical with superstructure in the mineralization time of 18 h. However, the shape of CaCO_3 had changed from an initial rough spherical structures to a kind of smooth spherical, and then to a complex cube-like superstructure in the microbial mineralization system with the absence of any additive, in mineralization times varied from 6 to 12 h and finally to 18 h, respectively (Figs. S1, S2, Supporting Information). From this we can see that SF has a huge effect on the shape of calcium carbonate.

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