Organic membranes determine the pattern of the columnar prismatic layer of mollusc shells

Antonio G. Checa[1,2]*, Elena Macías-Sánchez[1], Elizabeth M. Harper[3], Julyan H. E. Cartwright[2]

[1]Departamento de Estratigrafía y Paleontología, Universidad de Granada, Granada 18071, Spain,

[2]Instituto Andaluz de Ciencias de la Tierra, CSIC-Universidad de Granada, Armilla 18100, Spain,

[3]Department of Earth Sciences, Cambridge University, Cambridge CB2 3EQ, United Kingdom.

*To whom correspondence should be addressed: Antonio G. Checa

Departamento de Estratigrafía y Paleontología, Facultad de Ciencias, Universidad de Granada, Avenida Fuentenueva s/n, 18071 Granada, Spain

Tel. +34 958243201; +34 635332245

Fax: +34 958248528

Email: acheca@ugr.es

Abstract

The degree to which biological control is exercised compared to physical control of the organization of biogenic materials is a central theme in biomineralization. Here we show that the outlines of biogenic calcite domains with organic membranes are always of simple geometries, while without they are much more complex. Moreover, the mineral prisms enclosed within the organic membranes are frequently polycrystalline. In the prismatic layer of the mollusc shell, organic membranes display a dynamics in accordance with the von Neumann-Mullins and Lewis laws for 2D foam, emulsion, and grain growth. Taken together with the facts that the organic membrane is produced first, and that the same organic membrane pattern can be found even without the mineral infilling, our work indicates that it is the membranes, not the mineral prisms, that control the pattern, and the mineral enclosed within the organic membranes passively adjusts to the dynamics dictated by the latter.

1. Introduction

In the biomaterials making up the shells of molluscs, the question of biological versus physical control has usually focused on the interactions between organic macromolecules and their influence on the mineral phase [1,2], crystal morphology and orientation [3–5]. At a microscopic level, the mutual relationships of the organic matrices and forming minerals have been studied in nacre [6,7], and other microstructural aggregates [8,9]. These studies have led to the proposals of models of self-organization for particular microstructures which help to explain their complex arrangement on simple physical grounds. This is the case of the interlamellar membranes of nacre [7] and the horizontal membranes filling in the cuttlebone chambers [10], both of whose patterning has been explained by liquid crystallization. Similar physical explanations have been recently provided for the calcitic prismatic layers of *Pinna*. These belong to a family of similar materials found in many representatives of the extensive order Pteriomorphia of the class Bivalvia (including pen-shells, pearl oysters, oysters, saddle oysters and some scallops). They are strictly termed of the calcitic columnar prismatic or CCP type and are formed by prismatic grains of calcite with regular polygonal outlines, which elongate and grow perpendicular to the shell surfaces. The mineral prisms are surrounded by relatively thick (0.5–3 μm) organic membranes. They constitute important functional materials acting in bending, their high degree of flexibility being provided by the organic envelopes. The evolution in depth of the calcitic prisms of the outer layer of the pen shell *Pinna nobilis* has been recently explained by the normal grain growth theory [11] because those prisms which initially exhibited lower transverse surface area or a lesser number of sides were more prone to be outcompeted during growth. In this view, the organic sheaths should passively adjust to the changes in dimensions of the prisms perpendicular to the growth axis [12]. Here we have studied in detail the prismatic calcite layers of several molluscan taxa, particularly focusing on the irregularities observed during the evolution of the polygonal pattern. We conclude that when the organic network is considered, it is found to evolve according to the predictions of the von Neumann-Mullins law (which also applies to the normal grain growth theory), therefore being comparable to a soap froth or emulsion. Conversely, several features of the calcite grains do not fit into the normal grain growth theory. The main implication is that the organic network is responsible for the cellular pattern observed. This is a good example of the preponderance of the organic over the mineral phase.

