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Abalone nacre insoluble matrix induces growth of flat and oriented aragonite crystals

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Abstract

Mollusc shell formation takes place in a preformed extracellular matrix, composed of insoluble chitin, coated with proteins and dissolved macromolecules. The water-soluble matrix is known to have a strong influence on the growth of $CaCO_3$, whereas the role of the insoluble matrix on mineralization is unclear. Therefore, we mineralized the EDTA (ethylenediaminetetraacetic acid) insoluble organic matrix of abalone nacre with a modified double-diffusion set-up, where the diffusing solutions were constantly renewed. Control experiments were performed with cellulose and chitosan foils. The mineralized matrices/foils were analyzed with SEM. We show that the insoluble matrix of abalone nacre induces the growth of flat and roughly polygonal $CaCO_3$ crystals. In some of the experiments with the insoluble matrix, the growth of three-dimensional parallel sheets of densely packed platelets inside the insoluble matrix was observed. XRD on these samples revealed that they consist of oriented aragonite.

Keywords: Biomineralization; Nacre; Crystallization; Mineralization; Insoluble matrix; $CaCO_3$; Aragonite; Composite

Nacre, the iridescent inner surface of mollusc shells is a highly ordered polymer–mineral composite of extraordinary toughness [1]. The growth of this biomineral is regulated by only a small amount of organic material (2–5% w/w, [2]). The organic material exerts complete control over nucleation, polymorph selection, and morphology—from nanoscale to macroscale—at a degree, which is far beyond today's technological capabilities.

In nacre roughly hexagonal aragonite (one of three $CaCO_3$ polymorphs) platelets (0.5 μm in height, 5–15 μm in diameter) are arranged in layers, separated by sheets of the so-called interlamellar organic matrix in vertical direction. Between individual platelets of the layers, organic material is intercalated, referred to as the intertabular matrix. Single aragonite platelets are strongly oriented, such that the (001) plane of aragonite is parallel to the plane of the interlamellar organic matrix [3].

The growth of nacre takes place in a preformed three-dimensional extracellular matrix, soaked with the extrapallial fluid (short: EPF, consisting ions of a defined concentration [4] and dissolved macromolecules, mainly acidic proteins [5,6]). The EPF is secreted by the mantle epithelium of the animal. For the researcher, the organic matrix is accessible after demineralization, leaving the water-soluble matrix fraction and the water-insoluble, interlamellar, organic matrix (short: insoluble matrix). The interplay of these two matrix fractions during the growth of the material is not fully understood. Many studies have been performed on water-soluble matrix proteins. It is known that most of these proteins interact strongly with growing $CaCO_3$ crystals. The entirety of the water-soluble matrix is able to suppress crystallization in solution [7],[1] but also to promote the growth of (001) oriented aragonite on (1014) calcite surfaces [8]. The insoluble matrix is thought to act as nucleation

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[1] The cited authors examined the soluble matrix of bivalve nacre. Results from our laboratory show that this is also true for gastropod nacre, which we treat here.

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surface and predefined mold, determining size and orientation of the crystals.

An individual sheet of the insoluble matrix has a thickness of approximately 40 nm [6] (abalone nacre) with a core of highly ordered β-chitin [9,10] and a periphery consisting of silk-fibroin-like protein [10,11][2] as well as negatively charged, aspartate-rich proteins [13]. It has been shown that the insoluble matrix is permeable for ions and macromolecules [14]. Histochemical results indicate that there is a fine structure underlying each aragonite platelet, with a central aspartate-rich nucleation zone [13].

In this article, we focus on the influence of the insoluble matrix on growing calcium carbonate crystals. To make sure that possible matrix–crystal interactions are detectable, a method for slow and steady surface controlled mineralization has to be used.

In the widely used ammonium-carbonate method [15], the pH rises over 9.0, a value where $CaCO_3$ precipitation is quite rapid and surface charges of the insoluble matrix may differ from the native state (the pH value of the EPF is about 7.5, [4]). Common double-diffusion methods with two vessels separated by the insoluble matrix seem also inappropriate, because the precipitation of $CaCO_3$ is accompanied with a decrease in pH, which changes surface properties of the matrix and leads in combination with the non-constant concentrations to a non-constant precipitation rate.

