UNUSUAL MORPHOLOGY OF CALCIUM CARBONATE CONTROLLED BY AMINO ACIDS IN AGAROSE GEL

CHENG-LI YAO^{a*}, CHUN-XIA QI^a, JIN-MIAO ZHU^a, WANG-HUA XU^b

^aDepartment of Chemistry, Hefei Teachers College, Hefei, Anhui 230061, China

^bDepartment of Chemistry, Anqing Teachers College, Anqing, Anhui 246011, China

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ABSTRACT

The polar amino acid, such as aspartic acid (L-Asp) and arginine (L-Arg) are the primary active molecules of the glycoprotein on the organic/inorganic interface of biomineralized tissue. In this study, aspartic acid and arginine were used as the organic template in inducing the nucleation and growth of calcium carbonate. With the analysis of X-ray diffraction we investigated the relationship between amino acid concentration and the precipitation phase crystal structure of calcium carbonate. SEM was employed in the analysis of the morphological characteristic of the precipitation. To interpret the cause of the hierarchic structure was proposed from the view of the small biomolecules polarity that can control over the growth of the crystals and the aggregation of the micro crystals. The conclusion also provides a new material synthesizing strategy.

Keywords: Calcium carbonate, biomineralization, aspartic acid, arginine, agarose gel

INTRODUCTION

Calcium carbonate (CaCO_3) is the most abundant mineral formed by organisms, followed by silica, calcium phosphate, and others. Biomineralization results in complex materials that are characterized by a remarkable level of molecular control of the particle size, structure and morphology, aggregation, and crystallographic orientation of the calcium carbonate phases.^{1,2} The variety of biomineralizates can be expressed by the fact that approximately 128000 species of molluscs exist.³ The majority of them produce shells and capsules from CaCO_3 having different sizes, shapes, and color patterns.

Many shells are constructed within frameworks that may be lamellar, columnar, or reticular. In each case, there are two important features of assembly. First, a relatively inert structural frame is built from insoluble macromolecules.^{4,5} Second, acidic proteins, such as L-Asp, are assembled on the hydrophobic scaffold. Mineralization takes place at the interface between the acidic proteins and the aqueous environment. Also, the oyster shell matrix contains 20-30 mol % aspartate.^{2,6}

Amino acids/protein-template synthesis of inorganic crystals and inorganic/organic hybrid structures has been highlighted in recent years. One of the most widely studied systems is CaCO_3 . Among amino acids, L-Asp particularly demands attention because some Asp-rich peptides are known to control the polymorphism and structure of CaCO_3 during the process of biomineralization.^{7,8,9} Most previous papers have reported the effects of Asp on the calcite surface morphology during crystal growth and dissolution.^{10,11,12}

Agarose is a linear polysaccharide extracted from marine red algae, and consists of alternating 1,3-linked β -D-galactose and 1,4-linked 3,6-anhydro- α -L-galactose. It forms a gel when a homogeneous solution is cooled to below the ordering temperature ($\sim 35^\circ\text{C}$) and an infinite three-dimensional network of agarose fibers develops; the melting of agarose gel occurs at a comparatively higher temperature ($\sim 85^\circ\text{C}$), leading to a setting-melting hysteresis behavior.¹³ Agarose gel is widely used in the food and pharmaceutical industries and as an important support for chromatography analysis.¹⁴

The aim of the present work is to investigate the effect of polar amino acid on the growth of carbonate crystals in the agarose gel/ amino acid system and to attempt to answer the following question: Does polar amino acid affect the nature, or the particle size of the calcium carbonate forming phases?

The structure of CaCO_3 precipitation was investigated by a wide-angle X-ray diffract meter (XRD). Scanning electron microscope (SEM) was employed to investigate morphologic features of the porous aggregate of the precipitation of CaCO_3 . Finally, a possible assumption was proposed for simulating organic control crystal growth and particle aggregation.

MATERIALS AND METHODS

Material

Agarose was purchased from Sigma. L-Asp and L-Arg were obtained from Institute of Biological Products of Shanghai (Shanghai, China). CaCl_2 and Na_2CO_3 are all of analytical reagent grade. Deionized ultrapure water was

used throughout the experiment.

The synthesis of calcium carbonate

In a typical synthesis, CaCl_2 (100 mL, 0.05 mol·L⁻¹) solution was heated to 75–80 °C and 10% (w/w) of agarose was added, which was further heated to 85–90 °C to ensure dissolution of agarose, then adding various amounts of L-Asp (80 mg, 160 mg, 320 mg) and L-Arg (80 mg, 160 mg, 320 mg), respectively. The calcium chloride/agarose/ amino acid solution was poured into a culture dish and allowed to cool at the room temperature. The cooled agarose gel was cut into cubic pieces about 1 cm³ in volume, which were soaked in Na_2CO_3 (100 mL, 0.05 mol·L⁻¹) solution at 30 °C. After 48 h of incubation, the gel was rinsed in water to get rid of the residual inorganic salt. Finally, the resulting CaCO_3 crystals were recovered by heating the gel containing CaCO_3 crystals in hot water ($\sim 90^\circ\text{C}$) to dissolve agarose followed by washing and filtration. The preliminary experimental result showed that the short period of heating in hot water did not affect the crystal morphology considerably.