2. Results and Discussion

Several groups of molluscs gastropods [13–15], belemnoid cephalopods and a few bivalves [16, 17], some brachiopods [18] and even vertebrate eggshells [19], secrete external calcitic shell layers organized into prisms or fibres that are not accompanied by any organic membranes. In all these cases the calcitic crystalline domains grow perpendicular to the shell surfaces, and display very irregular outlines (figure 1a–c). These vary from irregular polygonal (figure 1a, b) to fully dendritic (figure 1c). There is good evidence that each domain is a single crystal [18,20] (Supplementary figure S1). Similar outlines are also found in abiogenic sedimentary calcite, e.g., speleothems [21,22] (figure 1d). The irregularity shown by these examples is in stark contrast to the prisms found when calcitic domains are surrounded by organic membranes.

In the CCP layers of pteriomorphs, the mineral prisms can be a single crystal, or be polycrystalline. Evidence that the prisms contained within the organic membranes of some species of the pearl oyster *Pinctada* are polycrystalline is particularly clear. This is formally demonstrated with electron diffraction [23,24] (figure 2), but is also revealed by slight etching [25] (figure 1e). In *Pinctada margaritifera* prisms begin as single crystals with fluctuating crystallographic orientations, but soon begin to split progressively into different crystalline domains [24]. However, throughout this behaviour of the mineral phase, the organic membranes remain unaltered and the prisms develop with the prisms having a smaller surface area or fewer sides becoming preferentially extinguished (Supplementary figure S2). Except for rare instances of polycrystalline prisms (figure 1f), the prisms of *Pinna* are monocrystalline and retain consistent crystalline orientations throughout growth [24], such that it is in *Pinna* impossible to differentiate prism dynamics from that of the organic membranes. Polycrystalline prisms have been observed in the CCP layers of oysters [23] and other pteriomorph bivalves (figure 1g) and, interestingly, are also commonly encountered in calcitic prismatic pearls attributed to *Pinna* (figure 1h). In all cases prism walls meet at 120°, and many walls are curved to meet this Plateau border condition. Nevertheless, this condition is not fulfilled for crystal domains meeting across prism boundaries (figure 1e, inset, figure 3e). Thus, judging from the cases in which the prisms are polycrystalline, organic membranes set the boundaries of mineral prisms and not the other way round.

Examination of the internal shell surfaces of *Pinctada* reveals membranes running towards the prism interiors that incompletely divide a prism (figure 3a–f). Not infrequently membranes may even be isolated within prisms (figure 3b, e). Decalcification shows that these are receding membranes (figure 3f), which, in at least some instances, result from membranes formerly forming complete boundaries that have split and separated during growth (figure 3g, h). At a triple junction formed by an incomplete membrane with two other wall segments the dihedral angles tend to be similar (~120°), although, in general, the angle between the other

wall segments tends to increase with shortening of the receding membrane (figure 3c-e). When the receding membrane becomes very short or disappears, the angle between segments of the adjacent boundary membrane tends to the rectilinear (∼180°) (figure 3c, d, f). With a reduction in the number of sides of a prism, many membranes become strongly curved (figure 3e). In all cases examined, the ends of the receding membranes coincide with domain boundaries of a prism, as revealed after slight etching (figure 2, and figure 3c–e). This is explicable as the two formerly separated crystalline domains progressively closing the gap left upon recession of the membrane. Note that when the process is complete, the crystals do not fulfil the Plateau law any more, whereas this continues to hold for the membranes (figure 3c–e). All this process is sketched in figure 3i.

