Orientated Growth of Hemispherical Calcium Carbonate Beneath Protein Langmuir Monolayer

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Abstract

Oriented calcium carbonate crystals have been grown beneath bovine serum albumin (BSA) Langmuir monolayers at the air-solution interface from supersaturated calcium bicarbonate solutions. The calcium carbonate prepared was characterized by XRD, SEM, TEM and SAED. It was found the crystal morphologies changed from amorphous calcium carbonate (ACC) to regular hemispherical calcite crystals with the prolonging of the time. These observations suggest that the process is step by step, during which the orientation of the crystalline phase was controlled by the BSA Langmuir monolayer template. These results provide new insight into the proteins-biomineral interaction, which can be extended for shape control over other minerals or inorganic-organic hybrid materials.

Subject Areas

Composite Material

Keywords

Hemispherical Calcium Carbonate

1. Introduction

Self-assembled two-dimensional protein layers play an important role in biomineralization processes. A well-studied example is mollusk shell nacre where the protein-rich layer is dominated by β-sheet secondary structures [1], and it is assumed that the acidic residues in this two-dimensional protein structure provide an epitaxial match to the (001) face of aragonite, which is thus favored in the crystallization of $CaCO_3$ [2] [3]. In addition, the secondary protein structure

seems to be associated with the CaCO₃ polymorph where a change from aragonite to calcite is coupled with denaturing of the antiparallel β-sheet structure [4]. Also, the secondary structure of amelogenin was suggested to control the architecture of the hydroxyapatite phase in human enamel [5], so that changes of protein secondary structures appear to be a highly attractive additional handle for crystallization control in biomimetic mineralization approaches.

Thin membrane-like films at the air-solution interface (Langmuir monolayers) provide a more ideally flat organic surface with controllable chemical composition and molecular density [6]. These surfaces have been used as templates to direct the crystal nucleation and growth of ice, semiconductor particles, and a variety of minerals including calcium carbonate, and barium sulfate [7] [8] [9] [10]. However, they all used small surfactant molecules such as fatty acids etc. as templates to control the crystal nucleation and growth of materials. Although these surfaces can control the crystal nucleation and growth, it is disadvantageous for studying the mechanism of biomineralization.

Proteins normally have a high molecular weight, a large relative size, and a very flexible structure that can change conformation according to the experimental conditions. In addition, they can contain a high number of polar, hydrophilic groups that can make the molecule interact with the ions in the subphase. They also contain a large number of nonpolar groups that dislike being in contact with water. This combination of different groups makes a great number of proteins thermodynamically stable at the air/water interface [11] [12] [13] [14]. A lot of documents indicated the bovine serum albumin can form stable monolayers by spreading it at the air-water interface, which holds α-helical structure [15]. Thus, BSA Langmuir monolayer with a specific secondary structure can be used as a model of biomineralization to study the nucleation and growth of crystals.

2. Experiments

2.1. Materials

Analytical grade CaCO₃ was obtained form Institute of Biological Products of Tianjin (Tianjin, China). Analytical grade bovine serum albumin (Sigma, USA) was used in all experiments. BSA contains 582 amino acid residues and has a molecular weight of 67,000 g·mol⁻¹. It is also well known that it has an isoelectric point at pI 4.7. Amyl alcohol was analytical purity obtained from the sigma. All solutions were prepared with triply deionized water, its conductivity was resistance of 18.2 MΩ·cm⁻¹ and its pH was 7.0.
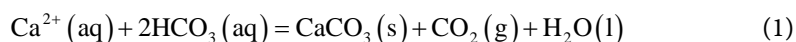
The preparation of the protein Langmuir monolayers was performed using a commercial LB trough (KSV mini-trough, Finland) at room temperature. The pressure/area isotherms were recorded using a computer-controlled Langmuir film balance. The experimental errors for both molecular area and surface pressure values are smaller than 5%. The monolayers deposition process at the air/solution interface was carried out using a microsyringe. The protein solutions were prepared with the deionized water, but to improve the spreading

process a 0.05% (v/v) solution of amyl alcohol was added in all cases [16].

