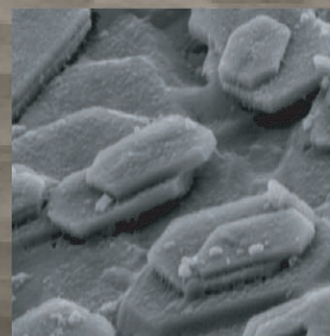
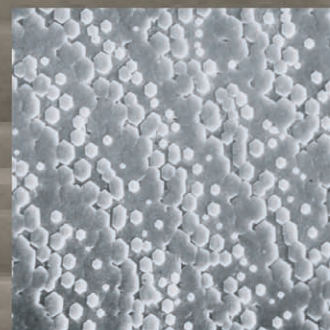
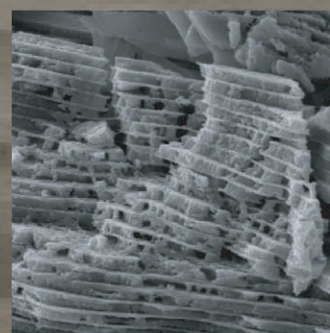
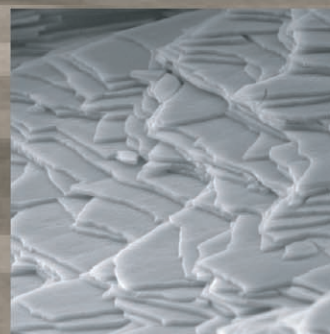




*Mollusk Shell Formation:  
A Source of New  
Concepts for  
Understanding  
Biom mineralization  
Processes*



## Mollusk Shell Formation: A Source of New Concepts for Understanding Biomineralization Processes

Lia Addadi,\* Derk Joester, Fabio Nudelman, and Steve Weiner<sup>[a]</sup>

**Abstract:** The biological approach to forming crystals is proving to be most surprising. Mollusks build their shells by using a hydrophobic silk gel, very acidic aspartic acid rich proteins, and apparently also an amorphous precursor phase from which the crystals form. All this takes place in a highly structured chitinous framework. Here we present ideas on how these disparate components work together to produce the highly structured pearly nacreous layer of the mollusk shell.

**Keywords:** biomineralization · crystal growth · gels · mollusk shell · nacre

### Introduction

The formation of crystals from supersaturated solutions is only rarely, if ever, a straightforward spontaneous process governed only by solubility considerations. Until just a few years ago everyone thought that biogenic crystals form by crystallization from a supersaturated solution, because they form under ambient conditions in an environment full of water. The surprise, however, is that at least in some biological mineralization processes, the systems have evolved incredible means of control, with the result that even the simplest of concepts, such as the presence of a supersaturated solution, are not obvious. Our thinking about biomineralization mechanisms has radically changed as a result of new observations, many of which were made while studying the formation of mollusk shells. Here we describe these results

and present new concepts that we think may well have implications to other biomineralization processes.

Mollusks, like many other mineralizing organisms, including the vertebrates, first isolate their environment of mineral formation from the outside world.<sup>[1,2]</sup> Mollusks use a highly cross-linked protein layer (periostracum) and the epithelial cells of the mantle, the organ directly responsible for shell formation. They then elaborate a matrix within this space comprising various macromolecules. This matrix is the framework in which mineral forms. The major components of the matrix are the polysaccharide  $\beta$ -chitin, a relatively hydrophobic silk protein, and a complex assemblage of hydrophilic proteins, many of which are unusually rich in aspartic acid.<sup>[3]</sup> The final stage of the process is the formation of the mineral itself within the matrix. Some of the acidic proteins are also occluded within the mineral phase as it forms. The mineral in mature mollusk shells is most often aragonite, sometimes calcite, and in certain taxa, the same shell may have layers of calcite and layers of aragonite (reviewed in references [1,3,4]).