Characterization of crystal phase and morphology of precipitation

The sizes and morphologies of CaCO_3 precipitates were characterized by using SEM on a DSM 940 A (Carl Zeiss, Jena) microscope. Calibration of CaCO_3 pellets (in a proportion of 1% in KBr powder) was performed and recorded with a Nicolet 870 Fourier Transform Infrared Spectrometer between 4000 cm⁻¹ and 400 cm⁻¹ with a resolution of 4 cm⁻¹. The XRD measurements were made by an MAPI8XAHF X-ray Diffractometer at a scanning rate of 4 °/min, using a monochromatized CuK α radiation ($\lambda = 0.154$ nm).

RESULTS AND DISCUSSION

SEM images

Figure 1: Morphologies of mineralized CaCO_3 with different L-Asp/L-Arg concentrations in the agarose system: (a) without L-Arg or L-Asp; (b) with L-Arg 0.8 $\text{mg}\cdot\text{mL}^{-1}$; (c) with L-Arg 1.6 $\text{mg}\cdot\text{mL}^{-1}$; (d) with L-Arg 3.2 $\text{mg}\cdot\text{mL}^{-1}$; (e) L-Asp 0.8 $\text{mg}\cdot\text{mL}^{-1}$; (f) L-Asp 1.6 $\text{mg}\cdot\text{mL}^{-1}$; (g) L-Asp 3.2 $\text{mg}\cdot\text{mL}^{-1}$

Figure 1a~g showed SEM pictures of CaCO_3 particles precipitated in 10% (w/w) of agarose and different concentrations amino acids. Figure 1a showed the tree branch-shaped crystals formed in the agarose without any amino acids. Well-dispersed spherical CaCO_3 aggregate particles were produced in the system with L-Arg (0.8 $\text{mg}\cdot\text{mL}^{-1}$) and the particle size was about 5~6 μm (as shown in Fig. 1b). With the increase of L-Arg concentration, spherical CaCO_3 aggregate particles were still gained, but the amount became less. At the same time, there were many irregular particles formed which composed of little rhomboidal particles (Fig. 1c). Surprisingly, when L-Arg concentration is 3.2 $\text{mg}\cdot\text{mL}^{-1}$, the spherical CaCO_3 aggregate particles disappeared (Fig. 1d). Fig. 1e~g showed SEM micrographs of CaCO_3 particles obtained in the presence of L-Asp with different concentrations (0.8 $\text{mg}\cdot\text{mL}^{-1}$ □ 1.6 $\text{mg}\cdot\text{mL}^{-1}$ □ 3.2 $\text{mg}\cdot\text{mL}^{-1}$) in the agarose gel system, respectively. Similar to L-Arg, L-Asp had great influence on the morphology of CaCO_3 particles and pine flower-like aggregate particles were obtained (as shown in Fig. 1.e). However, with L-Asp concentrations from 1.6 $\text{mg}\cdot\text{mL}^{-1}$ to 3.2 $\text{mg}\cdot\text{mL}^{-1}$, unusual, maize cob-pine flower shaped CaCO_3 aggregates radially aligned crystals were obtained (Fig. 1f-g). These results indicate that L-Arg and L-Asp had significant influence on the morphology of CaCO_3 in the agarose gel system.

IR spectra and XRD

Figure 2: FT-IR spectra of CaCO_3 with different L-Asp/ L-Arg concentrations in the agarose system: (a) without L-Arg or L-Asp; (b) with L-Arg 1.6 $\text{mg} \cdot \text{mL}^{-1}$; (c) L-Asp 1.6 $\text{mg} \cdot \text{mL}^{-1}$.

Infrared spectra of CaCO_3 crystals produced in synthesized in the presence and absence of amino acid were presented in Fig.2. Fig.2a displayed the absorption peak of crystal at 860 cm^{-1} and 710 cm^{-1} , while 860 cm^{-1} between 875 cm^{-1} and 856 cm^{-1} (the CO_3^{2-} out-of-plane deformation mode of the aragonite).¹⁵ The absorption peak of 710 cm^{-1} showed the calcite formed. It showed that the product was the mixture of calcite and aragonite. From Fig. 2b we could see that FT-IR absorption bands at 875 cm^{-1} and 710 cm^{-1} , in which 875 cm^{-1} is the CO_3^{2-} out-of-plane deformation mode of the calcite,¹⁶ which indicated the presence of calcite. From the Fig.2c we also could find the absorption peak of 877 cm^{-1} , 744 cm^{-1} , which indicated the vaterite formed. These results were confirmed by XRD.

Figure 3: XRD patterns of CaCO_3 with different L-Asp/ L-Arg concentrations in the agarose system: (a) without L-Arg or L-Asp; (b) with L-Arg 1.6 $\text{mg} \cdot \text{mL}^{-1}$; (c) L-Asp 1.6 $\text{mg} \cdot \text{mL}^{-1}$.

