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Abstract

The aim of this work was to study the tiling mode of nacre tablets in the 'brick and mortar' array of sheet nacre. For that purpose, incipient shell nacre *(Pinctada margaritifera*) was analysed by electron microscopy (SEM) and Raman spectroscopy. Experimental observations pointed out the key role of the stairs-like growing front in sheet-like nacre not only for its long range ordering but also as controlling the hierarchy of local mechanisms.

A morphogenesis sequence is proposed taking into account the dynamics of the environment. First, the mantel cells are organised to synthesise and discharge alternatively the extrapallial fluid as batches. Because of the stairs-like feature of the growth front, the extrapallial fluid organizes as successive 'biological films', each of them delayed from the underlying one by 10 to 15µm. Each film is a compartment to prefigure a nacre layer. Then, after individualisation, this film undergoes nucleation and crystallisation of tablets. Finally, the biological film transforms progressively as mature nacre following self assembly mechanisms.

The resulting tablets have a shape which responds to a Voronoi growth model, this is shown for the first time: aggregation at the same speed in all directions around single growth centers. This is an efficient model to understand the growth mechanism and rationalise all the experimental observations we have obtained.

1. Introduction

The active role of proteins in biomineralisation is fundamental (Falini *et al.*, 1996; Belcher *et al*., 1996; Feng *et al.*, 1999); it represents a source of inspiration for future nanotechnology with a bottom-up approach. The "brick and mortar" ordering of nacre (mother of pearl, MOP) has already inspired the toughening of ceramic materials by co-processing rigid ceramic as silicon carbide and compliant interlayers as boron nitride (Naslain *et al.*, 1998; Smith, 1998) and many other examples (Simkiss and Wilbur, 1989).
The interdigitating brickwork array of tablets of sheet nacre, is not the only interesting aspect of the nacre structure. The biocrystal itself is a composite. It has not only the mineral structure of aragonite but possesses an intracrystalline organic content (Schmidt, 1924; Watabe, 1965; Weiner and Traub, 1984) having a role of an organic template (Wada, 1972) already evidenced by *in vitro* experiments.
The long range orientation from layer to layer is another intriguing feature to understand. The spatial organisation of the biomineralisation mechanism of the crystal growth is the main focus of the present paper. The original idea is to harden the soft tissues whilst maintaining intact the interface with the hard mature nacre. This new vision of the mechanism of the mineralisation zone yields information on the hierarchy of the phenomena taking place during the biomineralisation.

2. Materials and methods

2.1. Preparation of growing shell surfaces

A six month old *Pinctada margaritifera* oyster, maintained at a depth of 7-12 m in French Polynesia, was sacrificed. It was fixed in 70% ethanol, then dehydrated very progressively and finally the fat was removed with xylene. At this stage the oyster was embedded in methyl methacrylate. Some sections were cut across the animal (100µm thick). Then the shell was removed as shown in Fig.1.

2.2. Direct observation of the hard part of the shell

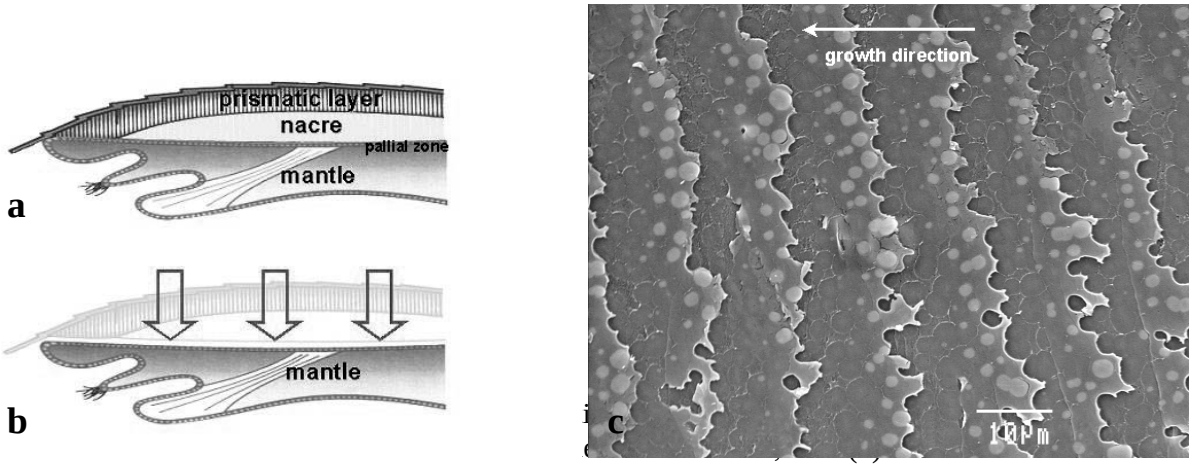
A second type of observation can be qualified as a direct observation of the nacre surface. In this case, the animal is sacrificed and dipped in alcohol. At the laboratory, the soft tissues are gently removed from the shell which is dried, gold coated and then analysed by SEM (second protocol).

