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Hierarchical architecture of the inner layers of selected extant rhynchonelliform brachiopods

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ABSTRACT

In spite of several attempts for a best knowledge of the phylum, brachiopods remain, compared with molluscs, among those least analysed in terms of biomineralization. The lack of economic impact for extant species is probably liable for that situation. Much attention has been on the microstructure of calcite biomaterials (rhynchonelliforms and craniiforms). Here, we emphasize the sub-micrometric structure of selected examples of rhynchonelliform shells using Atomic Force Microscopy (AFM) to complement Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray (EDX) analyses. The hierarchical organization of the shell layers (secondary and/or tertiary elements) is highlighted for species non-yet observed from this point of view, and is compared to a few already mentioned in the literature. Previous analysis revealed that granules are composed of a complex aggregation of sub-units in intimate relation with an intracrystalline matrix. Their shape, size and probably early orientation depend on the species as well as age and living environments of the specimens studied. The control of the inorganic part of the composite fibrous elements is constrained by the deposition of nearly arched shape or polygonal protein membranes at the inner boundary of the primary layer, prior to the deposition of the first granules, membranes becoming proteinaceous sheathes progressively enshrining fibres. The diverse orientations of the granules in fibrous neighbours thus further increase arguments in favour of the tendency to improve the shell strength.

1. Introduction

As for many marine invertebrate organisms, representatives of the phylum Brachiopoda, emerging since the early Cambrian, protect their soft body by means of a mineralized skeleton. The bivalved shells of extant brachiopods present a variety of features in terms of shape, colour and/or ornamentation. The shell of rhynchonelliform brachiopods, (one of the three subphyla based on the shell mineralogy, microstructure and loop morphology; Williams et al., 1996), is mainly formed by two layers of low-Mg calcite beneath the periostracum (the external organic layer): a thin external acicular layer and a thicker fibrous secondary layer. In some cases, an additional tertiary layer thickens and reinforces the shell posteriorly with regard to the commissural edge (Williams, 1968a, 1968b; MacKinnon and Williams, 1974; Williams et al., 1996; Boullier et al., 1986; Gaspard, 1986, 1991). Selected rhynchonelliform brachiopods of the order Terebratulida (Terebratulidina and Terebratellidina) illustrate this study with the purpose of emphasizing the sub-micrometric architecture in these calcite biomaterials and trying to decipher, at these scales, the control exerted on the formation of these biogenic crystals during the biomineralization processes within the subphylum. Comparisons have also been introduced with extant Rhynchonellida.

How the shell is built, and how are the different layers, especially the inner layers, processed to shape the shell, are key questions that have yet to be deciphered. Previous work of Williams (1956) and Rudwick (1959) introduced the debate. Detailed observations of the structure of brachiopod shells reveals levels of hierarchy in shell architectural construction as for many other organisms (Currey, 2005; Weiner and Dove, 2003; Weiner and Vekilov, 2003; Gal et al., 2015) and the control exerted at the sub-micrometric scale (Addadi et al., 2015). Here, we focus mainly on the inner (secondary and tertiary) layers. Fibrous elements appear, at first sight, as single crystals but they are complex composite biomaterials organized step by step. Their crystallographic texture has been discussed (Schmahl et al., 2004; Griesshaber et al., 2007). A parallel has been made with the elements of the tertiary layer: prismatic (Williams, 1956, 1966; Boullier et al., 1986; MacKinnon and Williams, 1974) *vs* columnar (Goetz et al., 2009) on relevant species. It appears that

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these carbonate biominerals are biologically controlled as for many phyla, including molluscs (Marin and Luquet, 2004; Marin et al., 2008; Nouet, 2014) this includes the presence of granules (Gaspard, 1986, 1991), and also the control over shape, size and crystallographic orientations (Checa et al., 2009, 2014; Cusack et al., 2008b). One wonders also about the likely presence of a transient amorphous calcium carbonate (ACC) phase (Griesshaber et al., 2009) as in some other phyla (Weiner et al., 2003; Politi et al., 2004). However, brachiopods present differences in the deposition of the diverse layers and the data are less abundant than for other phyla including molluscs.

Herein, the levels of organization in the shell are emphasized by the combined use of localized characterization techniques, such as Atomic Force Microscopy (AFM), Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray Analysis (EDX), the purpose being to point out the observations at a sub-micrometric scale and approach crystal growth propensities and their position as controlled by organic matrices.

