Effect of Serine and Arginine on the Phase Transition from Amorphous CaCO₃ and CaCO₃·6H₂O to Calcite Film

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Calcium carbonate, ubiquitous throughout nature, is one of the most biologically significant minerals. It is known that the organic matrix of biological materials controls the phase transition of CaCO₃, but much remains undiscovered regarding its pathway from an amorphous to crystalline solid. In this study, examination of the initial formation of CaCO₃ films has been proposed as a new methodology to identify the phase transition of CaCO₃. We have identified a significant role for both serine and arginine in the synthesis of CaCO₃ and have found them to be important for the stabilization of amorphous calcium carbonate and CaCO₃·6H₂O. X-ray diffraction, scanning electron microscopy, and transmission electron microscopy have been used for the identification of crystalline phase and surface structure. This study presents information useful for understanding the phase transition of CaCO₃ and the function of organic molecules in the formation of biological materials.


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1. Introduction

Biological materials including bone, mollusk shells, and others are synthesized by many different organisms.¹,² They have received significant interest in the fields of chemistry and materials science due to their higher material properties. Mineral materials, including calcium- and silicon-based biomaterials, are commonly produced by a wide range of phyla, including archaea, bacteria, fungi, lower and higher plants, and the Chordata. Calcium carbonate (CaCO₃), which forms the exoskeleton of mollusk shells and sponges, has been a subject of study due to its wide availability and excellent material characteristics.³,⁴ In particular, the shell formation of bivalves has been used as a means to understand the mechanisms of biomineralization.

In natural systems, CaCO₃ generally exists in six forms: three different polymorphs (vaterite, aragonite and calcite), two different hydrated phases (monohydrocalcite, CaCO₃·H₂O and calcium carbonate hexahydrate, CaCO₃·6H₂O), and amorphous calcium carbonate (ACC).¹ Among these forms, the formation of stable amorphous calcium carbonate is fascinating; its transition to the crystalline phase is not only thermodynamically favored but also kinetically fast.⁵ The occurrence of ACC in nature, such as in bivalve shells or cellular systems, is uncommon. However, the initial biomineralization process of bivalve shell formation indicates the presence of ACC. In bivalve shells, the CaCO₃ crystalline phase starts as ACC, changes to aragonite at the planktonic larval period, and is followed by the transition to calcite after attachment to a substrate.⁶ Thus far, key factors related to the production and retention of ACC have rarely been examined because the biomineralization process has been conducted in closed systems. In these systems, mass transfer is restricted by an organic membrane or cell, and synthesized biological crystals exist on the micro scale. Therefore, the crystalline phase must be analyzed by X-ray diffraction or other spectrometric techniques.⁷,⁸ This study presents methodology that is able to identify the role and effect of amino acids on the synthesis and retention of both ACC and CaCO₃·6H₂O in the aqueous state. Using CaCO₃ thin films to understand the mechanism of CaCO₃ phase transitions in nature, we have studied the stabilizing role of amino acids (serine and arginine), a key feature in the transformation pathway of CaCO₃.

2. Experimental Methods

The method for CaCO₃ film synthesis has been previously described.⁹ CaCO₃ films were obtained under atmospheric conditions (room temperature and 1 atm.). The concentration of amino acids was used to control the ACC (Amorphous Calcium Carbonate) retention period along with the characteristics and morphology of the CaCO₃ thin films. Distilled water (DW), in the absence or presence of amino acids (25 mmole), was mixed with Ca(OH)₂ and stirred at 400 rpm for 1 min. The solution was placed in a 100 ml beaker. The synthesized CaCO₃ films were analyzed after 10 min, 30 min, 1 h, 2 h and 24 h. The synthesized CaCO₃ films were analyzed by X-ray diffraction (DMAX 2200 PV, RIGAKU), field emission scanning electron microscopy (FEI, Nova230 FE-SEM), and transmission electron microscopy (FEI, Tecnai G2 F30 FE-TEM). In the XRD analysis, a Si-low background sample holder was used to decrease the effect of the glass holder. Detailed information on the low-background sample holder is given in the supporting materials (Fig. A2). Appendix contains the crystal patterns of CaCO₃·H₂O and CaCO₃·6H₂O, the Si-low background sample holder information, and the synthetic steps for the CaCO₃ films.
3. Results and Discussion

