Communication

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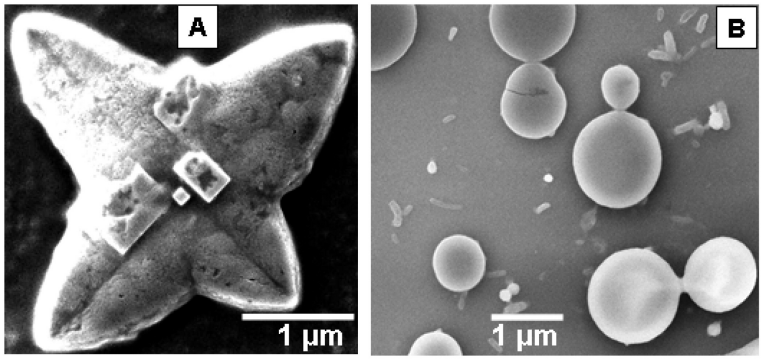

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Biosynthesis of $CaCO_3$ Crystals of Complex Morphology Using a Fungus and an Actinomycete

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Synthesis of inorganic materials of complex morphology, texture, and controllable crystallography is an important goal in crystal engineering with relevance to design of catalyst supports, membranes in separation methodologies, and biomedical applications such as bone implants, drug delivery systems, and DNA transport.[1] Biological systems provide a number of examples of inorganic materials such as $CaCO_3$, magnetic iron oxide, and amorphous silica of exquisite morphology with structural and functional value.[2] Elucidation of general biomineralization principles has followed from studies into the growth of minerals in biological organisms[3,4] and on biomacromolecules extracted from organisms.[5] The important biomineral $CaCO_3$ has received considerable attention, and these principles have been used in the development of a number of biomimetic templates such as Langmuir monolayers,[6] self-assembled monolayers,[7] lipid bilayer stacks,[8] vesicles,[9] and functionalized micropatterned surfaces[10] for its synthesis.

Morphology variation and polymorph selectivity of $CaCO_3$ crystals may also be achieved by growth in solution in the presence of suitably designed additives such as proteins extracted from $CaCO_3$-rich bioorganisms[5] or synthetic molecules such as polymers.[11] Laboratory processes for the synthesis of $CaCO_3$ crystals have hitherto relied on very specific proteins from calcareous microorganisms and an external source of CO_2 for reaction with Ca ions. In this communication, we address the following questions. Can the morphology and crystallography of $CaCO_3$ crystals in solution be modulated by proteins/biomolecules not normally associated with calcareous microorganisms ? Many fungi and actinomycetes are known to produce reasonable amounts of CO_2 during growth.[12] Can the CO_2 released from such microorganisms be used to react with Ca ions and synthesize truly biogenic $CaCO_3$ crystals? Some of us have recently shown that enzymes secreted by plant microorganisms such as *Fusarium oxysporum* (a fungus)[13] and *Rhodococcus* sp. (an actinomycete)[14] may be reacted with metal ions to yield nanocrystals of the corresponding metal or metal sulfide. In this communication, we show that biogenic $CaCO_3$ crystals may be grown by simple exposure of aqueous Ca^{2+} ions to *Fusarium* sp. and *Rhodococcus* sp. While cruciform-shaped calcite particles are obtained with *Fusarium* sp., the highly unstable vaterite polymorph in a disklike morphology is obtained with *Rhodococcus* sp.

In a typical experiment, 20 g each of *Fusarium* sp. and *Rhodococcus* sp. biomass were suspended in 10^{-3} M aqueous $CaCl_2$ solution in conical flasks after thorough washing and centrifugation under sterile conditions.[15] These flasks were then plugged with cotton and incubated at 27 °C. Aliquots of the aqueous component were separated from the mycelia periodically and subjected to analysis. Figure 1 shows scanning electron microscopy (SEM)[16] images recorded from drop-cast films on Si (111) substrates of the aqueous $CaCl_2$ solution after exposure to the fungus, *Fusarium* sp.

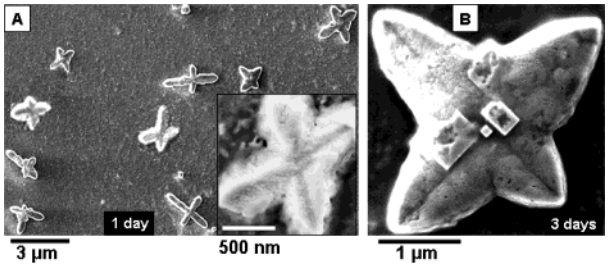

Figure 1. (A) SEM picture of $CaCO_3$ crystals after 1 day of reaction of Ca^{2+} ions with *Fusarium* sp. The inset shows a magnified image of one of the crystals. (B) SEM picture of a single mature $CaCO_3$ crystal after 3 days of reaction of Ca^{2+} ions with *Fusarium* sp.