Nacre comprises uniformly thick layers of aragonite crystals separated by interlamellar layers of organic matrix (Figure 1). This very simple geometry greatly facilitates structural investigations. In 1984, Weiner and Traub<sup>[5]</sup> presented a model of the interlamellar matrix structure in nacre. The model was based mainly from transmission electron microscope (TEM) observations of forming nacre<sup>[6]</sup> and X-ray and electron diffraction studies of mature nacre.<sup>[7,8]</sup> The mechanistic implications of this model were that the chitin is buried inside layers of silk fibroin, and hence is a passive entity. The silk fibroin is the substrate on which at least some acidic proteins are located that are responsible for the epitaxial nucleation of aragonite crystals. The evidence for epitaxy was based on the observed alignment of the crystallographic  $a$  axes of aragonite with the chitin fibrils.<sup>[5]</sup> One weakness of the model was that there was no direct evidence that the silk itself was structured, let alone aligned with the chitin. This was implied by the fact that in the model it forms a layer between the crystal and the chitin and the diffraction patterns showed that the chitin and the

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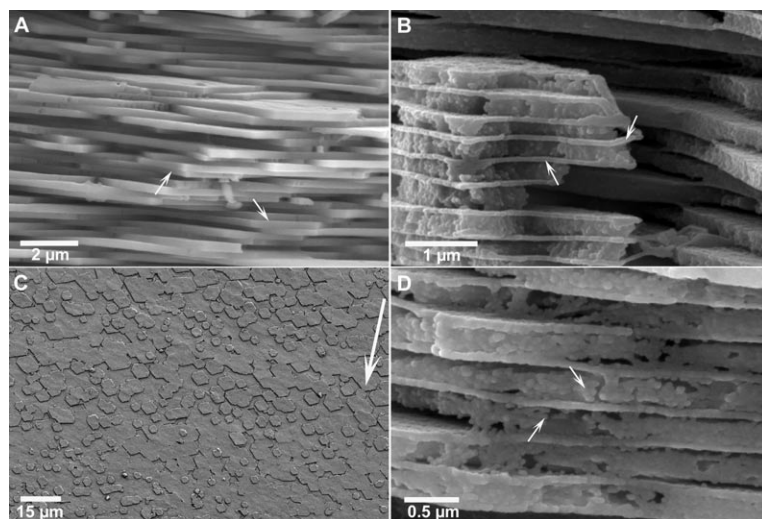


Figure 1. Scanning electron microscope (SEM) images of nacre from the bivalve *A. rigida* (A, C) and the cephalopod *N. pompilius* (B, D). Nacre is a mineralized tissue type deposited by many mollusk species to build the inner layers of their shells. Bivalves and cephalopods are two of the major mollusk classes. Both build nacre following essentially the same blueprint. A) Fracture section of *Atrina* nacre, perpendicular to the shell. Mature nacre consists of thin (~30 nm) layers of matrix alternating with thicker (~500 nm) layers of the calcium carbonate mineral aragonite (lamellae) laid down parallel to the inner shell surface as well as to the layer of shell-building cells in the mantle. The matrix layers are so thin that they cannot be detected in the section (arrows indicate the location of the interlamellar matrix). A significant difference between the ultra-structures of nacre formed by different mollusk classes is the persistence of the orientation of the crystals in the direction perpendicular to the shell surface: in bivalves the aragonite tablets of juxtaposed layers are almost never in register, while in cephalopods they form stacks of ~50 aligned tablets in the vertical direction. B) Fracture section of *Nautilus* nacre, after slight etching with EDTA, fixation and critical-point drying (CPD). During etching and subsequent CPD, the interlamellar sheets expand. They are thus clearly visible as 60–70 nm layers between the etched mineral tablets (arrows). C) SEM image of the nacre growth front on the inner shell surface (*A. rigida*). Individual aragonite tablets are nucleated on the underlying matrix sheet (not visible in this preparation) and grow rapidly in a direction perpendicular to the shell surface (the crystallographic *c* axis). Growth parallel to the lamina follows after the tablet has reached its maximum thickness. The growing crystal tablets are hexagons. They only assume irregular polygonal shapes when they grow and merge together in a continuous layer. The image shows three characteristic, well defined terraces of superimposed growing layers. Arrow indicates the growth direction. The mantle cells were presumably juxtaposed to the terraces in the living organism. D) EDTA-etched and CPD-dried aragonite tablets exhibit a texture of colloidal particles (50–100 nm, arrows) that is typical of crystals grown from amorphous precursors.

crystal are themselves aligned at the molecular scale. The observed relatively weak  $\beta$ -sheet structure could well have been derived from the acidic proteins.