2. Materials and methods

2.1. Materials

Several species of extant rhynchonelliform brachiopods were observed, they are: adult *Aerothyris kerguelenensis* (Davidson) collected during the cruises MD 03, MD 08, and MD 30 with the oceanographic research vessel 'Marion Dufresne', off Crozet islands (Apôtres, Cochons, Pingouins, Possession; 119–380 m deep) and Poker II cruise with the trawler 'L'Austral' on the Kerguelen Plate (119–333 m deep) in the Southern Indian Ocean, entrusted by the Muséum National d'Histoire Naturelle (MNHN, Paris) for study (Fig. 1A); representatives of the genus *Macandrevia* King: *M. africana* Cooper sampled during the Walda cruise (station CY14, off Angola, 3431 m deep) entrusted by IFREMER (Brest) and *M. cranium* (Müller), off Norway (Skarnsundet, 70–100 m deep, courtesy of the Trondheim Museum) (Fig. 1C–D), as well as: *Liothyrella neozelanica* Thomson from New Zealand (Doubtful Sound, 120 m deep) (Fig. 1B). Comparisons have been made with some species previously observed: *Gryphus vitreus* (Born), Mediterranean Sea, *Terebratulina retusa* Linné, Firfth of Lorn (Scotland) and *Dallina septigera* (Lovèn) (Bay of Biscay) as well as with a rhynchonellid: *Notosaria nigricans* (Sowerby) from Tikoraki Point, New Zealand.

2.2. Methods

2.2.1. SEM

Fixed in 12.5% glutaraldehyde, etched median longitudinal sections (several seconds in 12.5% RDC, Laboratoire Moderne) were observed using a JEOL LTD SEM after shell embedding in an epoxy resin, sectioning and polishing prior to gold coating (Fig. 2), completing natural internal surface views after mantle withdrawal.

2.2.2. X-ray maps

Maps of elements distribution (particularly Mg and S) have been realized on polished carbon-coated mirror sections (compared to sections for SEM observations) using the SEM-EDX (ZEISS SUPRA 55 VP) operating at 15 kV and a 8 nA current, with the purpose to highlight the major growth rhythms (Fig. 3).

2.2.3. AFM

Analyses of median longitudinal sections, as for the SEM, after light etching (a very few seconds: 1–3, even 6 s. in 0.1% formic acid) or without etching according to samples cleanliness, were performed using a Veeco Dimension 3100 Atomic Force Microscope (AFM) in Tapping mode (Veeco-Bruker©). The OTESPA probes used allow an exact positioning of the probe on the surface sample, the tip being located at the very end of the cantilever, the purpose is the exact setting over a precise point of interest on the sample. In this mode, both height and phase signals are respectively recorded.

Height signal provides an image of the topography of the surface sample. Phase signal is an error signal, which gives insights on surface properties. The phase lag between the period of the oscillation imposed to the cantilever and the recorded period is due to various interactions that can occur between the tip and the sample surface: strong phase lag contrasts therefore reveal variations in elastic deformation or surface adhesion effects. In biomaterials, these viscoelasticity contrasts are mainly associated to variations in organic-inorganic contents (Nouet et al., 2012), giving insights to structural features at a sub-micrometric scale.

AFM brings a more precise appreciation of the topographic height of the granules and, best of all, reveals viscoelasticity contrasts. Moreover, compared to SEM observations, no coating is necessary. As well, 2D and 3D representations are easily obtained.

However, it is essential to get convenient polished surfaces, using progressive grinding papers and diamond polycrystalline or aluminium oxide suspensions. It must be point out that the use of aluminium oxide suspension can occasionally introduce foreign sub-micrometric spherules that obscure the results, even when repetitive ultrasonic cleanings are used. In these cases, an additional very light etching (a few seconds in 0.1% formic acid) was applied.

Note that microboring organisms were occasionally observed in the shell thickness resulting in casual, light or heavy diagenetic modifications at the micro and sub-micrometric level. We carefully avoided these sectors in our AFM investigations.

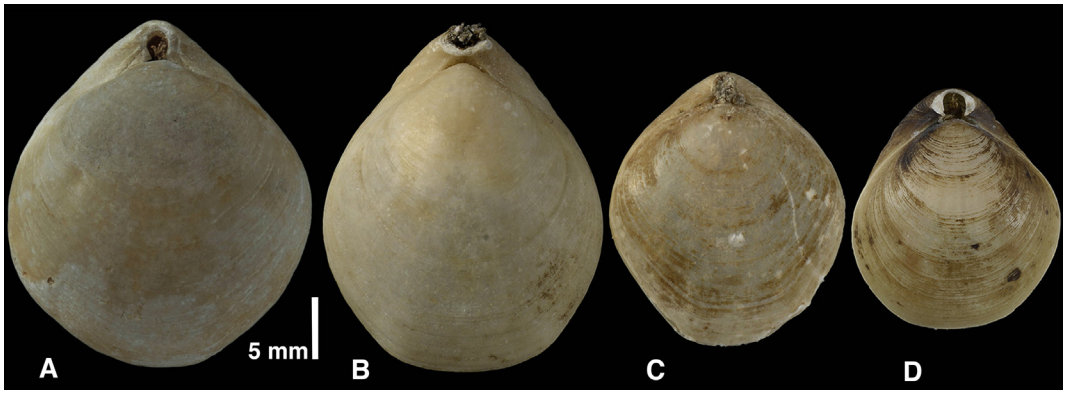

Fig. 1. A. *Aerothyris kerguelenensis* (Davidson); B. *Liothyrella neozelanica* Thomson; C. *Macandrevia africana* Cooper; D. *Macandrevia cranium* (Müller).