2.3. SEM examination

Observations with SEM were made systematically after gold coating on the JEOL JSM-840A of the Common Service of Electronic Microscopy of the laboratories of the Life Sciences (Muséum National d'Histoire Naturelle). A Hitachi 4500 FEG was also used at lower voltage for some more details.

2.4. Raman spectroscopy

Specimens for Raman Microscopy were analysed as following with a DILOR XY spectrometer : objective x50 ; green argon laser excitation at 514.5nm ; laser power at source : 100 to 200mW ; CDD multichannel detection in the range of 130–1200cm^{-1} ; slit width 100-150µm ; 5 counts of 20 seconds and peak position calibration corrected to standard diamond at 1332 cm^{-1}.



Fi m imaged by SEM on the mantle side (empty arrows: SEM incident electrons), (c) incipient nacre layers at the surface of the mantle.

3. Biomineralization sequence observed on mantle side

Contrary to usual SEM observations, Fig.1c gives a vision of the growing nacre layers but on the side of the epithelial tissue. After fixation, dehydration and mounting of the whole animal, the shell was removed from the animal (fixed and hardened by the preparation). Thus what is shown in Fig.1c is a plane view of the mantle surface, after the removal of the mature nacre-layer. This side will be referred to as *mantle side* in the following. As far as is known, it is the first time that plane sections of the growing front of nacre have been imaged. The opposite side, removed from the animal, is the shell covered with the mature nacre. It will be referred to as the *shell side* and it is this side which provided the regular SEM pictures usually produced in the literature.

3.1. Interface in-between hard and soft tissues

It is important to note first that this SEM preparation is a fracture surface. In other words, the surface which is observed in Fig.1c, results from the crack propagation produced by the removal of the mineral shell at the end of the preparation. The crack has separated the hardened tissues of the mantle from the mature nacre. The first comment concerns the way the fracture propagates at the interface. Most of the time the crack propagates parallel to the layering in the interlaminar matrix, but this crack is very regularly deflected to the next interface. It can be seen that the location of the deflection corresponds systematically to the zone where the film contains a critical amount of white round tablets. This can be interpreted as a region with a higher rigidity (critical volume fraction of rigid filler in the supple film). The result is the “garden terrace” appearance revealed by this fracture referred to here as the “stairs-like” front of the biomineralisation in the sheet nacre.