Levi-Kalishman et al.<sup>[9]</sup> revisited this problem using cryo-TEM which avoids the introduction of drying artifacts. They found that the interlamellar matrix was composed almost entirely of  $\beta$ -chitin, with little or no evidence for silk-like proteins. Another level of complexity was added when Weiss et al.<sup>[10]</sup> showed that in mollusk larvae, mineralization of the aragonitic shell occurs by means of the initial deposition of an amorphous calcium carbonate precursor phase. Nassif et al.<sup>[11]</sup> recently demonstrated that the nacreous tablets of adult mollusk shell nacre are coated by a thin surface layer of amorphous calcium carbonate. Even though it is not yet known that adult mollusks form their shells through a transient amorphous phase, the report that adult echinoderms use the transient-phase strategy for forming their calcitic skeletons,<sup>[12]</sup> shows that this is likely.

## Discussion

Here we propose an updated synthesis of nacre structure that can serve as a working model to be tested and improved. We first review the state of our knowledge of the major structural elements of the matrix.

**Chitin:** The chitin is not in the more common  $\alpha$ -form with anti-parallel chains, but rather in the  $\beta$ -form with parallel chains.<sup>[13]</sup> It is clearly highly ordered and has a preferred orientation over length scales of several microns, as evidenced from X-ray and electron diffraction patterns<sup>[7,8]</sup> and from the lattice images obtained from cryo-TEM.<sup>[9]</sup> Only in unusual cases does the preferred orientation extend to longer distances. The chitin fibers are well aligned under individual crystal tablets in nacre.

**Silk:** In nacre the silk is usually the major protein fraction. It is rich in Gly and Ala, or just in Gly.<sup>[14]</sup> When the mineral of the mature shell is dissolved in EDTA, most of the silk remains insoluble.<sup>[15]</sup> Cryo-TEM studies on the structure of EDTA-deminerated nacre sheets failed to reveal a recognizable structure for the silk.<sup>[9]</sup> Furthermore, assemblies of chitin and silk in vitro do not provide either images or diffraction patterns any different from chitin alone, again implying that the silk fraction is not ordered.<sup>[9,16]</sup> The sequence of a Gly-Ala-rich protein from nacre matrix showed that the nacre silk is similar to arthropod silk, and more specifically to spider silk.<sup>[17]</sup> It is thus relevant to note that spider silk in the silk gland is highly concentrated, hydrated, and not ordered.<sup>[18]</sup> Spider silk only becomes fibrillar and ordered during its extrusion from the silk gland.<sup>[19]</sup> Reconstituted silk forms hydrogels of disordered and entangled peptide strands.<sup>[20]</sup>

Pereira et al.<sup>[21]</sup> reported that ~0.05% wt/wt<sub>mineral</sub> of protein with the composition of silk exudes from nacre ground into powder and suspended in water. This silk fraction inhibits calcium carbonate crystallization in vitro,<sup>[22]</sup> similar to the inhibitory effect of reconstituted silk-worm fibroin.<sup>[23]</sup> We recently visualized the proteinaceous material exuding from the nacre of the bivalve *Atrina rigida* by using the environmental scanning electron microscope under wet conditions