The reverse process, by which a new membrane extends to separate two crystalline domains previously in contact, is also observed. Observation of the external shell surface reveals the existence of prisms that contain two, three or, rarely, more crystalline domains (figure 4a). Incipient membranes may initially extend, or not, between these different domains. As the prismatic layer thickens, the membranes sometimes extend along the domain boundaries, until they may meet and fuse (figure 4b, c). In this way, an initial prism is divided into two, each of a single domain. Upon extension, the growing membrane deforms the parental membrane until producing a triple junction at or close to 120° (figure 4b). Calcitic prismatic pearls of *Pinna* are particularly interesting because, as usual, some prisms disappear, but, owing to the increase in surface area with pearl growth, prismatic units rather tend to split with the insertion of new organic membranes (figure 4d, e). The final pattern (figure 4e, g) tends to be much more irregular than in the *Pinctada* shell. Without exception, the tips of growing membranes continue into the boundaries between crystallographic domains (figure 4e-g), which are seen by the presence of nanometric membranes (figure 1h, inset). Some membranes may even begin in the interior of prisms, isolated from previous membranes, but always precisely at the boundaries between domains (figure 4f). Some particularly intricate and densely reticulated patterns are recorded in *Pinctada margaritifera* (figure 4h) in which, perhaps owing to the abundance of available organic matter, membranes intrude through the boundaries of the intraprismatic crystalline domains until almost all of them are surrounded by thick organic walls. The disappearance or production of membranes and, eventually, of new prisms brings about a local reshaping of the network topology, which clearly influences the sizes of crystalline domains adjacent to the intervening membranes. A model for either situation is depicted in figure 5. In order for the organic membranes locally to displace the crystalline domains they must include (presumably proteinic) components able to inhibit mineral growth. In summary, it is the organic membranes, and not the mineral domains, that

produce the prismatic pattern typical of this biomineral composite. One further piece of evidence strengthens our conclusion: membranes can be found without accompanying mineral domains during shell regeneration (Supplementary figure S3a-c) or abnormal shell secretion (Supplementary figure S3d) (see also [26]).

In a recent study on the calcitic prismatic layer of *Pinna* [11], the development of the prismatic biomineral aggregate was explained based on normal grain growth theory, by assuming that the increase of thickness of the layer is proportional to the passage of time. According to that hypothesis, the organic membranes, and not the mineral grains, are the passive elements [12]. We are proposing for the same biomaterials that, contrariwise, the main patterning agent is the development with time of the organic network. The organic material is made up of a two-dimensional fluid network within the extrapallial space between the shell and the soft body of the organism, otherwise filled with the extrapallial liquid. It has, therefore, the dynamics of a 2D emulsion, which, like grain growth, belongs to the class of phenomena termed a 'fluid foam' or 'cellular fluid' that includes foams, emulsions, magnetic garnets and grain boundaries in crystals. This dynamics is governed by the von Neumann-Mullins topological law [27,28], which states that those elements with a number of sides greater than six will grow at the expense of those that have less than six sides. We find concomitantly that the prismatic layers of distant molluscan species obey the Lewis law [29]; the average area of a polygon of n sides is proportional to n (figure 6).

3. Conclusions

The mantle cells of the pearl oyster *Pinctada radiata* are in close proximity with the forming mineral prisms and the organic membranes [30]; that is, the extrapallial space thickness is nanometric. Let us perform some order-of-magnitude calculations to see how long it would take calcium and carbonate to diffuse across the extrapallial space, on the one hand, compared to how long it would take an initial homogenous mixture of two immiscible phases to self-organize into structures of prism size, on the other. We have a layer —the extrapallial space — of height h~100 nm (10^{-7} m). Let us consider a structure that forms in this space —the prism size— of characteristic size d~10 µm (10^{-5} m). The diffusion time $t_D \sim L^2/D$, where D is the diffusion constant. The surface-tension-driven velocity will scale as $\sim\gamma/\mu$ where γ is the surface tension and μ is the viscosity. This gives a timescale to compare against the diffusion time: a surface-tension-driven time $t_\gamma \sim \mu L/\gamma$. Now let us compare: for $CaCO_3$ crossing the extrapallial space, the diffusion constant is $D \sim 10^{-9}$ m^2 /s (as for any small molecule or ion) and the diffusion time is $t_D \sim 10^{-5}$ s. The surface-tension driven time for the two phases to self-organize on a scale