To identify the phase transition of CaCO$_3$, films synthesized at the interface between DW and air under atmospheric conditions (room temperature and atmospheric pressure) were observed. Figure 1 shows the difference between CaCO$_3$ crystalline phases with 25 mM serine, 25 mM arginine (Figs. 1(b) and 1(c), respectively), or without additive (Fig. 1(a)). The star (*) indicates the main peaks of CaCO$_3$·6H$_2$O, and the C denotes the characteristic peaks of calcite. ACC is a broad peak ranging from approximately 20 to 40°. In the absence of amino acid, the characteristics of ACC were not present. In the presence of serine (Fig. 1(b)), the characteristics of ACC were clearly present compared to the absence of amino acid. In the presence of serine (Fig. 1(b)), the characteristics of ACC were clearly present compared to the absence of amino acid.

CaCO$_3$·6H$_2$O was identified up to 2 h (25 mM serine) or 1 h (25 mM arginine) post mixing. The crystalline information for CaCO$_3$·6H$_2$O was obtained from previous research\textsuperscript{10} (Appendix Fig. A1). To eliminate the influence of the amorphous phase in the glass sample holder (also ranging from 20 to 40 degree), a Si-Low background sample holder was used (Appendix Fig. A2).

The retention time of ACC (the broad peak ranging from approximately 20 to 40°) was 2 h with the addition of amino acid (Fig. 1). In the CaCO$_3$ crystallization system of CaCl$_2$ and NaCO$_3$, the complete transition of ACC to calcite has been reported to occur in approximately 4–16 h.\textsuperscript{11} The transition of ACC to calcite found in natural environments (e.g., mollusk shell formation\textsuperscript{6}) or sea urchin larval spicules\textsuperscript{12}) shows an increase in the proportion of calcite within an individual species over several days. According to previous research, the intensity of calcite in either the absence or
presence of amino acid increased according to the increase in time after mixing Ca(OH)\textsubscript{2} and DW. The difference in the retention time of the CaCO\textsubscript{3} crystalline phase, ACC and CaCO\textsubscript{3}·6\textsubscript{H}2O\textsubscript{2} was determined by the type of amino acid. To understand these results, correlation of the structural properties between the amino acids (serine and arginine) and the CaCO\textsubscript{3} phase (ACC and CaCO\textsubscript{3}·6\textsubscript{H}2O) ought to be considered. However, a fundamental obstacle faced by structural chemistry has been the lack of a structural model for amorphous CaCO\textsubscript{3}.

Based on the peak ratio of calcite (the final product of the CaCO\textsubscript{3} film), the preferred orientation was identified and is listed according to the amino acid present (Table 1). As shown in Table 1, serine did not develop any specific crystal face compared to the absence of amino acids or the presence of arginine. Arginine developed several crystal faces, including (006) and (018). In particular, the (006) face is equivalent to the (001) face, which is reported to have favorable protein interactions.\textsuperscript{13,14} The experimental results (Fig. 1 and Table 1) showed that the addition of arginine increases the preferred orientation of the (001) face in CaCO\textsubscript{3} films with a calcite crystalline phase. The difference in the preferred orientation has a notable effect on the morphology of CaCO\textsubscript{3} films (Appendix Fig. A3). Moreover, the peak ratio of calcite in the absence of amino acids was very different from the hexagonal type (PDF 05-0586), which could be due to the morphology differences between burr-like and cubic crystals.

According to the TEM analysis of CaCO\textsubscript{3} synthesized in the absence of amino acids with a 10 min reaction time (Fig. 2), sphere-like particles approximately 1 \textmu\text{m in size were identified as ACC (Fig. 2(b)). The TEM results are consistent with the XRD pattern of CaCO\textsubscript{3} (Fig. 1(a)).

To understand the nature of the starting material for CaCO\textsubscript{3} films, morphological differences were analyzed according to reaction time, ranging from 10 to 30 min (Fig. 3). As shown in Figs. 3(a) and 3(b), the growth and aggregation of particles increased with increasing reaction time (from 10 to 30 min). The shape of the starting material for a film is a hemispherical structure, not a spherical structure (Fig. 3(c)). This result indicates that the source of calcium ions exists only in DW and that the key source of carbonate ions is aqueous CO\textsubscript{2}.