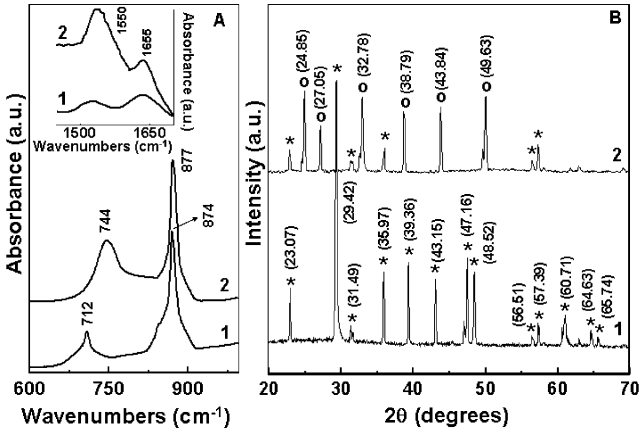

Figure 2. (A and Inset) FTIR spectra of $CaCO_3$ crystals by the reaction of Ca^{2+} ions with *Fusarium* sp. (curve 1) and *Rhodococcus* sp. (curve 2) in different spectral windows. (B) XRD patterns of $CaCO_3$ crystals on glass substrates synthesized using *Fusarium* sp. (curve 1) and *Rhodococcus* sp. (curve 2, text for details).

for 1 day (A) and 3 days (B). After 1 day of reaction, a large number of cruciform-shaped crystallites are observed. The inset of Figure 1A shows a magnified view of one of the crystallites that appears rather porous. After 3 days of reaction, mature crystals exhibit the same cruciform structure (Figure 1B) wherein the porous nature of the crystals is more clearly seen. Secondary nucleation of rhombohedral crystals has taken place on the underlying cruciform-shaped crystal.

Figure 2A shows the Fourier transform infrared (FTIR)[16] spectrum of powders of the cruciform $CaCO_3$ crystals in a KBr pellet (curve 1). Absorption bands centered at 874 and 712 cm^{-1} characteristic of the calcite phase of $CaCO_3$ are seen.[5] Curve 1 in Figure 2B is a plot of the X-ray diffraction pattern of the *Fusarium* sp. biogenic $CaCO_3$ crystals.[16] The 2θ values of the Bragg reflections are listed in the figure and correspond excellently with those reported for calcite.[7,17] The presence of proteins within the calcite crystalline framework is indicated by strong amide I and II signatures in the FTIR spectrum (curve 1, inset of Figure 2A) and

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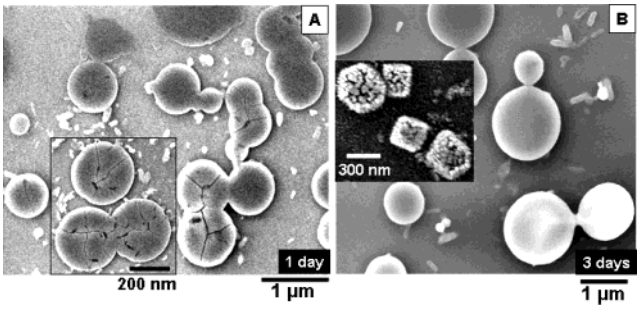

Figure 3. (A) SEM micrograph of $CaCO_3$ crystals after 1 day of reaction of Ca^{2+} ions with *Rhodococcus* sp. The inset shows a magnified image of an assembly of the crystallites. (B) SEM micrograph of $CaCO_3$ crystals after 3 days of reaction of Ca^{2+} ions with *Rhodococcus* sp. The inset shows SEM images of the vaterite crystals after calcination.

N and S signals that are present along with the expected Ca, C, and O signals in the energy dispersive analysis of X-rays (EDAX) spectrum of the cruciform crystals (Supporting Information, **S1.A**, curve 1).