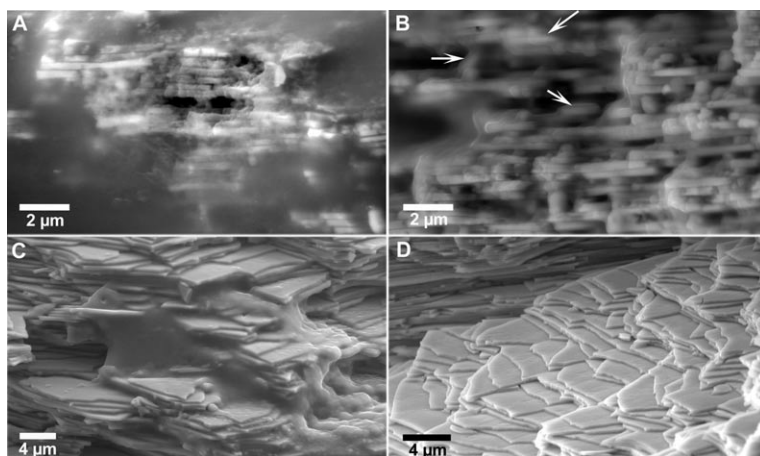


Figure 2. Wet-mode environmental SEM (ESEM) images of fracture surfaces perpendicular to the lamina of nacre of *A. rigida*. Samples were mounted under water and slowly dried at a vapor pressure of 6 Torr, below the dew point of water, then observed at the dew point of water at 5°C. Approximately 2 h elapsed between the mounting and the first image (A). During this time, the silk-like proteins exude from in between the mineral layers and are visualized as a diluted gel material that gradually condenses into films. A) Sample is still mostly covered with water, but the laminar structure and the aragonitic tablets can already be detected. A selected area (top center) was dried under the electron beam, revealing the nacre tablets beneath a very hydrated material. B) With further drying (area different from that depicted in A), tablets appear covered with a hydrated, gel-like organic material (arrows) that is progressively condensing. C) Completely dry sample after lowering the pressure to well below the dew point of water for a prolonged time. The gel-like substance dried into a film covering the tablet edges. D) Protease-treated sample observed using the same procedure as in A)–C) does not show any gel-like organic material. This shows that the material leaching from the shell fragments is protein. It is known from the work of Pereira-Mouries et al.<sup>[21]</sup> that the protein is silk-like.

(Figure 2). The material has gel-like properties, insofar as it is highly hydrated but the water is held within the gel at pressures well below the dew-point of water (Figure 2A and B). When the water is evaporated under more drastic conditions, the material dries as a film (Figure 2C). The gel-like material is sensitive to treatment with bleach and proteases (Figure 2D). We thus have good reason to believe that this gel-like material is composed of the silk proteins isolated by Pereira et al.<sup>[21]</sup> We also noted that the thickness of the interlamellar matrix layers after surface etching of the mineral phase is about twice that of the un-etched layers (Figure 1). This implies that the matrix expands in volume when the mineral is removed. Taken together, this information is consistent with the silk being in a hydrogel-like state also before mineral formation occurs. We surmise that some water is removed from the silk gel while the mineral forms. In mature nacre the silk remains compartmentalized between adjacent crystal polygons or between the crystal and the chitin matrix.

**Acidic proteins:** The acidic proteins assume the  $\beta$ -sheet conformation in the presence of calcium.<sup>[24]</sup> They may thus well be the proteins responsible for the observed protein  $\beta$ -sheet reflections in X-ray and electron diffraction patterns.<sup>[7,8]</sup> We also note that the measured  $d$  spacing between the  $\beta$ -sheets is larger than that expected in silk, and more compatible with proteins with bulkier side-chain groups, such as the Asp-rich acidic proteins. Many sequences of proteins from nacre have been obtained,<sup>[4]</sup> but to date none of these are

unusually acidic. The first sequences of really acidic matrix proteins are from prismatic calcitic layers.<sup>[25–27]</sup> They have well-defined domains, some of which are highly charged. We do know that some of the unusually acidic proteins in nacre are able to selectively nucleate aragonite rather than calcite in vitro, even in the absence of magnesium.<sup>[23,28,29]</sup> We have produced polyclonal antibodies against the aragonite-nucleating acidic protein fraction and have used these antibodies to show that some components of the acidic protein fraction localize in the center of the mineral polygons, at which crystal nucleation is believed to occur (Figure 3C).<sup>[30]</sup> Histochemical mapping of the matrix surface under a single aragonite crystal tablet shows that in the cephalopod *Nautilus* nacre the nucleation site comprises a core of carboxylate groups surrounded

by a ring of sulfate groups.<sup>[30,31]</sup> Interestingly, in the bivalve *Atrina* regions of size comparable to those of the chemically mapped areas in *Nautilus* are topographically delineated in the mature nacre tablets: these are outlined in relief on the side exposed to the mantle cells, while they are concave on the opposite side (Figure 3A and B).