Supersaturated solutions of calcium bicarbonate were prepared according to the procedures of *Kitano* [17]. Briefly, carbon dioxide gas was bubbled through a stirred aqueous suspension of $CaCO_3$ for 24 hours. The suspension was then filtered and filtrate purged with CO_2 gas for 0.5 hour to dissolve any remaining crystals. The resulting supersaturated solution had a pH of 7. Total Ca^{2+} concentrations (2.5 mM) were measured using EDTA titration. The spreading monolayers at the air-water interface were formed by spreading solutions of BSA (1×10^{-4} mol·L^{-1}) on the pure water surface or Supersaturated solutions of calcium bicarbonate. A 30-min lapse time was estimated to be sufficient to equilibrate the protein monolayers before compression. We used very low compression rates, ca. 3 mm/min, which have been shown to be appropriate to obtain reproducible BSA isotherms [18].

2.2. Crystallization under Langmuir Monolayers

In all experiments, crystallization was governed by the slow loss of CO_2 gas from unstirred supersaturated solutions according to the reaction shown in Equation (1).

$$Ca^{2+}(aq) + 2HCO_3(aq) = CaCO_3(s) + CO_2(g) + H_2O(l) \qquad (1)$$

The prepared supersaturated calcium bicarbonate solutions were poured into a Langmuir trough (KSV mini-trough), model 611 M; maximum working area, 220 cm^2), and the air-water interface was swept and aspirated before deposition of the surfactant solution. The surfactant solution (10 μL) was carefully deposited onto the solution surface, and the monolayers were left for 30 min prior to compression. Pressure-area isotherms were recorded while compressing the monolayers at rates of 3 mm/min until surface pressures corresponding to a target pressure were reached (10 mN·m^{-1}, 15 mN·m^{-1}, 20 mN·m^{-1}, 25 mN·m^{-1}). Each experiment was repeated three times with the same condition. Crystals as-grown in association with the monolayers were respectively removed after 6h by carefully horizontally dipping hydrophilic glass slides through the air-water interface. The crystal face growing into the solution is therefore directly deposited on the glass slide.

2.3. Characterization of Crystal Phase and Morphology of $CaCO_3$

The sizes and morphologies of $CaCO_3$ were characterized by using SEM on JSM-5600LV scanning electron microscopy (Jeol. Ltd. Japan) with operating at 30 kV. The slides supporting the crystals were mounted on copper sample stubs with conducting carbon tape and were sputter-coated with gold prior to viewing.

3. Results and Discussion

3.1. The ∏-A Isotherms of Spreading Langmuir Monolayer on Pure Water and Calcium Carbonate Solutions

Figure 1(a) shows the surface pressure-area isotherms of BSA spreading on a

pure water surface as well as on the subphase of supersaturated calcium bicarbonate. BSA formed a Langmuir monolayers structure on a pure water surface as reported previously [19] [20]. When spreading BSA on the subphase of supersaturated calcium bicarbonate, on the one hand, larger changes were observed. The isotherms on the subphase of supersaturated calcium bicarbonate solution became condensed and showed a much lower initial surface pressure compared with that on pure water subphase. The limiting area was about 750 Å^2 molecule^{-1} on calcium bicarbonate subphase. It is also well known that BSA has an isoelectric point at pH 4.7, however, the pH value of supersaturated calcium bicarbonate solution is about 7, which made BSA Langmuir monolayers with negative charges, this result strongly electric interaction between BSA Langmuir monolayers and Ca^{2+} ions in the subphase. At the same time, we study the dependence of barrier position on time through the curve of time-barrier position (as shown in Figure 1(b)) when the subphase is a supersaturated calcium bicarbonate solution. It indicates that the barrier position increases with time when the surface-pressure keep at different values, in other words, the area between the two barriers gradually reduces with time, which could further account for the interaction-existing between the BSA Langmuir monolayers with Ca^{2+} ions in the subphase solution, which make the area of monomolecular diminish. On the other hand, although the limiting area per molecular and initial surface pressure have a larger change, the shape of surface pressure-isothermal held a same trend with onto the pure water subphase, which may indicate the BSA Langmuir monolayer keep the same secondary structure onto two subphases of pure water and supersaturation calcium carbonate.