Therefore, we remineralized the insoluble matrix of abalone nacre in a modified double-diffusion device where the diffusing calcium carbonate-salt solutions were constantly renewed by a pump. In comparison, we crystallized on cellulose and chitosan membranes. We show for the first time that the insoluble matrix induces the growth of flat and oriented aragonite crystals on the matrix surface. In some of the experiments with the insoluble matrix, we observed the growth of parallel sheets of aragonite platelets inside the insoluble membrane. The in vitro grown material has the same appearance as natural nacre.

Materials and methods

Preparation of nacre pieces. Shells of the gastropod *Haliotis laevigata* (obtained from Abalone Exports, Laverton North, Victoria Australia) were cleaned with water and a brush. Calcitic outer parts of the shell were removed by sand blasting. The remaining nacreous shell was crushed with a hammer in pieces of approximately 2 cm diameter. The pieces were incubated for 2 min in a solution of 1:1 sodium hypochlorite (NaOCl, Merck) and ultrapure water (Milli–Q Academic, Millipore) to remove organic contaminations from the surface. Hypochlorite and dissolved organic compounds were removed by extensive washing with ultrapure water.

Demineralization of nacre. Dialysis tubes (Spectra/Por RC, MWCO 3500, Spectrum Laboratories) were prepared by heating up to 100 °C in 3 mM ethylenediaminetetraacetic acid (EDTA, AppliChem) to remove the glycerol coating, inactivate proteases and eventual contaminations by heavy metal ions. The clean nacre pieces were demineralized by dialysis against 100 mM EDTA with 0.02% sodium azide (NaN_3, Fluka) at pH 5 constantly stirring at 4 °C. The outer solution was exchanged every 1–2 days. Complete removal of mineral parts was indicated by the absence of CO_2 bubbles after 20 days. After 30 days, the demineralization dialysis was stopped. Total demineralization of the insoluble matrix was confirmed by light microscopy, scanning electron microscopy (SEM) of the surface and inner parts, and by using Feigl's aragonite staining method [16].

For storage, the insoluble matrix was dialyzed against 10 mM sodium bicarbonate ($NaHCO_3$, Sigma) with 0.02% NaN_3, pH 8.4, at 4 °C.

Generation of chitosan films. For the generation of chitosan films we used a modified protocol, as described in [17].

Chitosan 0.5% (m/v) (from crab shells, >85% deacetylated, Sigma) was dissolved in 3% acetic acid and filtered (12–25 μm, black ribbon, Whatman). A clean glass slide was covered with the filtrate and put in an oven at 50 °C for 5 h to evaporate the solution. To separate the chitosan film from the glass, it was dipped for 5 h in 1 M NaOH. The resulting films were approximately 0.1–0.2 mm thick, transparent, homogeneous, and robust.

Crystallization experiments were carried out by double diffusion across an ion permeable membrane. The diffusing solutions were constantly renewed with a peristaltic pump to keep concentrations and pH constant and prevent crystallization (Fig. 1)[3] in solution. Three types of membranes were used: The insoluble matrix of nacre,[4] chitosan films, and regenerated cellulose dialysis membranes (Spectra/Por RC, MWCO 3500, Spectrum Laboratories, prepared as described above in the section "Demineralization of nacre"). Membrane pieces of 14 mm diameter were created with a punch and placed on a neoprene ring of 14 mm outer, 12 mm inner diameter and 0.48 mm height. In case of very thin membranes, a second neoprene ring was placed on the other side of the membrane as a spacer. The two sides of the crystallization box were flown through by 20 mM $CaCl_2$ (pH 7.3) and 20 mM $NaHCO_3$ (pH 8.4), respectively, with a speed of 0.5 ml/min/side up to a total volume of 480 ml/side (≈10 h). At the end of the experiment the membranes were cut into halves and dried at room temperature on a glass cover slide with different sides to the top. As a control identically treated excess membrane pieces (except mineralization) were kept and investigated with SEM.

Scanning electron microscopy (SEM). For scanning electron microscopic investigations, the specimens were gold coated with a sputter coater (Emitech K550) and investigated at 20 kV using a Camscan Series 2 (Cambridge Instruments) SEM in secondary or back-scattered electron imaging mode.