Crystal growth occurs through the aggregation of hemispherical particles. The morphology of calcite thin films depends on the presence of organic molecules. Different shapes, with an aggregated burr- or flower-like structure, were identified depending on the amino acid present (Fig. 4). It is interesting to note that the morphology in the presence of arginine showed a flower-like structure and the absence of amino acids showed a burr-like structure. This difference in morphology may be related to the different degree of preferred orientation in the final crystal structure. In the absence of amino acids, crystal face (202) forms, compared to the presence of amino acids and calcite (Table 1). The correlation between crystal face (202) and burr-like structure could be hypothesized from the degree of preferred orientation. Interestingly, rod-type crystals from urchin teeth showed a developed set of symmetry-related \{110\} planes.\textsuperscript{15} The \{110\} plane, including crystal face (202), could be an excellent candidate to develop into stable faces in the burr-like structure of calcite. The presence of amino acids, crystal faces (006) and (018) developed. As previously mentioned, crystal face (001) is a typical crystal face related to shell formation, composed of calcium carbonate. Nucleation may be associated with amino acids (serine or arginine). In the case of arginine, nucleation may be followed by CaCO\textsubscript{3} growth that develops the more stable (001) and (018) faces in burr-like structures.

Table 1 Degree of preferred orientation. The main peak (104) of calcite was set as a standard for calculation. The degree was calculated to (hkl)*100/(104). The highest value is indicated in bold.

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*PDF (05-0586)

Fig. 2 TEM images of the starting material for a CaCO\textsubscript{3} film. The material has a spherical structure that is approximately 1 \textmu\text{m in size (a). The diffraction pattern is characteristic of ACC (b).
ACC is thermodynamically more unstable than calcite but it is widely found in nature. Analyses of biological calcium carbonate have identified the presence of phosphate anions and magnesium cations associated with ACC and poorly ordered calcite.

In general, bivalves use calcium and carbonate ions from seawater for their shell formation. The phase transition of CaCO$_3$ flows from ACC to aragonite at the stage before attachment and then to calcite at the attachment stage. It has been reported that various macromolecules (e.g., nacrein, lustrin A, and aspein) can play a key role in the bivalve CaCO$_3$ phase transition.

Initially, ACC and CaCO$_3$·6H$_2$O were produced and the phase transition to calcite was identified. The presence of serine produced a longer retention time of CaCO$_3$·6H$_2$O than of arginine (Figs. 1(b) and 1(c)). Serine and arginine are thought to prevent crystal nucleation and growth by interfering with the formation of an ordered calcium carbonate crystalline structure. Macromolecules, secreted from epithelial cells, have been reported to inhibit CaCO$_3$ crystallization, thereby maintaining the ACC state. Organic molecules shown to promote stable ACC may bind to a specific part of ACC that controls crystallization growth. Detailed investigations of these transformations require the interpretation of ACC and the crystallographic data for amino acids. Evaluating the crystal build of the amorphous phase will be a bottleneck for understanding phase transitions.

Previous research on sea urchins has indicated that transient forms of ACC typically contain very little molecular water, whereas most stable forms are hydrates commonly having the formula CaCO$_3$·H$_2$O. However, the characteristic XRD peak of CaCO$_3$·H$_2$O was not identified during the initial retention time (from 10 to 30 min), compared to previous crystalline information (Appendix Fig. A3). CaCO$_3$·H$_2$O and CaCO$_3$·6H$_2$O have negative enthalpies of formation with respect to calcite, but are unstable with respect to the same phase at ambient conditions, based on free energy. The entropy change per water is 32.0 for CaCO$_3$·H$_2$O and 33.5 J K$^{-1}$ mol$^{-1}$ for CaCO$_3$·6H$_2$O. These entropy values indicate that the ACC and CaCO$_3$·6H$_2$O to
calcite transitions are not thermodynamic in nature. Interactions with additives, kinetic factors, and the competition of CaCO$_3$·H$_2$O and CaCO$_3$·6H$_2$O need to be considered as well.