Figure 3a-c showed the XRD patterns of CaCO_3 particles obtained in amino acid / agarose gel system. Using the reflection peaks at (104) plane for calcite, (221) plane for aragonite and (110) plane for vaterite, we can find that there are not only sharp calcite but aragonite reflections in figure 3a, confirming that without any amino acid led to the formation of calcite and aragonite. While polar amino acid with different isoelectric point (pI) led to the formation of calcite or vaterite. As figure 3b had shown, only the diffraction peaks from

calcite appeared, this provides that it is easily to form calcite in the agarose gel system with L-Arg. Figure 3c had shown there only vaterite were formed in the agarose gel system with L-Asp.

The formation mechanism of CaCO_3 crystal in the amino acids / agarose gel system

It is known that agarose is a neutral polysaccharide that mainly carries hydroxy functional groups. It has been documented that whereas the matrices of acid proteins including gelatin can exert significant effects on the CaCO_3 crystallization,^{17,18,19} the matrices of polysaccharides such as chitosan/chitin usually do not solely exert direct effects on CaCO_3 crystallization.^{20,21}

There many research show that amino acids with different isoelectric point (pI) have different ability to induce the formation of CaCO_3 . Manoli, Kai and Tong^{12,22,23} found minerals adsorbing amino acids with charged R groups much more than with uncharged R groups. According their research we learned the uncharged polar amino acids in CaCO_3 precipitates tend to stabilize the vaterite phase, and the vaterite content in CaCO_3 precipitates is correlated to the concentration of amino acids in the precipitates. When pI of amino acids was higher than pH (7.0) of solution and positive charge was taken on its surface. It could attract CO_3^{2-} strongly, and result in the CO_3^{2-} ions being enriched; the higher supersaturation in the region was in favor of CaCO_3 crystals to nucleate and grow. Electrostatic matching, structural and stereochemical complementarity made the amino acids offer nucleation sites for CaCO_3 crystal, reduced interfacial energy and stabilized vaterite formation.

Therefore, it is expected that in the present case, agarose molecules do not directly affect the crystallization of CaCO_3 and agarose gel just play a role of reaction media that control the diffusion process of ions in the media and hinder the crystal growth when the crystal size reaches the pore size of the agarose network. Considering that the pore size for the 1% agarose gel is around 140 nm ,²⁴ considerably smaller than the initial rhombohedral calcite crystals, the main role the agarose gel play on the CaCO_3 crystallization could be the control on the diffusion process of reactant ions rather than the hindering of crystal growth. Therefore, a possible mechanism for the formation of the tree branch-shaped crystals is as follows: At first, Ca^{2+} ions were distributed uniformly in the agarose gel matrix. When CO_3^{2-} ions diffused into the gel, many small crystals formed suddenly, leading to a quick decrease of the reactant ion concentration nearby. With gradual diffusion of CO_3^{2-} ions into the gel, the nuclei grew the fastest since the agarose network causes a stagnant diffusion of ions to accumulate reactant ions for spatial reasons. With the prolongation of time, the tree branch-shaped crystals gradually extruded and radially extended (Figure 4).

Figure 4: The possible formation mechanism of unusual morphology calcium carbonate.

With the addition of polar amino acid, the spherical, maize cob-pine flower shaped and other shaped CaCO_3 particles were gained. The different shaped CaCO_3 particles owed to the agarose gel matrix, but also amino acid with different isoelectric point. It is not difficult to find CaCO_3 particles formed in the alkaline conditions in the experiments. The isoelectric point of L-Arg and L-Asp are $\text{pI}=10.76$ and $\text{pI}=2.97$, respectively. Irregular particles with little rhomboidal calcite particles formed in the L-Arg / agarose gel system and maize cob-pine flower shaped vaterite formed in the L-Asp / agarose gel system may be explained by the reasons below.

First, L-Arg possesses the R-helix conformation under alkaline conditions and coordinate with Ca^{2+} ,²⁵ which is a suitable template for CaCO_3 mineralization. Second, CaCO_3 is easier to deposit in an alkaline environment. Third, the agarose gel released Ca^{2+} slowly; hence the formation of calcium carbonate will towards easy and stable direction. However, alkaline condition is unsuitable for molecular conformation of L-Asp to fully stretch and the negative charges on its surface were surplus in the alkaline condition. It would attract Ca^{2+} strongly because of electrostatic interactions and offered bonding site. When CO_3^{2-} was added to the system, the CaCO_3 nucleus was

formed fast. However, the agarose gel afforded the Ca^{2+} slowly, which led to the accumulation of quasi-circular CaCO_3 particles to all directions and the formation of maize cob-pine flower shaped CaCO_3 at last.

CONCLUSION

In this study, the method of directly precipitation was used to obtain CaCO_3 crystal. In these processes, additives such as L-Arg and L-Asp were added into the agarose gel to control the crystallization of CaCO_3 . The spherical□maize cob-pine flower shaped and other shaped CaCO_3 particles were successfully produced in experiments. Different isoelectric point of amino acids will effect the morphology and structure of CaCO_3 . Amino acids such as L-Arg were helpful for the growth of calcite and L-Asp for vaterite. These results indicated that amino acid played essential importance role in the process of biomineralization of CaCO_3 .

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