X-Ray diffraction (XRD). A *z*-axis diffractometer equipped with bent collimating Göbel mirrors, motorized slits, and a fast NaI scintillation counter (Cyberstar, Oxford Instr.) controlled by SPEC (Scientific Corp.) was used. The mirrors were optimized to select Cu Kα radiation.

Results and discussion

In the following experimental results are described and discussed. Images of some performed crystallization experiments are shown in Fig. 2.

Crystallization on cellulose and chitosan membranes (control)

In the double-diffusion crystallization experiments with cellulose membranes (Fig. 2A), we obtained calcite

[2] Which might exist in a gel-like state during the growth of nacre [12].

[3] Therefore, membrane surface charges are kept constant and crystallization can occur at a constant rate directly on the membrane, possibly showing interactions. A similar device has been previously published [18].

[4] To remove potential mineral remnants, which may influence crystal growth, we incubated some specimens of the insoluble matrix in 6% acetic acid for 1 min and washed them intensively with ultrapure water afterwards before performing double-diffusion crystallization. There was no effect on the resulting mineral phase.

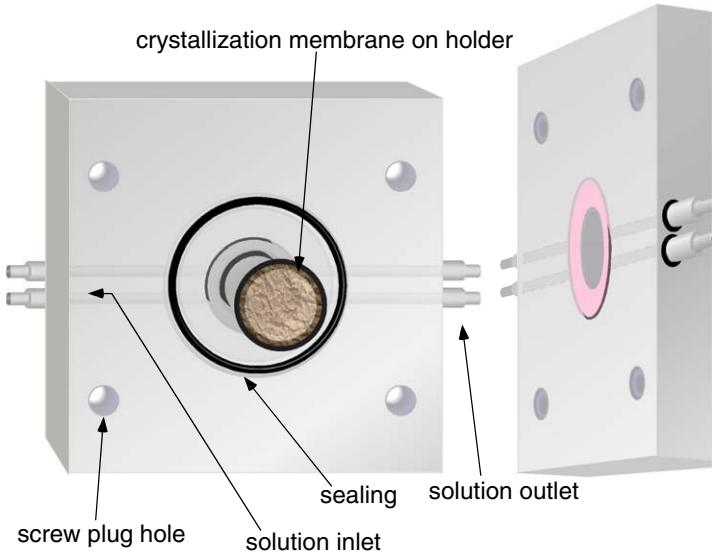

Fig. 1. Double-diffusion crystallization box (opened). An ion permeable membrane separates the constantly pumped carbonate and calcium ion containing solutions, which mix by diffusion through the membrane. Two flow channels per side were used, to get a better radial distribution of ions.

