Organic macromolecules bind a significant proportion of the precursor calcium for nacre formation.

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Abstract

The mechanism of nacre formation in gastropods involves a vesicular system that transports organic and mineral precursors from the mantle epithelium to the mineralization chamber. Between them lies the surface membrane, a thick organic structure that covers the mineralization chamber and the forming nacre. The surface membrane is a dynamic structure that grows by the addition of vesicles on the outer side and recedes by the formation of surface membranes on the inner side. By using a combination of electron microscopy imaging and spectroscopy, we have monitored the journey of the vesicles from the mantle epithelium to the mineralization chamber, focusing on the elemental composition of the organic structures at each stage. Our data reveals that transport occurs in lipid bilayer vesicles through exocytosis from the mantle epithelium. After release within the surface membrane, chitin undergoes a process of self-assembly and interaction with proteins, resulting in progressive changes of the internal structure of the surface membrane until the final structure of interlamellar membranes is acquired. Finally, these detach from the inner side of the surface membrane. Elemental analysis revealed the transport of a considerable amount of calcium bound to proteins, likely forming calcium-protein complexes.

1. Introduction

Nacre is a biomineral that covers the inner surface of the shells of many mollusks, and which shows a characteristic "brick and mortar" arrangement, where the bricks are constituted by calcium carbonate platelets of the polymorph aragonite, and the mortar is constituted by organics. Unlike conventional human-made brick walls, the parallel membranes that separate and provide structure to nacre, also called the *interlamellar membranes* (ILMs), are formed *prior* to the crystallization of the aragonite platelets (Nakahara 1979, Cartwright and Checa 2007). ILMs are mainly composed of glycoproteins and polysaccharides, specifically chitin in β-sheet conformation (Goffinet 1969, Weiner *et al.* (1980), Weiner *et al.* 1991). Constituting only 5% of the shell dry weight, this organic component confers the biomineral a precisely controlled hierarchical organization, that results in a resistance to fracture significantly superior to its inorganic counterpart (Jackson *et al.* 1988).

Two out the four nacre-forming groups, bivalves and gastropods, have evolved different nacre production mechanisms. In the case of bivalves, nacre precursors are secreted by the ectodermic cells of the mantle epithelium to the *extrapallial space*, a narrow cavity (~50-100 nm) between the tips of the mantle cell microvilli and the shell growth surface. This enclosed space provides the controlled environment necessary for the self-assembly of the shell components.

However, gastropods have a transient extrapallial space, every time the soft body of the animal is extruded from the shell. The formation of the ILMs derives from a dense superficial layer, called the *surface membrane*, which lines the external part of the mineralization compartment (Nakahara 1979). The side of the surface membrane adjacent to the mantle epithelium is composed of a dense organic material, formed by the addition of vesicles (Checa *et al.* 2009). However, in the innermost region, facing the mineralization compartment, the structure changes towards an arrangement of seemingly welded membranes (Bevelander *et al.* 1969, Nakahara 1979) from which fresh ILMs progressively detach in a zipper-like fashion (Cartwright *et al.* 2007, Checa *et al.* 2009) and are positioned with a typical spacing of about 500 nm (Nakahara 1991).

In recent decades, attention has focused on the transport and transformation of amorphous phases for the formation of biominerals. The case of nacre is not different, and the presence of amorphous calcium carbonate (ACC) has been demonstrated in early stages of mineralization of gastropod nacre (DeVol *et al.* 2015). However, little attention has been devoted to the

structure-function relationships of the organic structures (interlamellar membranes, surface membranes) that precede mineralization. Using electron microscopy and spectroscopy we have studied the early stages of mineralization in three marine gastropods, *Phorcus turbinatus, Steromphala pennanti* and *Steromphala* sp. (Trochidae, Vetigastropoda).

We have monitored the vesicular transport system, from the mantle epithelium to the self-assembly of the polysaccharide/protein scaffold at the surface membrane inner side. Our data demonstrate that a significant amount of calcium is transported bound to organic macromolecules in the form of calcium-protein complexes, which then associate with β-chitin to provide the organic framework for aragonite platelet mineralization.

2. Methods

2.1 Critical point drying for SEM observations

Specimens of *Steromphala pennanti* and *Steromphala* sp. were caught alive in Almuñécar (36º44'02''N, 3º41'28''O), in the coast of Granada province (Spain), fixed alive (glutaraldehyde 2.5% in cacodylate buffer pH 7.4) and prepared by critical point drying. SEM investigation was carried out in a Zeiss Gemini at the Scientific Instrumentation Center of the University of Granada (CIC-UGR).