Figure 3 shows SEM images recorded from solution-cast films of the aqueous $CaCl_2$ solution after exposure to *Rhodococcus* sp. for 1 day (A) and 3 days (B). Even after 1 day of reaction, the $CaCO_3$ crystals exhibit a morphology completely different from those biosynthesized using *Fusarium* sp. (Figure 1). The crystals are circular in cross section, the porous nature of which is reduced significantly in the mature crystals (Figure 3B). Such circular morphology of $CaCO_3$ is indicative of the formation of the highly unstable vaterite polymorph.[5] The FTIR spectrum recorded from the circular $CaCO_3$ crystals is shown in Figure 2A, curve 2. The absorption bands at 744 and 877 cm^{-1} are characteristic of vaterite.[5] The XRD pattern of these $CaCO_3$ crystals is shown in Figure 2B, curve 2. The Bragg reflections identified by "o" agree excellently with those reported for vaterite.[17] A small percentage of calcite is also observed in the XRD pattern (peaks labeled with *). As in the case of calcite synthesized using *Fusarium* sp., proteins are present in the *Rhodococcus* sp. biogenic vaterite crystals as well (FTIR: inset of Figure 2A, curve 2, and EDAX: Supporting Information, **S1.A**, curve 2).

A control experiment was performed wherein the *Fusarium* sp. and *Rhodococcus* sp. biomass was suspended in water for 2 days and the aqueous component with secreted proteins was separated by filtration and reacted with 10^{-3} M $CaCl_2$. CO_2 was bubbled very slowly through this solution, and the $CaCO_3$ crystals formed were analyzed by SEM (Supporting Information, **S2**). The SEM images show the formation of circular vaterite (**S2.A**) and cruciform calcite crystals (**S2.B**), indicating that the proteins secreted by these microorganisms are responsible for the morphology control and polymorph selectivity. This conclusion is strengthened by the fact that $CaCO_3$ crystals grown by bubbling CO_2 in pure $CaCl_2$ solution exhibit characteristic rhomobohedral calcite morphology (**S2.C**).

It is clear that specific (and as yet unidentified) proteins secreted by the two microorganisms play a crucial role in defining the morphology and, indeed, crystal structure of the $CaCO_3$ crystals formed. While the exact nature of binding of the proteins with specific crystallographic faces needs elaboration, the location of the proteins in the crystals (surface adsorption vs uniformly intercalated in the crystals) may be indirectly determined by removal of the proteins by calcination. The inset of Figure 3B shows an SEM image of biogenic vaterite crystals grown using *Rhodococcus* sp. after heating at 300 °C for 3 h. Upon calcination the originally compact crystals are transformed into highly porous and more faceted crystals. Increase in porosity indicates removal of proteins uniformly intercalated into the crystalline framework, while the morphology change suggests possible variation in crystal structure. N and S signals were below EDAX detection limits in these crystals (Supporting Information, **S1.A**, curve 3). The FTIR spectrum of the calcined vaterite crystals exhibited absorption bands at 712 and 874 cm^{-1} (Supporting Information, **S1.B**), clearly showing that the calcination-induced morphology variation seen in the SEM images is due to transformation of vaterite into calcite. The protein−$CaCO_3$ composite structures may arise from aggregation of the secreted proteins mediated by Ca^{2+} ions and is followed by reaction with CO_2 yielding a templated $CaCO_3$ mosaic crystal.

The exciting possibility of biosynthesis of $CaCO_3$ crystals of variable morphology and with polymorph selectivity by challenging microorganisms not known for calcareous biofilm formation with Ca^{2+} ions has been described. The source of carbonate ions is the microorganisms themselves, thus significantly enhancing the application potential of the work with important implications in crystal engineering, catalyst design, etc.

Supporting Information Available: EDAX (**S1.A**) and FTIR spectra (**S1.B**) of biogenic and calcined $CaCO_3$ crystals; SEM pictures of $CaCO_3$ crystals in different control experiments (**S2**). This material is available free of charge via the Internet at http://pubs.acs.org.

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(15) See refs 13 and 14 for details of culturing of the two microorganisms.
(16) XRD measurements of drop-coated films of the biogenic $CaCO_3$ crystals on glass substrates were carried out on a Phillips PW 1830 instrument operated at a voltage of 40 kV and a current of 30 mA with Cu Kα radiation. FTIR spectroscopy measurements of the purified and dried biogenic $CaCO_3$ powders in KBr pellets were carried out on a Perkin-Elmer Spectrum One instrument at a resolution of 4 cm^{-1}. For SEM analysis, drop-coated films of biogenic $CaCO_3$ crystals were made on Si (111) substrates. SEM measurements were performed on a Leica Stereoscan-440 instrument equipped with a Phoenix energy dispersive analysis of X-rays (EDAX) attachment.
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