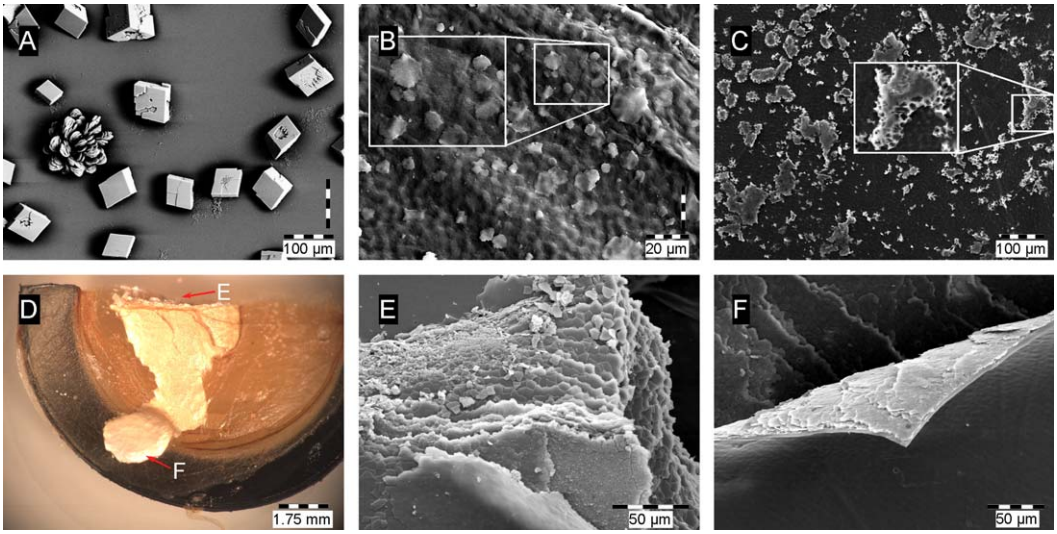

Fig. 2. SEM (A, B, C, E, and F) and optical (D) images of specimens after $CaCO_3$ crystallization experiments in the double-diffusion device. (A) Calcite (rhombs) and vaterite (florets) crystals grown on cellulose. (B,C) Flat crystals grown on the surface of the insoluble matrix show a high similarity to tablets of growing nacre. (D) Macroscopic assembly, grown in the water-insoluble matrix of nacre with iridescent appearance. (E) The fracture region of the specimen displayed in (D) shows a microstructure almost identical to that of nacre (the detail can be seen on (D) as a small, nearly transparent fragment). (F) Attached sheets of platelets inside the insoluble matrix and no mineral on the surface in a region where the matrix cracked during preparation.

rhombohedra and vaterite florets. When we used chitosan membranes, we received mostly vaterite florets and a minor component of calcite. This is in accordance with similar experiments [18,19] indicating that without dissolved additives usually only calcite and—by rapid crystallization—vaterite crystallizes.

Surface crystallization on the nacre insoluble matrix

Crystals, grown on the surface (Figs. 2B and C) of the nacre insoluble matrix, were mostly flat and roundish or roughly polygonal. The average diameter of the crystals is estimated to be 10–15 µm. Sometimes single crystals were joined to form assemblies. The boundary of the crystals was often congruent with the outline of the underlying honeycomb structure (intertabular matrix) of one or several platelet imprints. This indicates that preferred nucleation occurs on the surface of the insoluble matrix between the intertabular matrix layers. In contrast to that, in some of the experiments the honeycomb-like structure was mineralized (see zoom-in box in Fig. 2C), whereas the inside of the honeycomb-like structure was not mineralized or less mineralized. We suggest two possibilities for that. Either the intertabular matrix holds back high amounts of nucleating

proteins, which were pushed away during the growth of the original material, or the structures are artifacts, produced by flaking off of charged crystals under the influence of the electron beam.

The flattened crystal geometry indicates a strong interaction of the surface with the growing crystal. Whether this interaction is heteroepitaxial (for example: nucleation guided by precise alignment of carboxylic groups according to the position of the Ca^{2+} ions of the desired crystal face [11]) or directed by electrostatic interaction of a charged surface with specific crystal planes (for example, the highly polar (001) aragonite plane [20]) is still unclear but the latter seems more likely considering the mineral bridge theory [14] and the flexibility of carboxylic groups of the insoluble matrix surface [21].

Space-filling crystallization inside the nacre insoluble matrix

In a few of the experiments with the insoluble matrix, we received macroscopic, space-filling mineralization inside the membrane with the characteristic iridescent appearance (Fig. 2D). SEM investigations on these samples showed that the mineralization morphology was nearly identical to that of natural nacre. Sheets of platelets formed inside the insoluble matrix (fracture region displayed in Fig. 2E). In Fig. 2F, a region is displayed, where the insoluble matrix surface cracked during the preparation uncovering sheets of platelets under the surface. In Fig. 3, an XRD measurement of a space-filled sample compared with XRD on natural nacre and nacre powder (both: *H. laevigata*) is shown. All samples show aragonite peaks. The space-filled sample and the natural nacre have very similar peak intensities. Compared to the powder sample the intensities are different, with some peaks completely suppressed, indicating preferred crystal orientation. In the remineralized insoluble matrix at $2\theta = 29.4°$ also the main calcite peak is visible, showing that not only aragonite was nucleated.

It is unclear why space-filling crystallization was only obtained sometimes. It might be that the inner matrix structure often collapsed during demineralization process due to the debonding of repulsive, charge carrying groups and making tree dimensional mineralization impossible. This would agree to the proposed model [20] of attached soluble acidic proteins on the matrix surface, acting as crystallization inhibitors in solution (because of chain flexibility) and nucleators when bound to chitin. It is also possible that the proposed silk-fibroin gel between the sheets of the insoluble matrix during growth of bivalve nacre [12] is necessary for sufficient spacing of the insoluble matrix sheets.