of d is $\mu\, d/\gamma$ where the surface tension is $\gamma \sim$ 0.1 N/m (this is water-air, but surface tensions will be this or less) and the viscosity (of water) is 10^{-3} kg/m/s so $t_\gamma = 10^{-7}$ s. Thus surface-tension-driven processes are much faster than diffusive processes, the unmixing of the initially homogenous mixture is much faster than the diffusion time; so a self-organized mechanism is possible. Another possibility is that crystals and membranes are formed by direct deposition from the mantle cells. Given the relative sizes of prisms (tens of micrometres), membranes (1–3 µm) and mantle cells (5–10 µm) [30], every mantle cell must be secreting both organic and inorganic material, depending on exactly which components it is in contact with. This contact is not permanent during shell secretion, since the mantle is periodically extruded and withdrawn. This implies that a recognition process must operate for mantle cells to continue the production of the prisms and membranes of the microstructure. Such a mechanism may explain how the new organic membranes extend exactly at the boundaries between crystalline domains: mantle cells must somehow be able to perceive the boundaries between the crystallographic domains and use them as signals to either extend previous or produce new membranes. This signal might well be the tiny organic pellicles trapped between crystallographic domains in *Pinctada margaritifera* previously defined [25] or recognized here in the pearls of *Pinna* (figure 1h, inset). The two mechanisms need not necessarily be competing hypotheses. During membrane production the mantle cells only secrete, but do not influence the pattern of the organic network, which is only guided by physical laws. In a similar way, the crystals will also interact and compete for space, but only within the volumes set up by the organic cavities. In summary, besides physical constraints, there is a well-orchestrated subjacent cellular strategy. Finally, the fact that organic membranes advance, recede or remain stationary depends on the local availability of organic material. The organic component we suppose is liquid when it is laid down; hence we assimilate it to an emulsion. When we observe it under the microscope, the organic component is now a solid, possibly due to a solidification/polymerization process, currently unknown. What is certain is that the membranes are very elastic and highly deformable. The mineral phase could also have had a liquid precursor (polymer induced liquid precursor, PILP [31,32]), which has been assumed to be generated by acidic biopolymers during biomineral formation [32,33]. Acidic proteins are usually occluded within biogenic calcite prisms (e.g. [34]) and there is good evidence for the occurrence of PILP-like intermediates during prism formation in *Pinna* [35]. The observations of internal surfaces and growth lines demonstrate that the organic membranes and the calcitic prisms are always growing at the same level [26]. Therefore, the conditions for an emulsion between the organic and mineral (plus intracrystalline organic) phases to have developed could have been fulfilled during growth of the CCP layers.

CCP layers form extensive flanges or lamellae that act in bending upon closure of the shell, in order to provide a tight seal of the shell, effective against predators or water loss [36] (Supplementary figure S4). This ability to flex is provided by the deformability of the organic fraction [37]. CCP is possibly the richest in organic matter of all molluscan shell microstructures, with values around 10% (thermogravimetry data on *Pinna nobilis*; Supplementary figure S5). Since the metabolic cost of producing the organic fraction is much higher than that of the mineral component [38,39], this microstructure is particularly expensive. The organic membrane network evolves to lower the free energy of the system by reducing the area of the membranes. From the organismal viewpoint this implies saving part of the energy required for its production.

This is a good example of how organisms make use of simple physical processes to adjust their physiological and adaptive requirements.

4. Methods

Samples and Sample Preparation. Samples of *Pinctada margaritifera* (Pteriomorphia, Pteriidae) came from different sources, mainly Olango Island (Philippines) and French Caledonia. The prismatic pearls of *Pinna* (Pacific coast of Mexico) were provided by KCB Natural Pearls (San Francisco, CA, USA). All specimens are housed in the Department of Stratigraphy and Paleontology, University of Granada (Spain). Partial or complete decalcification was carried out by immersion in 2-4% EDTA.