2.2 Chemical fixation

Specimens of the nacreous gastropod *Phorcus turbinatus* were collected in Benalmádena (Málaga, Spain 36°35'42''N, 4°30'56''W). Immediately after capture, specimens were fixed in cacodylate-buffered glutaraldehyde 2.5%. Fragments of the surface membrane area were fractured under the binocular microscope, postfixed in 1% osmium tetroxide, dehydrated in ethanol series and embedded in epoxy (EMbed 812, Electron Microscopy Sciences).

2.3 Histology of the mantle

Phorcus turbinatus specimens were caught alive at the same spot, transported to the lab, anesthetized by intradermal injection of $MgCl_2$ and dissected with a scalpel to isolate the mantle. Mantle tissue was sectioned into 1-2 mm pieces and fixed in cacodylate-buffered 2% paraformaldehyde and 4% glutaraldehyde, postfixed in 1% osmium tetroxide, dehydrated in ethanol series and embedded in epoxy.

2.4 Flash-freeze and freeze-drying

Juvenile specimens of *Phorcus turbinatus* were caught alive in La Herradura (36°43'44''N, 3°43'35''W), in the coast of Granada province (SE Spain), frozen *in situ* in liquid nitrogen and stored in a vacuum flask until the next day. Freeze-drying was carried out in a Flexi-Dry MP (CIC-UGR). The initial temperature (−170 °C) was raised slowly at an average pressure of 75 mTorr for 2 days. At the end of the drying cycle (20 °C), the specimens were removed and stored at 4 °C. This process involves exclusively the elimination of unbound water (sublimation phase or primary drying).

Specimens were cut with a diamond saw and pieces from the surface membrane area were selected for embedding with epoxy (Embed 812, EMS), increasing the proportion of embedding medium to pure ethanol in three steps (1:2, 1:1, and 2:1). Osmication was applied to half of the samples and avoided for the rest. Ultrathin sections were cut (PowerTome Ultramicrotome) at a small angle relative to the surface (< 10º), thus maximizing the visualization likelihood of the surface membrane and interlamellar membranes. The slices were laid in copper grids with lacey carbon to stabilize them under the electron beam.

2.5 Transmission Electron Microscopy (TEM) Imaging

Transmission Electron Microscopy imaging was carried out at 200 kV using either a Philips CM200 equipped with a field emission gun and a Gatan UltraScan 4000 GIF camera or a double Cs corrected JEOL JEM-ARM200F equipped with a Gatan Oneview camera (Fritz Haber Institute of the Max Planck Society, Berlin).

2.6 Energy Dispersive X-Ray Spectroscopy (EDS)

EDS mapping was acquired at 200 kV using a beryllium holder on a Cs corrected FEI Titan (ThermoFisher) equipped with a field emission gun and 4 silicon drift detectors for EDS analysis (Super-X Detection System) (CIC-UGR). Spectra were exported to Digital Micrograph for further analysis using the script "Read Brucker EDS spectrum text file" of Dave Mitchell.

2.7 Electron Energy Loss Spectroscopy (EELS)

EELS spectra were obtained at 200 kV by means of a post-column energy filter spectrometer (Ultrascan 4000 GIF camera, Gatan) using a beryllium double tilt holder. Emission current was set to 5 µA to increase energy resolution and reduce the total dose. EELS spectra covering C Kα, Ca L_2-L_3, N Kα and O Kα core edges (from 240 to 650 eV) were recorded using a dispersion of 0.2 eV/channel, with a collection semi-angle β = 20 mrad and a 2 mm spectrometer entrance

aperture. The energy resolution was ~1.4 eV measured by the full width at the half maximum (FWHM) in the zero-loss peak.

For spectra covering one edge (Ca L_{2-3}), we used a dispersion of 0.05 eV/channel, with a collection semi-angle of $\beta = 20$ mrad and a 2 mm aperture. The energy resolution was ~0.95 eV at FWHM in the zero-loss peak.

Sample thickness was computed using the relative log-ratio method (Mitchell *et al.* 2005) and in all cases $t/\lambda < 0.2$ (between 0.14 and 0.2), and therefore plural scattering contribution can be considered negligible. In any case, the background was subtracted using power-law fitting before each edge and plural scattering was removed using a Fourier-ratio deconvolution, both available in Digital Micrograph 3.1 software (Gatan Inc.). Data plotting and normalization were performed using Origin Pro 9.