3.2. The stairs-like growing front in sheet nacre

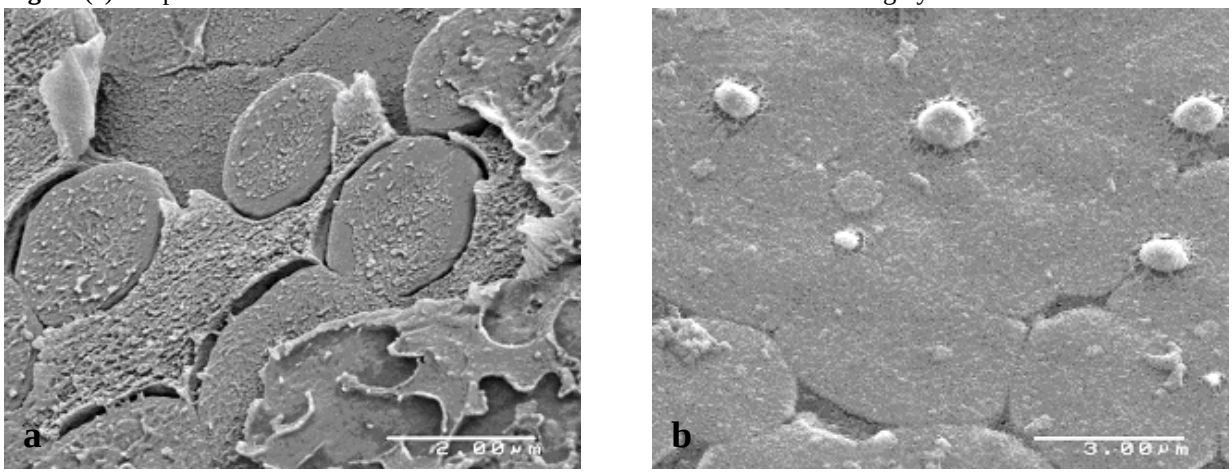
First point revealed by this surface fracture is that nacre growth does not occur layer by layer. *All the layers are growing at the same time, at their stairs-like front.* Each step is a growing layer front with a spatial offset compared to its neighbours (above and below) with a significant distance from one step to the other.

It is also clearly evident from the inspection of this preparation that each layer is initially secreted under the form of the extrapallial fluid: a liquid “precursor” which forms a film and then undergoes a progressive crystallisation in order to become mature nacre. The complete sequence of biomineralisation can be summarised as follows: (i) the secretion of the precursor fluid; (ii) a shaping as a gelatinous-like film (compartment); (iii) nucleation of circular tablets; (iv) growth and progressive transformation into polygons up to the complete biocrystallisation of the layers (v).

3.3. Nucleation signal

The nuclei are located at the level where the intercrystalline matrix of the underlying layer forms, that is to say when tablets of the underlying layer get in contact (Fig.2.). The signal for nucleation is likely to come from the underlying layer. The state of maturation of the underlying layer is critical to control the nucleation process itself, *i.e.* signal to start nucleation in the overlying layer. It is very simply launched in time, thanks to the offset from one row to the next.

Fig. 2. (a) Incipient nacre on the shell side: detail of the fracture surface revealing cylindrical nuclei in the film



(SEM) (b). direct observation of the shell after the animal was sacrificed in alcohol and the soft tissues removed.

4. Mineralisation of the film as seen from the shell side

4.1. Growth of aragonite tablets

Fig. 2 shows the main features observed by SEM on the opposite side, *i.e.* the shell side. With these additional observations, it is possible to observe the remainder of the series of bio-mineralisation stages, *i.e.* platelets growing up to mature nacre.

In Fig. 2a, we find again the film and the isolated round nuclei. It is worthy of note that aragonite tablets acquire first their final thickness and then extend laterally with a cylindrical shape until they become in contact with each other when the film becomes residual and the tablets acquire a polygonal shape. Most of the time the surface is covered by a thin coating which remains stuck on this side. In Fig. 2b, direct osbservation of the shell is necessary to locate nuclei regarding the underlaying row : they are located upon the intercrystalline organic matrix.

4.2. Raman characterization of aragonite nuclei

Fig. 3a is a SEM view at high magnification of a growing step on the shell side. It is easy to distinguish aragonite tablets from the surrounding film at the same level, and polygonal mature aragonite on the layer below. The locations of two comparative Raman analyses are presented in (b) these were obtained on growing white tablets (star) and on the film in the surrounding area (circle). The two spectra exhibit the same peaks of characteristic aragonite. The film also shows the presence of significant fluorescence: a continuous background emission here increasing with wavenumber.