**The mineral:** Mineral tablets from mature nacre diffract as single crystals of aragonite.<sup>[32,33]</sup> The average volume of a tablet is  $10 \times 10 \times 0.5 \mu\text{m} = 50 \mu\text{m}^3$ . To deposit this volume of aragonite (with a density of  $2.96 \text{ g cm}^{-3}$  and a solubility constant of  $10^{-8.22} \text{ M}^2$ ), the volume of saturated calcium carbonate solution needed would be at least  $10^5$  larger than the mineral volume deposited. This clearly represents a logistical problem at the mineralization site, both in terms of transporting sufficient mineral to the site and removing large volumes of water. One possible solution to these problems is to form the initial mineral phase elsewhere and then transport it to the mineralization site.

Mineral-containing vesicles within specialized cells have been observed in many different tissues in mollusks (reviewed in Watabe et al.<sup>[34]</sup>), including blood (hemolymph) cells thought to be involved in mineralization.<sup>[34,35]</sup> Watabe et al.<sup>[34]</sup> report that the mineral phase in most of these vesicles is amorphous calcium carbonate (ACC) or vaterite, both of which are highly unstable. In their study of shell regeneration, they concluded that the mineral phase is dissolved and transported to the site of shell formation and these granules therefore function as ion storage sites. Other



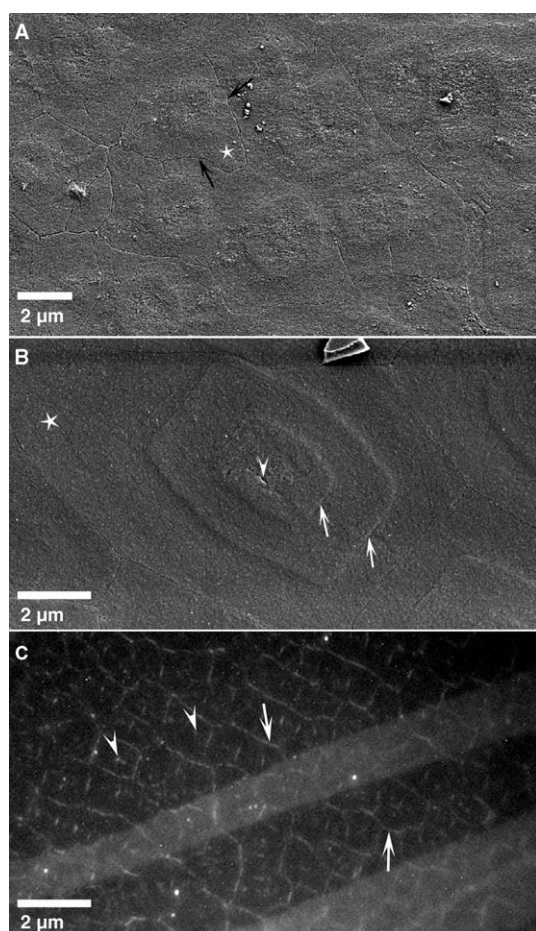


Figure 3. Distinct regions can be detected both on the aragonite tablets and on the organic matrix of *Atrina* which appear to reflect the stages of tablet formation. A,B) SEM images of a surface of *Atrina* nacre, fractured parallel to the shell layers, showing the topography of the tablets. The images were taken at different magnifications on two different fracture surfaces, both facing the mantle (the nucleation side). At least four regions with different topographies can be detected: a central rough indented region of ~600 nm (arrow heads) is surrounded by a higher terrace of hexagonal shape (white arrow). A lower terrace with the same hexagonal profile surrounds the latter (white arrow). From the boundary of this terrace to the boundaries of the polygons (black arrows) the filling material (stars) is at a still lower elevation. A negative imprint of the growth ring structure is observed on the back side of the tablets (not shown). C) Fluorescence micrograph of interlamellar sheets from decalcified nacre of *A. rigida*. Sheets were stained with polyclonal antibodies raised against the acidic aragonite-nucleating fraction of a nacre extract. The proteins are primarily localized in the center of the crystal imprint, which is likely the nucleation site (arrow heads), and the intertabular matrix, that is, the border between to adjacent tablets (arrows).<sup>[30]</sup>

