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Highly oriented aragonite nanocrystal–biopolymer composites in an aragonite brick of the nacreous layer of *Pinctada fucata*

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Received (in Cambridge, UK) 28th November 2003, Accepted 26th February 2004
First published as an Advance Article on the web 18th March 2004

^{13}C CP/MAS NMR and FE/TEM measurements of the aragonite brick of the nacreous layer of *Pinctada fucata* indicate that it assembles with highly oriented aragonite nanocrystals, which are regulated by biopolymers.

Natural $CaCO_3$ architectures, such as eggshell, coral reef, pearl, *etc.*, are mediated and regulated by a small amount of biopolymer ligands.[1] As these biopolymer ligands strongly bind to $CaCO_3$ crystals and control crystal growth, the resulting $CaCO_3$ architectures have highly controlled shapes and sizes under ambient conditions.[2]

A pearl, one example of $CaCO_3$ architectures, is composed of a thin nacreous layer (30 to 1000 μm thickness) on a nucleus with a diameter of 3 to 8 mm. The nacreous layer is composed of aragonite bricks and biopolymer matrix sheets – much like a brick wall. It is important that a pearl oyster produces aragonite crystals under ambient conditions, although the aragonite phase is stable under high-pressure conditions.[3] Previously, Wada suggested that aragonite bricks crystallize in the "compartment" formed by the biopolymer matrix sheet such as proteins (conchiolin *etc.*) and/or glucosamines, and the biopolymer controls the polymorph and the crystal axis of the aragonite bricks.[4] Each aragonite brick was thought to be a single crystal because each showed the characteristic diffraction of aragonite crystals. In the present study, we demonstrate that each aragonite brick in the nacreous layer of *Pinctada fucata* (Japanese pearl oyster) is not a single crystal but rather is composed by the integration of nano-order aragonite crystals, utilizing ^{13}C cross polarization/magic angle spinning (CP/MAS) NMR, field emission/transmission electron microscopy (FE/TEM) and elemental mappings using electron energy loss spectroscopy (EELS).

^{13}C CP/MAS NMR measurements† of the nacreous layer of *P. fucata* were undertaken to obtain *in situ* chemical information about biopolymers. ^{13}C CP/MAS is a useful technique for detecting polymer ligands in $CaCO_3$ composites.[5] In the CP/MAS experiments, the proton magnetization transfer to a carbon nucleus occurs during the contact time (ct) period. The effectiveness of magnetization transfer depends on the distance between the protons and carbon nuclei. The CP should be effective for the organic components, which have many protons, and the intense signal of carbonate carbons in the aragonite would be reduced by ineffective CP. Figure 1a shows the ^{13}C CP/MAS spectrum of the nacreous layer of *P. fucata*. The signals obtained from biopolymers were observed at around 22, 44, and 51 ppm (Fig. 1a), together with the broad carbonyl signals at 173 and 179 ppm, except for the sharp signal of carbonate ($^{13}CO_3^{2-}$) at 171 ppm (Fig. 1b). Previous reports assigned these carbonyl signals at 173 and 179 ppm (Fig. 1b) to the amide carbonyl and carboxylate of biopolymers (proteins), respectively.[5–7] During CP study to find the optimum duration of ct, we found that the carbonate carbon in the aragonite layer exhibits anomalous efficiency of magnetization transfer. As shown in Fig. 1d, the $^{13}CO_3^{2-}$ signal in the aragonite was still observed even at very short ct (0.2 ms). The $^{13}CO_3^{2-}$ signals of pure calcite crystals (Fig. 1f) and $CaCO_3$ composite in the presence of polymer ligand[6] (Fig. 1e) disappeared completely at the same ct, although the sharp $^{13}CO_3^{2-}$ signal appeared at a long ct (1 ms), *e.g.*, pure calcite (Fig. 1c). The crystal size of each $CaCO_3$ crystal is quite similar (data not shown). The T_1 value for each signal was measured by using Torchia's method.[8] The biopolymers have shorter T_1 values (5, 15, and 16 s for the signals at 22, 44, and 51 ppm, respectively), while the $^{13}CO_3^{2-}$ signal at 171 ppm has a longer one (90 s). The carbonate carbon in the nacreous layer has a shorter T_1 value (90 s) than that for pure calcite crystals (180 s). Taking into consideration the high efficiency of CP and the short longitudinal relaxation rate of carbonate carbon, the aragonite brick should have a proton magnetization donor very close to the carbonate carbons inside the crystals. These results strongly suggest that every aragonite brick in the nacreous layer is not a single crystal but rather has a finer structure.