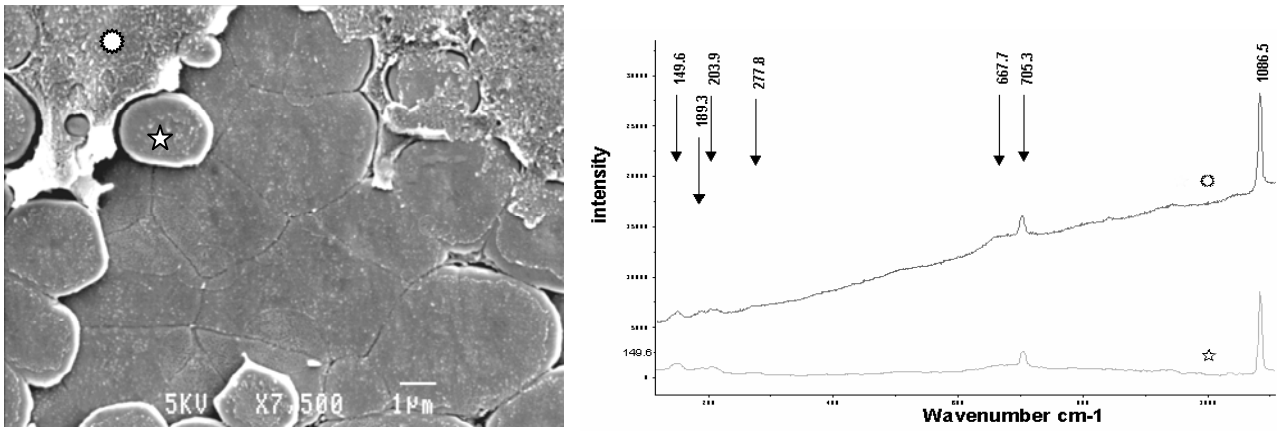


Fig. 3. Raman microscopic characterization of a nucleus (star) and the film (circle) on the shell side: a) analyses location imaged by SEM and b) Raman spectra. The film exhibits the same aragonite peaks but with an increasing background of fluorescence.

5. Modelisation: a Voronoi diagram as a model for tablet growth in the compartment

Some dynamic aspects of nacre growth were quantified. The offset from one step to an other is under control. We measured a distance of 10 to 15µm from one front to the other at the adult stage near the edge. A distance of 20 to 30µm is thus necessary for the complete mineralization involving the following steps: (i) secretion; (ii) shaping of the film; (iii) nucleation; (iv) growth and (v) polygonisation as a completely mature nacre layer, on the same step level. A growth rate of 3.4 $\pm$1.4 layers per day in the case of *P. margaritifera's* pearl was measured by Caseiro (1995).

SEM provides useful contrasts to locate nucleation and to measure its density within the film. As crystallisation proceeds, nuclei appear with a brighter contrast in the film. Nucleation of new tablets has to stay well-controlled in order to be statistically constant in density: ≈11 nuclei per 100µm^2. This makes 1 nucleus per approximately 10µm^2. Which is consistent with the mean size of the tablets in P. margaritifera and P. fucata *(Wada,1972).*

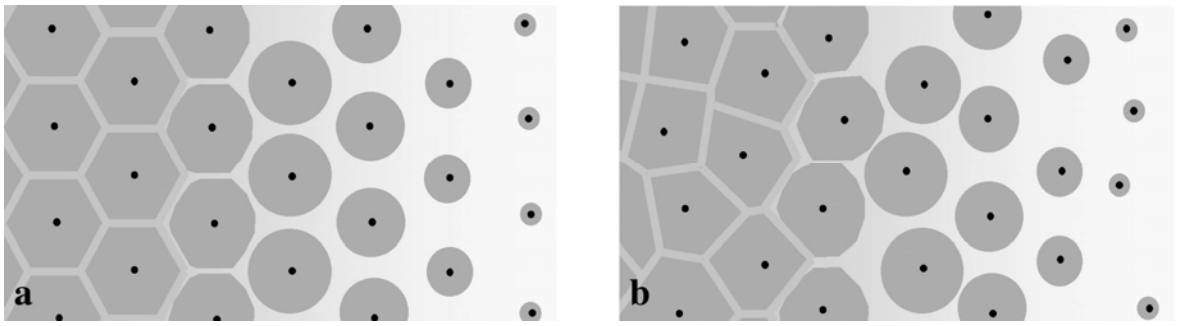


Fig. 4. *Modelization of the front of mineralization within the film of extrapallial fluid : a) a Voronoi diagram for a two-dimensional lattice of nuclei ; b) a more realistic case with the same amount of nuclei but less well ordered.*