forms of storage granules have been observed, including calcium-loaded phosphoproteins.<sup>[36]</sup> Neff,<sup>[37]</sup> however, observed mineral-containing granules in the epithelial cells of the mantle that are directly responsible for shell formation. These granules are much smaller than the storage granules, and it is conceivable that they may be transported intact to the site of mineralization. These granules did not produce electron diffraction patterns, and could therefore be composed of ACC.

It is not known whether the first deposited ACC mineral phase in mollusk larval shells forms there de novo, or is transported from some other site.<sup>[10]</sup> If the latter is true, and irrespective of whether the mineral phase is transported as a solid phase or a highly concentrated liquid phase, the logistic problems referred to above, would be greatly alleviated. Problems arise if the vesicles are loaded only with calcium, as a substantial input of carbonate would still be required from other sources. Although at least one acidic protein with carbonic anhydrase activity associated with nacre has been identified,<sup>[38]</sup> it is unlikely that such activity can be entirely responsible for providing carbonate to the crystallization sites. Besides, such an enzyme may regulate the carbonate/bicarbonate equilibrium, but does not create the carbonate ions from scratch.

The concept of a first-formed transient mineral phase different from the crystalline mature phase was first proposed for mollusks by Towe and Hamilton,<sup>[39]</sup> who observed hollow “crystals” in the developing nacre of the bivalve *Elliptio complanatus*, and suggested that “A possibility exists that the incipient calcification is not in the form of aragonite but rather in some other phase”. We have also noted that slight etching of nacre tablets brings out a bulk domain texture that is typical of etching of single crystals grown from an assembly of 50–100 nm colloidal particles (Figure 1B and D). This texture differs from the etch pits of single crystals grown from solution, which are normally delimited by well-defined crystallographic directions. These observations support the notion that in adult mollusks,<sup>[11]</sup> as well as larvae,<sup>[10]</sup> the first-formed phase is indeed ACC. Sea urchin larvae,<sup>[40]</sup> adult sea urchins,<sup>[12]</sup> and probably corals<sup>[41]</sup> and crustaceans<sup>[42]</sup> all use the transient amorphous calcium carbonate strategy to build their skeletons and shells.

**Proposed mechanisms of mineralization:** Although many issues are still open, the new information allows us to take some steps forward in understanding the mechanisms of nacre deposition. The main elements of our proposed mineralization scenario are:

- 1) The silk phase is a gel that pre-fills the space to be mineralized.
- 2) The chitin is the ordered structural phase that ultimately dictates the orientation of the mature crystals.
- 3) The matrix components are spatially differentiated.
- 4) The first-formed mineral is transient colloidal amorphous calcium carbonate (ACC).
- 5) Nucleation occurs on the matrix, and the crystal grows at the expense of the ACC phase.
- 6) During this growth phase some of the acidic proteins are occluded into the crystal.

We now discuss these processes in terms of four stages of shell formation:

- I) Assembly of the matrix.
- II) The first-formed mineral phase.

- III) Nucleation of individual aragonite tablets.  
 IV) Growth of the tablets to form the mature tissue

Figure 4 shows a schematic model of the matrix structure prior to and after mineral formation, following the scenario described above.