High-resolution FE/TEM analyses† were performed to investigate the possibility of finer structure in the aragonite brick. As the aragonite crystals do not have a cleavage plane, it is difficult to prepare a thin-sliced sample suitable for high-resolution FE/TEM analysis because the force of the scraper would disturb the fine structure of minerals. We utilized a focused ion beam (FIB) microprocessing system[9] in order to prepare a thin-sliced nacreous layer to avoid the damage caused by the force from the scraper. By using a Ga ion FIB, the nacreous layer was processed so as to prepare a small chip (*ca.* 10 × 6 × 0.05 μm) suitable for FE/TEM analyses. The image of the thin-sliced nacreous layer is shown in its entirety in Fig. 2a. The nacreous layers of *P. fucata* are composed of aragonite bricks that have *ca.* 0.5-μm thickness and biopolymer matrix sheets of *ca.* 30-nm thickness, like a brick wall. The existence of stripes on each aragonite brick due to the interference of the electron beam indicates $CaCO_3$ in the aragonite brick is highly oriented. Actually, the electron microdiffraction experiment performed with an aragonite brick showed the clear diffraction pattern of aragonite crystals. These results are consistent with previous works that studied the nacreous layer. As shown in Fig.

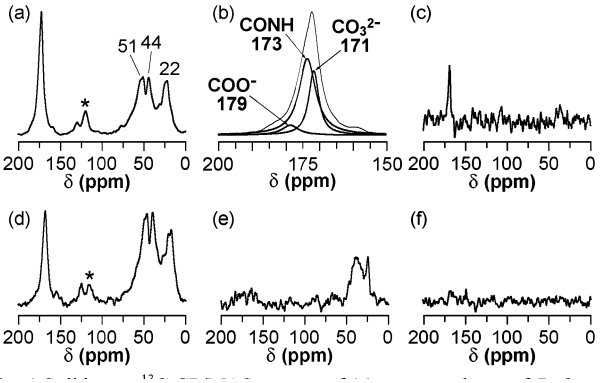

Fig. 1 Solid-state ^{13}C CP/MAS spectra of (a) nacreous layer of *P. fucata* (ct = 1 ms), (b) the expanded spectrum of (a) in carbonyl region (thin line) with curve fitting spectra (bold line), (c) pure calcite crystal (ct = 1 ms), (d) nacreous layer of *P. fucata* (ct = 0.2 ms), (e) the $CaCO_3$ composite in the presence of polymer ligand[6] (ct = 0.2 ms) and (f) pure calcite crystal (ct = 0.2 ms). Where: * indicates spinning side band.

DOI: 10.1039/b315478e

2b, however, the nanoscaled structure in the aragonite brick was observed at very high magnification (× 2,000,000). The aragonite brick is not a single crystal, but rather is composed of nanocrystals of $CaCO_3$ that have 5- to 15-nm diameter. Detailed analysis of a high-magnification FE/TEM image reveals that each nanocrystal in a brick has almost the same direction of the crystal axis and that the direction of the axis is different among the bricks (Fig. 2b). Additionally, the nanocrystals were observed in a biopolymer matrix sheet that exists between two aragonite bricks (Fig. 2b, arrows). Song *et al.* have reported a mineral bridge, which goes through the matrix sheet from one aragonite brick to another.[10] They suggest a somewhat tangled "brick–bridge–mortar" structure for nacre. Results of our FE/TEM analysis also support the existence of a mineral bridge in the biopolymer matrix sheet.

Until now, the aragonite bricks have been believed to be single crystals because of their translucent appearance and the results of diffraction studies. Our TEM analysis reveals that the ordered assembly of nanocrystals behaves as a single crystal in terms of diffraction analysis. To obtain information about the ordering mechanism of the nanocrystal axis, we carried out elemental mapping using EELS.† Figure 3 shows the elemental mapping of

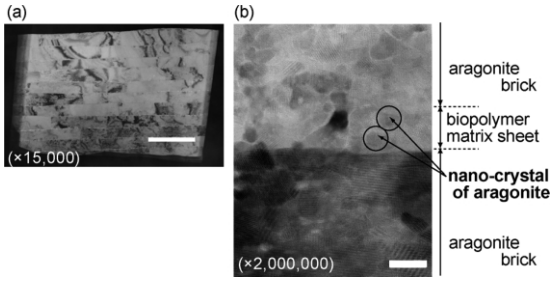

Fig. 2 FE/TEM images of nacreous layer of *P. fucata*; (a) whole image of thin-sliced nacreous layer (scale bar, 2 μm) and (b) its high-resolution image (scale bar, 15 nm).