3.2. SEM and TEM Images

It was observed that the almost similar morphologies of $CaCO_3$ could be formed when it is grown in BSA Langmuir monolayers at different times, as shown by the SEM images reproduced in Figures 2(a)-(d). However, the size of the $CaCO_3$ particles undergoes a series of change as shown in Table 1.
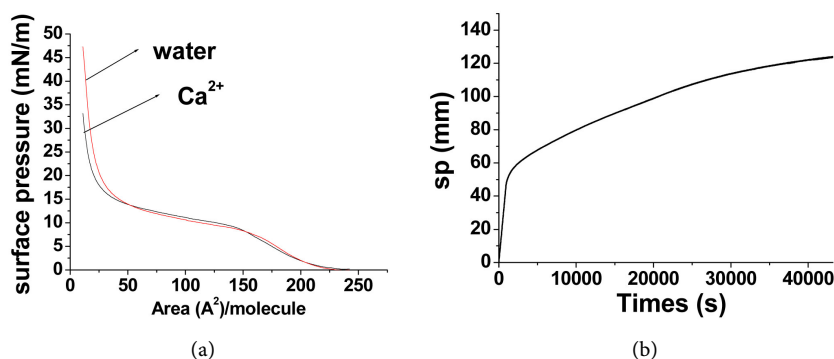

Figure 1. The surface pressure-area isotherm of BSA spreading on pure water subphase and subphase containing supersatured calcium bicarbonate (a), the barrier position-time curve of BSA Langmuir monolayers on calcium bicarbonate when compressing and keeping for 12 h (b).
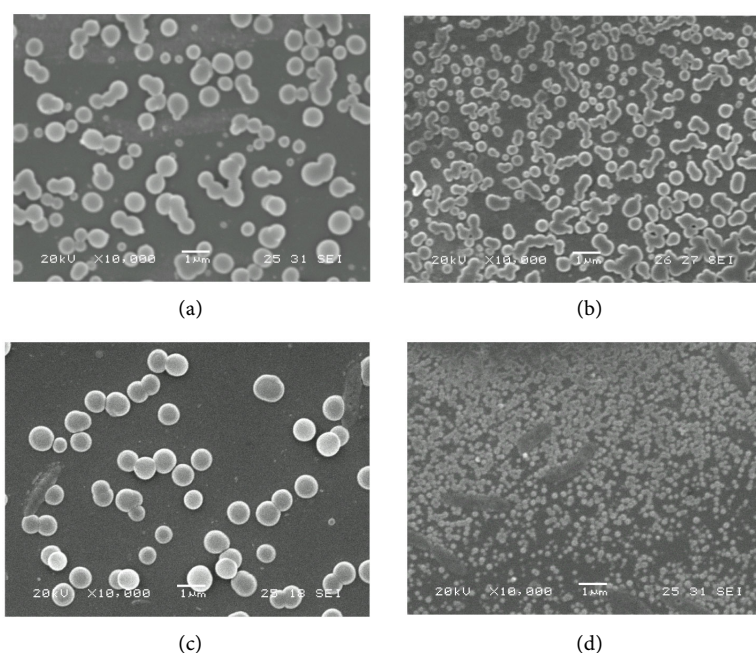
Figure 2. SEM images of CaCO₃ particles obtained in presence of BSA Langmuir monolayers a denotes 8 h, b denotes 12 h, c denotes 24 h and d denotes 48 h, respectively.

Table 1. The size of CaCO₃ particles formed beneath BSA Langmuir monolayer at different times.