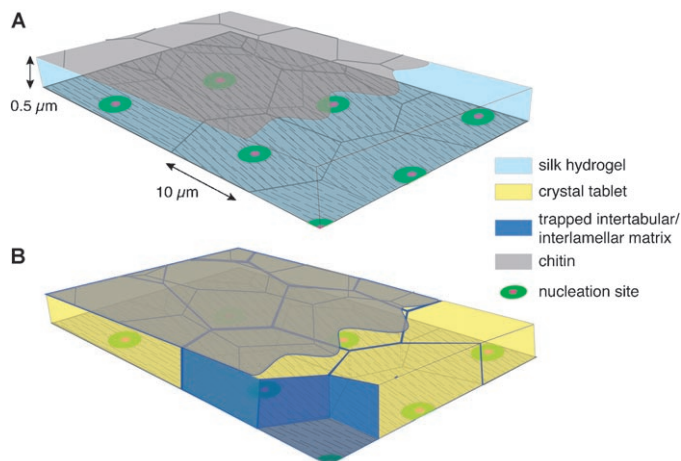


Figure 4. Schematic representation of the suggested model for nacre formation A) before mineralization and B) after mineralization. A) The assembled organic matrix prior to mineral deposition. The microenvironment is formed by two layers of  $\beta$ -chitin, with a gel comprising silk-like protein filling the space in between. Part of the upper chitin layer (upper right) has been removed to show the silk-like protein gel filling. The gel phase may inhibit crystallization and act as a space filler. The silk gel may already be loaded with colloidal mineral particles. Nucleating proteins are adsorbed on the  $\beta$ -chitin sheet. For clarity, the proportions of the spacing between chitin layers and between nucleation sites on the chitin have been altered. Note that the polygonal outlines of imprints are created only during mineralization and have been added to this scheme for added clarity only. B) Mineralized nacreous layer. Nucleation of aragonite (from colloidal particles) is induced on and by the acidic proteins. As the mineral grows, water and silk are displaced. The latter is eventually trapped between adjacent tablets and between the tablet and the chitin layer. Part of the upper chitin layer has been removed together with the underlying interlamellar matrix layer (upper right), to show the mineral tablet surface. A tablet fragment was removed (front corner) to allow visualization of the intertabular and interlamellar matrix.

*I) Assembly of the matrix:* Mantle cells certainly orchestrate the whole process of assembly.<sup>[1]</sup> Chitin is secreted by the cells into the extracellular space. It could well be, but is not proven, that one cell is responsible for the mineralization of one crystal tablet and its associated matrix. In some shells, such as the bivalve *Pinctada* and the cephalopod *Nautilus*, the chitin fibers are aligned laterally over distances much larger than individual cells.<sup>[43]</sup> Thus neighboring cells must be able to align the chitin patches that they form.

The mantle cells produce and release the other matrix components into the extracellular environment. Once released at least some presumably find their correct locations by self-assembly, in which case this pre-supposes that appropriate chitin-binding sites are built into their structures.<sup>[44]</sup> We would assume that the last stage of assembly is the in-

roduction of the silk gel, as in its presence other macromolecules would have difficulty diffusing through the system to find their appropriate locations. One function of this gel is probably space filling, namely to keep the successive interlamellar sheets separated at uniform distances from each other.

The growth of nacre occurs in terraces as can be seen from the locations of the smallest crystals on the inner surface of forming nacre (Figure 1C). Thus crystal nucleation and growth occur simultaneously at different levels. So it could be that the mantle cells are secreting mineral and macromolecules both through the chitin layers and laterally between layers.

*II) The first-formed mineral phase:* Our inclination is to believe that the amorphous mineral phase forms initially in vesicles within specialized cells and these mineral-loaded vesicles are delivered to the site of mineralization by the cells. Vesicle lipid membranes have the ability to stabilize ACC by isolating it from the aqueous environment.<sup>[45]</sup> It is intriguing to think that the membrane may be stripped from the mineral phase as it passes out of the cell and enters the mineralization site. In this way the ACC may be destabilized and thus become more amenable to crystallization. The silk gel phase is known to be a mild inhibitor of mineralization.<sup>[22]</sup> Maintaining the particles in the hydrophobic environment provided by silk may prevent water expulsion that is needed for crystallization to proceed. It may also limit the space for a critical nucleus to develop. Silk may thus help prevent uncontrolled crystallization until the particles are in contact with the nucleating site or with already formed crystalline material. The presence of other inhibitors, such as Mg, phosphate, or certain acidic proteins, may also function to prevent uncontrolled crystallization.