Once nucleation successfully started inside the insoluble matrix it seemed to be a self-enhancing process resulting in nacre-like platelet stacks of macroscopic dimensions. We suggest that this behavior results from a stretching of vertically collapsed matrix sheets, allowing lateral mineralization. In horizontal direction, nucleated crystals might grow across the matrix pores (mineral bridges) [14].

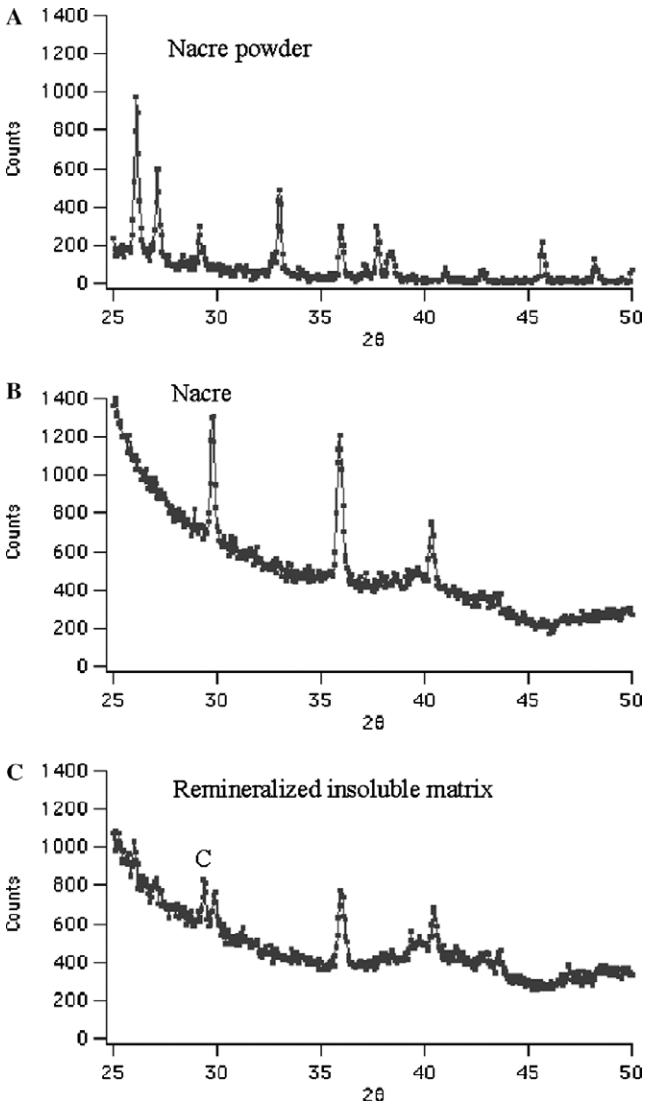

Fig. 3. (A) XRD graph of powdered nacre, showing aragonite powder peaks. (B) XRD graph of nacre from *H. laevigata* taken at 38° (surface, beam). Compared to the nacre powder most of the peaks are missing and show a strong dependency (not shown) on the specimen orientation related to the X-ray beam due to the high crystal orientation. (C) XRD graph of the remineralized nacre insoluble matrix (space-filled sample) taken at 38° (surface, beam) exhibiting almost identical behavior compared with natural nacre. In addition also calcite was present, indicated by the $2\theta = 29.4°$ calcite peak ("C" over peak). All measurements were taken with 25 s sampling time/step at angular steps of $\Delta 2\theta = 0.05°$.

Conclusion

We have shown by SEM and XRD that the insoluble matrix can act as a two- or three-dimensional template for the growth of aragonite crystals. The EDTA insoluble matrix of abalone nacre has therefore a strong influence on growth, morphology, and polymorph of $CaCO_3$. We obtained in some cases plate-like structures of aragonite on the insoluble sheets (acting as a two-dimensional template) and we obtained a nacre-like composite material with parallel sheets of densely packed aragonite platelets

on a three-dimensional template. Further studies on the structure of the insoluble matrix after EDTA demineralization will be performed for a better understanding of these processes.