Times (h)	8	12	24	48
Size (nm)	300	200	700	100
Number	few	more	few	more

Simultaneously, TEM image shows that the calcium carbonate formed spherical particles or aggregates with average size about 200 nm after 12 h, which is consistent with the results of SEM as shown in Figure 2(b), and the corresponding SAED indicates forming amorphous phase of calcium carbonate as shown in Figure 3(b), in this connection, the following XRD patterns give a uniform conclusion as shown in Figure 4(a). Amorphous calcium carbonate hemi-spherulites are transparent and become opaque with time. Transition of amorphous calcium carbonate hemi-spherulites into a crystalline phase was also detected from the following XRD patterns as shown in Figure 4(b), Figure 4(c).

3.3. XRD Patterns

Figures 4(a)-(d) show that XRD patterns of the resulting calcium carbonate at three different time. Figure 4(a) and Figure 4(b) show that the XRD pattern of calcium carbonate in the presence of BSA Langmuir monolayers, indicating that the amorphous CaCO₃ was formed before 12 h. When the crystallization time is 24 h and 48 h, it could be seen that there are only one peak corresponding to calcite (104) plane of rhombohedral phase (JCPDS 86-2342) as shown in Figure 4(c) and Figure 4(d), which shows a well preferential orientation.
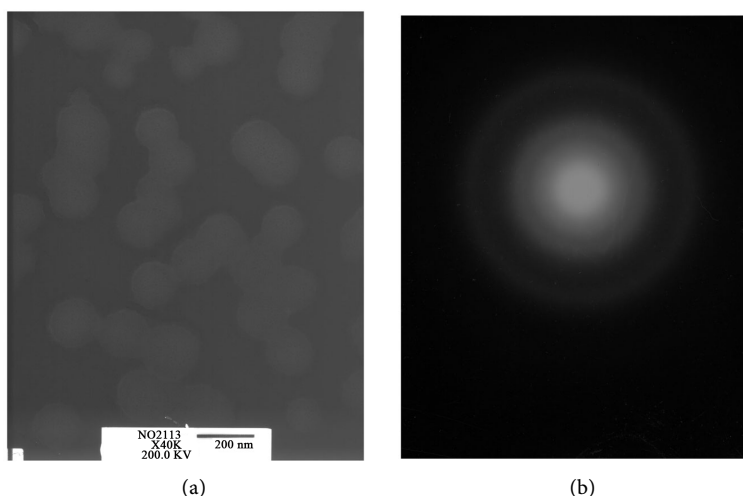
(a) (b)

Figure 3. TEM image (a), and corresponding SAED of CaCO₃ particles obtained (b) in presence of BSA Langmuir monolayers after 12 h.
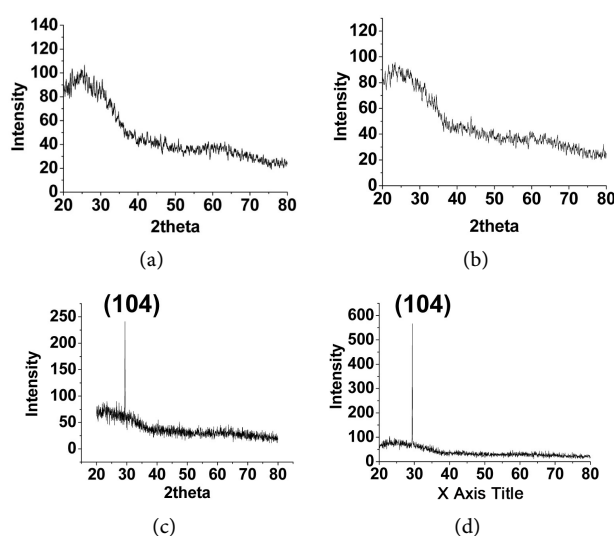

Figure 4. XRD patterns of CaCO₃ in presence of BSA Langmuir monolayers at different time, A, B and C denote the XRD patterns of CaCO₃ formed at 8 h, 12 h, 24 h and 48 h respectively.