In this scenario we see little function for the extrapallial fluid per se (the fluid between the outer mantle epithelial layer and the forming shell surface). It is often assumed that macromolecules and the ions necessary for mineralization can be secreted by the mantle cells into the extrapallial fluid, in which the former spontaneously self-assemble to generate the matrix and the latter crystallize in the matrix. We concur with others<sup>[1]</sup> that the cells must be juxtaposed to the mineralizing matrix where and when shell is being produced. The mantle of course can contract away from the mineralization site, and when this occurs the site presumably fills with extrapallial fluid. Furthermore, it is difficult to conceive how the matrix could self-assemble with such control over macromolecule location in the extracellular space without the direct intervention of cells.

*III) Nucleation of individual aragonite tablets:* For controlled nucleation to occur at one specific site, the matrix should have a well designed nucleation site that will induce crystal formation more effectively than at all other charged locations. In formed nacre the obvious location for this site is on the matrix surface underlying the center of each tablet. Histochemical studies of *Nautilus* nacre show that at these loca-

tions the matrix contains both sulfates and carboxylates.<sup>[31]</sup> Nudelman et al.<sup>[30]</sup> have shown that these do not co-localize, but that the very center is where the carboxylates are located and this zone is surrounded by a ring rich in sulfates. This arrangement is reminiscent of the structural model for nucleation proposed by Addadi and colleagues in which nucleation occurs on a highly structured carboxylate surface, presumably formed by certain Asp-rich proteins, and that the function of the unstructured sulfates is to attract calcium ions to the site.<sup>[46,47]</sup>

Aragonite nucleating proteins have been identified in the matrix extract after the mineral was removed.<sup>[29]</sup> Antibodies raised against a fraction enriched in the aragonite nucleating fraction did in part map into the matrix centers of crystal imprints, strongly supporting the notion that some of the proteins in this fraction do indeed function as nucleators.<sup>[30]</sup>

The above scenario is consistent with the idea that each aragonite crystal is nucleated de novo from a nucleation site on the matrix surface. It has also been suggested that in bivalves the intertabular matrix of the underlying layer provides a signal for the assembly of the nucleation site.<sup>[48]</sup> On the other hand, the fact that stacks of crystal tablets are very well aligned vertically,<sup>[33]</sup> supports the concept that each stack, be it of a few crystals in bivalves or as many as 300 or so in gastropods, nucleates once and that the single crystal formed propagates through holes in the matrix from one layer to the next.<sup>[49]</sup> It has also been proposed that the alignment of the crystals is a consequence of a passive selection process rather than of active epitaxial nucleation.<sup>[50]</sup> Whatever the mechanism for crystal alignment, controlled nucleation resulting in oriented crystals must occur at some stage. If both holes and structured nucleation sites exist side by side, then clearly the system has a built-in redundancy, which is by no means uncommon in biology.

*IV) Growth of the tablets to form the mature tissue:* The crystal first grows vertically along the fast growing *c* axis of aragonite, until it reaches the next delimiting sheet of chitin. The crystal then grows only laterally because there is no further possibility to assemble the particles vertically. During the growth process some of the acidic proteins are incorporated into the aragonite crystal, in which they presumably alter its mechanical and solubility properties.<sup>[47]</sup> Significantly the silk protein(s) are not occluded into the mineral phase, presumably because they are hydrophobic. They must be pushed ahead of the growing crystal and end up squeezed between adjacent crystals or between the chitin sheet and the crystal.

### Concluding Remarks

The microenvironment in which mineralization takes place is complex. We can now recognize both structured and gel-like domains, hydrophobic and hydrophilic surfaces, clear-cut spatial differentiation of charged functional groups on the matrix surface, and the participation of amorphous col-

loids as precursor phases for the mature crystals. There are still many gaps, some of them large, in our knowledge of the processes involved in vivo. The formulation here of what amounts to a working hypothesis, will hopefully stimulate more experiments that in turn will provide the hard-to-obtain facts needed for achieving a better understanding of this fascinating process.

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