The question arises immediately about the fit with a simple Voronoi diagram or any variation of this generic model. Each aragonite tablet is starting from a single centre : this is clearly confirmed here, after Wada (1972). The growth first evolves freely at the same rate in all directions. As a result the nuclei acquire rapidly a cylindrical shape. Then, when getting in contact with neighbours, the tablets turn into polygons (Fig. 4). The deviation from an ideal hexagon shape means that the nucleation location is not as perfectly repeated as in a lattice: this is simulated in Fig. 4. In Fig. 4a, nucleation follows a hexagonal array, thus Voronoi cells turn into perfect hexagons. In Fig. 4b the same amount of nuclei was used but with a small deviation from the regular lattice. As a result, the standard deviation of the tablets size increases and the shape turns into more realistic polygons sometimes with 5 or 7 (or more sides).

6. Conclusions

This work demonstrates that the front of biomineralisation in molluscs like *Pinctada margaritifera* adopts a unique feature : *the stairs-like front*. Its role is fundamental. The offset provided by this feature is responsible for the dynamics and the hierarchy of the processes. Sheet-nacre growth is not a cycled mechanism row-by-row as in columnar nacre (Wise, 1970; Nakahara *et al.*, 1982). This evidence of several simultaneous fronts of growing layers is in agreement with cross-sections obtained by equivalent techniques by other researchers (Wada, 1961; Towe and Hamilton, 1968; Wada, 1972; Nakahara, 1991). It is also in agreement with the different steps of development observed on the shell-side, from the hatching step after the metamorphosis of the oyster (*i.e.* 1 month) until the adult step (Mao Che *et al.*, 2001).

The experimental evidence obtained in the present work furnishes many arguments in favour of *the compartment theory* described by Bevelander and Nakahara (1969). The compartments are "open" : the systematic offset observed from one row to the other is an efficient way to organize interactions such as gradients of matter (*e.g.* water), pH variation, nucleation launching, or transmission of the crystallographic information by heteroepitaxy *etc.*…

An important point in this work is the Voronoi cell-type ordering around each nucleation site and the progressive mineralization in a continuous process from fluid to biomineral. The nacre layered structure is developed by a sequence of events (Fig. 5).

(i) The discharge of a mixture of organic and ionic species by some specialised cells of the mantle.

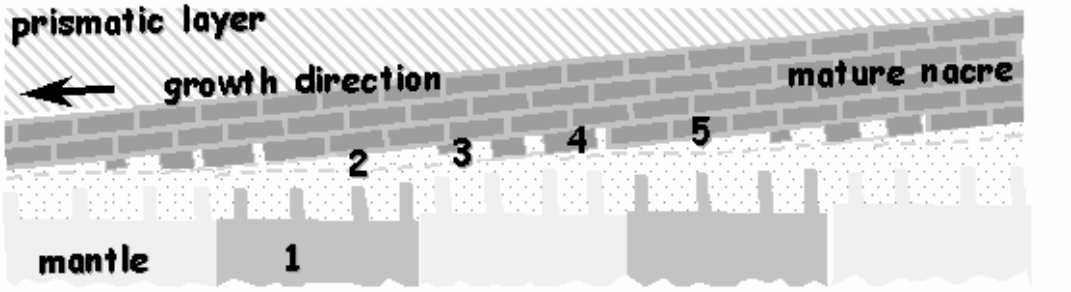
(ii) Each layer of this fluid forms a film at the interface between the mantle and the previous layer already formed (shaping of the film as a compartment).

(iii) This film further undergoes a self-ordering process until its complete mineralization. It starts with nucleation. The location and signal for nucleation comes from the underlying row of nacre: nucleation starts at the moment when the intracrystalline matrix forms in the preceding layer.

(iv) Next is the growth of nuclei in all directions until the total thickness of the film is reached. Then the tablets grow under the form of cylinders in Pinctada (7-12m in French Polynesia).

(v) Finally the tablets become in contact which each other. The layer is completed when the triple points are mineralized. This results in a Voronoi type 2D arrangement.

Fig. 5. Model for the stairs-like mineralization front in the sheet nacre. It shows the simultaneous multilayered growth of shell: 1) discharging epithelial cells, 2) extrapallial fluid organised as a film, 3) nucleation of tablet, 4) incipient nacre developing by self-ordering of the film, 5) mature nacre.



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