4. Discussions

4.1. Molecular Recognition between the BSA and Calcite

The results clearly shown that the BSA Langmuir monolayer could powerfully affect the nucleation and crystallization of calcium carbonate. The morphology of calcium carbonate does not at different times. With regard to the secondary structure of BSA molecular, it adopts α-helix structure, which arrays along certain direction at the interface at the interface of air-solution. The special nucleation sites were formed, which caused the characteristic morphologies of calcium carbonate. From the viewpoint of proteins-biomineral interaction, BSA moleculars may recognize and bind to specific crystal faces, affecting growth and morphology. In the whole process of crystallization, the BSA moleculars may be
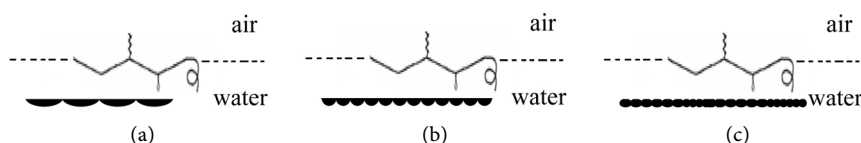
used as a process-directing additive to control the transformation from the ACC to calcite. Generally, geometrical matches and electrostatic attraction might play an important role in the nucleation and growth of calcium carbonate.

4.2. The Mechanism of Nucleation and Crystallization of Calcium Carbonate

It is well known that the crystals formation involves two steps: nucleation and growth. Let us look at the general nucleation process: the constituent molecules or ions in the solution may, on collision, join into groups of two or more particles to form dimmers, trimers, tetramers, and so forth. Before the embryos can reach a critical radius, r_c, they are unstable. To reach r_c, an energy barrier, the so-called nucleation barrier, needs to overcome. In which way and at which number the embryos reach the critical radius is main concern. When the nucleation barrier is overcome, the second stage of the phase transition begins: growth.

In our experiments, in the initial stage of nucleation of calcium carbonate, the transformation of calcium carbonate from the unstable amorphous calcium carbonate to the most stable calcite was proved by SAED and XRD patterns. According to above results, the process of nucleation and crystallization of calcium carbonate may be as following (Scheme 1): firstly, calcium ions binding to BSA monolayer, which is a prerequisite for subsequent nucleation, then formed ACC, at last transformed into the most stable phase calcite. Since ACC formed under conditions of sequential precipitation would be the most soluble phase, a lower energy barrier is expected according to the Ostward' law [21] [22]. So it should be the first solid phase formed during the crystallization and therefore could be prevalent in biomineralization [23] [24].

Dissection of the crystallization process into several stages could make the activation energy of each step lower than that of the one-step precipitation. A multistep crystallization process is plausible, especially in a biological environment in which temporal modifications of the crystallization kinetics may be prevalent. The existence of several phases would enable organisms to control mineralization through intervention with the kinetics. By selectively interaction with the mineral at different stages during the crystal forming process, the organisms could choose to manipulate both the polymorph and the orientation of the mineral to meet specific biological requirements. Given the great diversity in the phase, morphology, and orientation of biominerals, it may be possible that complicated multistep crystallization mechanism exits [25]. The results of this paper provided direct evidence in a model system for a multistep crystallization

Scheme 1. Proposed mechanism for the formation of dumbbell-shaped $CaCO_3$ particles.

process that may be an important pathway for biological mineralization. This

sequence of events stands in contrast to the prevailing view of epitaxy crystal, which is coincident to that of the calcium carbonate formed by many organisms [26] [27].

5. Conclusion

BSA Langmuir monolayer has played a critical role in the nucleation and crystallization of calcium carbonate. Calcium ions binding to the head groups of molecules at the interface of film-solution are a prerequisite of subsequent nucleation. Geometrical matches and electrostatic attractions between the monolayer and calcium carbonate were the main factors in determining the size and morphology of calcium carbonate formed. The observation of the transformation of calcium carbonate from ACC phase to the crystalline phase calcite provided direct evidence for the multistep-stage crystallization process in biomineralization. This work suggested a strategy for a synthesis of advanced materials under mild conditions.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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