A recent study of the biomineralization of sea urchin spicules suggests that hydrated ACC is the likely precursor of the anhydrous form, indicating the possibility that hydrated ACC plays a broader role than previously thought and that its transition may occur via multiple steps. In this study, it was demonstrated that serine and arginine play a significant role in the retention of CaCO$_3$ phases (ACC and CaCO$_3$·6H$_2$O) and also in the preferred orientation associated with crystal morphology. Although the amino acid impacts the retention of ACC and CaCO$_3$·6H$_2$O, the crystalline phase transition of ACC to calcite is related to the intrinsic characteristics of CaCO$_3$ crystallization, controlled by thermodynamic parameters and not the effects of amino acids.

On the other hand, the formation of aragonite, important in shell formation, suggests effects from organic molecules secreted by bivalves and not thermodynamic parameters.

Marine organisms, including bivalves, most likely utilize the effects of activation energy for the control of CaCO$_3$ polymorphs. For example, bivalves use organic molecules to synthesize aragonite, which is thermodynamically unstable compared to calcite. Unless the conditions that affect thermodynamic factors, such as temperature, pH value, and supersaturation are induced by an excess of amino acid, ACC can transform to calcite, which is thermodynamically stable and kinetically advantageous. The differences in the solubility of calcite ($\log K_{sp} = -8.42$) and aragonite ($\log K_{sp} = -8.22$) show that calcite is more stable under aqueous conditions than aragonite.

Due to the development of the main peak (104) and knowledge of the shell formation of bivalves, the key component in the calcite transition might be ACC and not CaCO$_3$·H$_2$O, though CaCO$_3$·6H$_2$O would also transform to calcite over time. To understand the transition pathway in more detail, both the crystal model of ACC and crystallographic information on the correlation between CaCO$_3$ and amino acids are necessary.

4. Conclusions

In the synthetic process of CaCO$_3$ thin films, serine (25 mM) and arginine (25 mM) show an effect on the retention time of amorphous calcium carbonate (ACC) and CaCO$_3$·6H$_2$O. This factor affects the preferred orientation and the morphology of CaCO$_3$ thin films synthesized under atmospheric conditions (room temperature and 1 atm). In the absence of amino acids, a burr-like crystal structure with crystal face (202) was identified, and a flower-like structure with crystal faces (006) and (018) was identified in the presence of arginine. ACC and CaCO$_3$·6H$_2$O to- calcite is a solid-state transformation, and ACC and/or CaCO$_3$·6H$_2$O are precursors involved in the phase change.

The methodology in this study is a new approach to overcome the limits of studying in vitro CaCO$_3$ crystallization. The data in this study serve to explain the effect macromolecule additives have on biomineralization, based on the morphological control of CaCO$_3$ and the interaction between ACC or CaCO$_3$·6H$_2$O.

Acknowledgements

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Appendix

Supplementary information shows the simulated X-ray pattern of CaCO$_3$·6H$_2$O and CaCO$_3$·H$_2$O. Figure A1 shows XRD pattern of CaCO$_3$·6H$_2$O. We simulated the pattern based on the previous information by using the software (VESTA (Visualization for Electronic and Structural Analysis) and RIETAN). The vertical bar means the Bragg position of X-ray diffraction calculated by RIETAN. The intensity of main peak (17.11°) was assumed to be 100 and all peaks has been normalized. The black arrows mean first and second peak of CaCO$_3$·6H$_2$O. We could identify the peaks at each initial retention time of Fig. 1.

Figure A2 shows the XRD result of blank measurement using Si-low background sample holder (Cu tube, 40 kV-40 mA, DS = SS = 1°, RS = 0.15 mm, 2θ/θ measurement).
also produces a very small, broad background attributable to continuous X-rays (DMAX 2200 PV, RIGAKU), although it is much lower than the background produced with an ordinary glass sample holder. Specifications on low-background sample holder is as follows;

1. Material: Single-crystal silicon for semiconductors
2. Cut-out direction: (911)
3. External dimensions: 35 (W) © 50 (H) © 2 (T) mm

Figure A3 shows XRD patterns of CaCO₃·H₂O. We simulated the pattern based on the previous information31) by using the software30) (VESTA (Visualization for Electronic and Structural Analysis) and RIETAN).

Only Ca(OH)₂ powder and DW (distilled water) were used. Figure A4 shows that CaCO₃ film was synthesized at the interface between solution and air.

REFERENCES