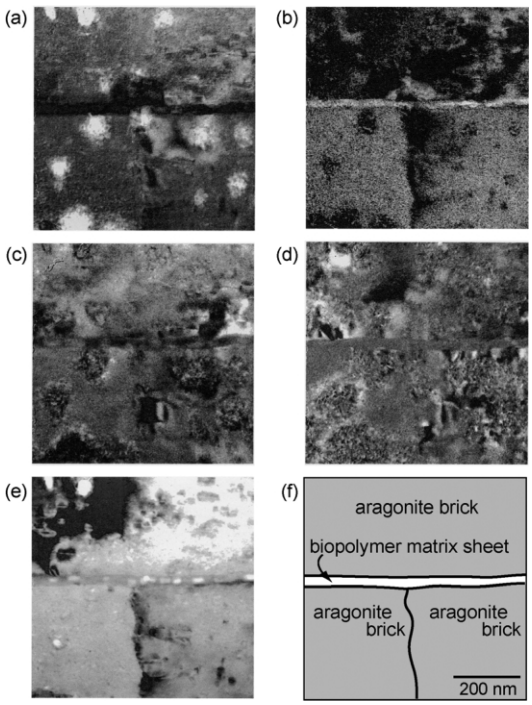

Fig. 3 Elemental mappings by EELS of nacreous layer of *P. fucata*; the elemental mapping of (a) Ca, (b) C, (c) O and (d) N, (e) zero-loss image and (f) schematic drawing of analyzed area.

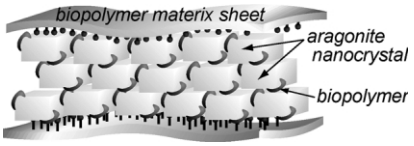

Fig. 4 Proposed structure of the single aragonite brick of the nacreous layer of *P. fucata*. Many aragonite nanocrystals are oriented in the brick.

the nacreous layer for Ca, C, O, and N. Each map is illustrated as a gray-scale image, and the white area indicates the high-concentration part of each element. As shown in Fig. 3a, there are some white spots of highly concentrated Ca ions in the aragonite brick. Concentrations of C and O are relatively low at the Ca spot (Fig. 3b and c). Carbon is highly concentrated in the biopolymer matrix sheet, although Ca also exists in the sheet. This Ca spot exhibits the described mineral bridge.[10] The inhomogeneity of Ca, C, and O also supports our determination that the aragonite brick is not a single crystal. The most striking feature of this analysis is that significant amounts of nitrogen were observed in the aragonite brick (Fig. 3d). This finding indicates that the biopolymers (likely proteins and/or glucosamines) could be incorporated into the aragonite brick, and if the biomineral is defined by the biologically produced organic–inorganic composites, an aragonite brick in the nacreous layer is itself a biomineral. These organic components would control the generation of nanocrystals of aragonite and also control the direction of the crystal axis. Thus, the biomineralization process in the nacreous layer of *P. fucata* is regulated at the nanometer or molecular level.

In conclusion, the aragonite brick in the nacreous layer of *P. fucata* is not a single crystal, but our results do show that the aragonite brick is highly oriented. The aragonite brick is composed of aragonite nanocrystals (Fig. 4), and the existence of nitrogen in the aragonite brick indicates the participation of biopolymers in the generation and orientation of aragonite nanocrystals. We believe that the clarification of the biomineralization process of the nacreous layer will lead to the creation of new nanomaterials.

We acknowledge the support of this work by a Research Fellowship of 21st cent. COE program "Integrated EcoChemistry" for Young Scientists [for K. T., 2002–2004], a JSJP Fellowship [for A. O.: Grant 2306 (1999–2002)] and a Grant-in-Aid for Scientific Research on Priority Area (No. 12304040) from the Ministry of Education, Culture, Science, Sports, and Technology, Japan.

Notes and references

† *Physical measurements.* ^{13}C CP/MAS spectra were obtained with a Chemagnetics CMX-300 spectrometer. FE/TEM analyses were taken on a Hitachi HF2000. A thin sliced sample suitable for FE/TEM was prepared with a Hitachi FB2000A. Elemental analyses by EELS were measured using Gatan Imaging Filter 678 on the HF2000 electron microscope.

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