Optical and Scanning Electron Microscopy. For optical microscopy we used a Nikon SMZ1000 binocular microscope equipped with a digital image acquisition system. Scanning electron microscopy observations were carried out in a desktop SEM Phenom Pro (Department of Stratigraphy and Paleontology, University of Granada) and in an Environmental SEM FEI Quanta 400 (CIC, University of Granada, Spain). Back scatter electron imaging was mainly used in order to enhance the contrast between the organic and mineral phases.

Electron back scatter diffraction. Prior to analysis, samples of the internal surfaces of the shells of *Pinctada margaritifera* were cleaned with 5% NaOCl for 2 min. In order to expose crystal boundaries, they were subsequently etched with 4% EDTA for 2 min. In order to relate surface features to crystal distribution, samples were analyzed unpolished. Since this technique is very sensitive to surface irregularities, the percentage of indexable patterns dropped drastically compared to polished samples, although the number of available data provided relevant information. To avoid excessive charging, samples were coated with a thickness of 2 nm of carbon in a Baltec MED 020 electron beam evaporator (CIC, University of

Granada, Spain). Samples of *Chama arcana* (Supplementary figure S1) were analyzed in a Zeiss Auriga Cross Beam work-station (operated at 20kV) equipped with an Oxford Instruments Nordlysnano EBSD detector of the Centro de Instrumentación Científica (CIC), University of Granada, whereas samples of *Pinctada margaritifera* (figure 2) were analyzed in an identical equipment (same conditions) of the (CITIUS, University of Seville. Spain).

Thermogravimetry. The organic matter content of samples of the prismatic shell of *Pinna nobilis* (Supplementary figure S5) was determined using a thermogravimetric analyzer (SHIMADZU TGA-50H) (CIC, University of Granada) for a temperature range from 25°C to 950°C (rate 10°C/min). Significant weight losses >200°C (when water loss is complete) and <600°C (when $CaCO_3$ decomposition into CaO begins) are attributed to the combustion of organic matter.

Numerical Methods. We processed SEM images using Mathematica.

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Figure 1. Biogenic (a-c) and abiogenic (d) calcite grains with complex morphologies, compared to the polycrystalline prismatic units of pteriomorph bivalves (e-h). a. *Tegula funebralis* (gastropod). b. Belemnoid rostrum (Oxfordian, Wittlesey, England). c. *Chama arcana* (bivalve). d. Speleothem (The Pyrenees, locality unknown). e. *Pinctada margaritifera*. The inset is a detail of a triple junction between mineral grains. f. *Pinna nobilis*. g. *Isognomon legumen*. h. Prismatic pearl of *Pinna.* The inset shows the organic pellicles which surround the crystalline domains. All surface views. e and g have been slightly etched.

Figure 2. Electron back scatter diffraction maps of the internal surfaces of *Pinctada margaritifera*. Secondary electron images and corresponding orientation maps of a wide area (a, b) and two subareas indicated in a (c-f). The loose membranes are receding membranes. Same specimen as in figure 4a. Different colours indicate different crystal orientations (colour key provided in g). The individual cells contain many crystalline domains and the receding membranes act as boundaries between domains. Not also that the tips of membranes continue into contacts between domains. Upon etching, some crystal boundaries are evident in c and e (arrows).