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References

[1] A.P. Jackson, J.F.V. Vincent, R.M. Turner, Comparison of nacre with other ceramic composites, J. Mater. Sci. 25 (1990) 3173–3178.
[2] P.E. Hare, P.A. Abelson, Amino acid composition of some calcified proteins, Carnegie Inst. Wash. Yearbook 64 (1965) 223–234.
[3] M. Fritz, A.M. Belcher, M. Radmacher, D.A. Walters, G.D. Stucky, D.E. Morse, S. Mann, Flat pearls from biofabrication of organized composites on inorganic substrates, Nature 371 (1994) 49–51.
[4] M.A. Crenshaw, The inorganic composition of molluscan extrapallial fluid, Biol. Bull. 143 (1972) 506–512.
[5] S. Weiner, Aspartic acid-rich proteins: major components of the soluble organic matrix of mollusk shells, Calcif. Tissue Int. 29 (1979) 163–167.
[6] H. Nakahara, G. Bevelander, M. Kakei, Electron microscopic and amino acid studies on the outer and inner shell layers of *Haliotis rufescens*, Venus 41 (1982) 33–46.
[7] A.P. Wheeler, J.W. George, C.A. Evans, Control of calcium carbonate nucleation and crystal growth by soluble matrix of oyster shell, Science 212 (1981) 1397–1398.
[8] J.B. Thompson, G.T. Paloczi, J.H. Kindt, M. Michenfelder, B.E. Smith, G.D. Stucky, D.E. Morse, P. Hansma, Direct observation of the transition from calcite to aragonite growth as induced by abalone shell proteins, Biophys. J. 79 (2000) 3307–3312.
[9] I.M. Weiss, C. Renner, M.G. Striegl, M. Fritz, A simple and reliable method for the determination and localization of chitin in abalone nacre, Chem. Mater. 14 (2002) 3252–3259.
[10] S. Weiner, W. Traub, X-ray diffraction study of the insoluble organic matrix of mollusc shells, FEBS Lett. 111 (1980) 311–316.
[11] S. Weiner, W. Traub, Macromolecules in mollusc shells and their functions in biomineralization, Philos. Trans. R. Soc. Lond. Ser. B 304 (1984) 425–433.
[12] Y. Levi Kalisman, G. Falini, L. Addadi, S. Weiner, Structure of the nacreous organic matrix of a bivalve mollusk shell examined in the hydrated state using cryo-tem, J. Struct. Biol. 135 (2001) 8–17.
[13] F. Nudelman, B.A. Gotliv, L. Addadi, S. Weiner, Mollusc shell formation: mapping the distribution of organic matrix components underlying a single aragonitic tablet in nacre, J. Struct. Biol. 153 (2006) 176–187.
[14] T.E. Schäffer, C. Ionescu-Zanetti, R. Proksch, M. Fritz, D.A. Walters, N. Almqvist, C.M. Zaremba, A.M. Belcher, B.L. Smith, G.D. Stucky, D.E. Morse, P.K. Hansma, Does abalone nacre form by heteroepitaxial nucleation or by growth through mineral bridges? Chem. Mater. 9 (1997) 1731–1740.
[15] L. Addadi, S. Weiner, Interactions between acidic proteins and crystals: stereochemical requirements in biomineralization, Proc. Natl. Acad. Sci. USA 82 (1985) 4110–4114.
[16] T. Kido, Identification of calcitic and aragonitic otoconia by selective staining methods, Acta Histochem. Cytochem. 29 (1996) 121–127.
[17] C.G. Aimoli, M.A. Torres, M.M. Beppu, Investigations into the early stages of "in vitro" calcification on chitosan films, Mater. Sci. Eng. C26 (2006) 78–86.
[18] A. Becker, M. Epple, A high-throughput crystallization device to study biomineralization in vitro, Mater. Res. Soc. Symp. Proc. 873.
[19] G. Falini, S. Fermani, A. Ripamonti, Crystallization of calcium carbonate salts into beta-chitin scaffold, J. Inorg. Biochem. (2002) 475–480.
[20] L. Addadi, S. Weiner, Control and design principles in biological mineralization, Angew. Chem. Int. Ed. Engl. 31 (1992) 153–169.
[21] N. Nassif, N. Pinna, N. Gehrke, M. Antonietti, C. Jäger, H. Cölfen, Amorphous layer around aragonite platelets in nacre, Proc. Natl. Acad. Sci. USA 102 (2005) 12653–12655.