Figure 3. Instances of receding membranes in *Pinctada margaritifera*. a. View of the transition of the normal cellular pattern to another pattern composed of wide cells with loose or incomplete membranes. The arrow points towards the shell interior. b. General view of a similar pattern. Note the abundance of incomplete membranes forming triple junctions with the membrane walls. There are instances of incomplete trifurcate membranes within the cell interiors (arrows). c, d. Close up views of two cells with incomplete membranes, some of them about to disappear completely (c). The tips of some of them continue into wavy crystalline boundaries (arrows). e. Particularly loose pattern. Some of the cells become particularly large (more than 100 µm). Note strongly curved walls (thick arrows). The normal pattern can be seen in the bottom left part of the image. The long thin arrows point towards triple junctions between mineral grains. f. Semidecalcified specimen showing a receding membrane that finally disappears towards the shell surface. Note how the cell wall changes from angled (arrow) to flat with the disappearance of the loose membrane. g. General view of a completely decalcified specimen showing abundant receding membranes. An instance of a residual nanomembrane that has survived decalcification is indicated (arrow). h. Details of splitting membranes (arrows) in similarly decalcified specimens. i. Sketch of the retraction and

disappearance of a membrane at a triple junction (thick lines). The receding membrane leaves behind the contact between two crystalline domains (wavy line). With retraction of the membrane, the angle between the other two segments becomes wider, until they form a single flat membrane. Note that the Plateau's law fulfils only for membranes but not for crystalline domains (Cr1, Cr2, Cr3). a to h are views of the internal shell (growth) surfaces.

Figure 4. Instances of advancing membranes in *Pinctada margaritifera* (a-c, h) and pearls of *Pinna* (d-g). a. General aspect of the external shell surface, with the periostracum removed. Some prismatic units contain two or three crystals (arrows), which are partly fused. b-c. Instances in which incomplete membranes meet and fuse during shell growth, thus delineating two different prismatic units. In c, one of the incomplete membranes has rotated and stuck to the cell wall (arrow). d. Radial fracture through a pearl of *Pinna*. During growth, some units wedge out (long thin arrow), but the pattern is dominated by the splitting of prismatic units due to the initiation of organic membranes (thick arrows). e. Oblique view of the fracture and surface of the same pearl as in e. The membrane seen on the fracture surface initiates at the position of the white arrow. The tips of the membranes and of their minor branches consistently coincide with boundaries between crystalline domains (black arrows). f, g. Two views of the growth surface of another pearl. As in e, the tip of every membrane branch always continues into a nanocrystalline domain boundary. Isolated membranes appear within the cells interior in f. The arrow in f points to the position of a very incipient subsidiary membrane. h. Locally developed meshwork, possibly delineating individual crystalline domains (arrows point to some crystal boundaries). a to c and are views of the external shell surfaces, and e to h are views of the growth surfaces.

Figure 5. Model for the interaction between membranes and crystals in the calcitic columnar prismatic layer of bivalves. Upper row, sequence for the disappearance of the membrane between two cells thus leading to their complete fusion onto a single one. Recession of the membrane causes the previously separated crystalline domains to come into contact. Lower row, sequence for the division of cells due to production of new membranes. These extend along the boundaries between crystalline domains. In the central cell, two membranes initiate at the opposite ends of a domain until they meet and fuse. Note how angles of triple junctions change with the origination or disappearance of membranes. Light grey indicates contracting crystalline areas and dark grey indicates expanding crystalline areas. See also Fig 2i.

Figure 6. The Lewis law $\langle A_n \rangle / \langle A \rangle = \alpha(n-6)+1$ in the calcitic columnar prismatic layer of bivalves where n is the number of sides of a prism, $\langle A_n \rangle$ is the average area of prisms of n sides, $\langle A \rangle$ is the average area of prisms. The species analyzed belong to different orders and superfamilies of the bivalve Subclass Pteriomorphia: Order Pteroida, Superfamily Pinnoidea [*Pinna rudis* (gradient α = 0.32), *Atrina pectinata* (α= 0.29)], Superfamily Pterioidea [*Pinctada margaritifera* (α= 0.27)*, Pteria hirundo* (α= 0.33)*, Pteria penguin* (α= 0.34), *Vulsella vulsella* (α= 0.26), *Isognomon isognomon* (α= 0.39)], Order Ostreoida, Superfamily Ostreoidea [*Ostrea edulis* (α= 0.38)]. The relationship is linear for the common prisms with good statistics whose number of sides is close to six.