3. Results

3.1 The system

The growth of the nacreous layer takes place near the shell aperture (Wise Jr 1970, Mutvei 1978). It is preceded by an aragonitic fibrous prismatic layer at the very shell edge and followed (and covered internally) by a platy aragonitic material with nacreous luster (Figure 1a-b, Supplementary Figure S1). In transversal cross-section, the surface membrane appears as a dense organic structure covering the growing nacre (Figure 1c, d). Interestingly, the tips of the incipient towers are always in contact with the surface membrane (Figure 1d) from where the growing tablet absorbs organic components (Checa *et al.* 2009). On the mantle side, the surface membrane appears covered by a multitude of vesicles that gradually integrate into the whole structure, whereas on the nacre side, the interlamellar membranes detach from it (Figure 1e). The spaces delimited by the interlamellar membranes where the crystals grow are filled by a silk-like protein with a gel-like structure containing acidic glycoproteins (Nakahara *et al.* 1982, Levi-Kalisman *et al.* 2001). When prepared by critical point drying, this organic filler material dehydrates and aggregates, appearing dispersed on the interlamellar membranes (Figure e-h).

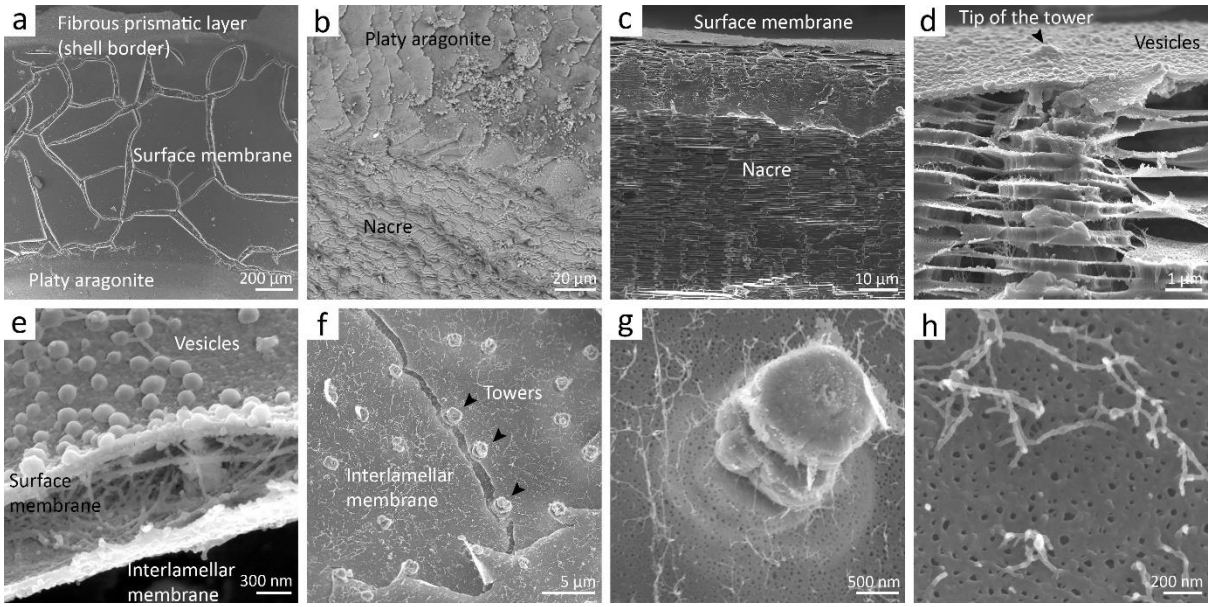

Figure 1. General view of the system. a) A fibrous prismatic layer grows on the edge of the shell, followed to the shell interior by the surface membrane, with a smooth and homogeneous appearance (the cracks are artifacts of sample preparation). A layer of platy aragonite grows next. b) Detail of the boundary between the platy aragonite and the underlying nacre. c) Cross section of the growing nacre. The material is growing actively in the upper zone, where the platelets are incomplete. d) The tips of the growing towers are always immersed in the surface membrane. Each platelet grows within the space limited by the interlamellar membranes. e) Vertical fracture through the surface membrane area, showing the two last formed platelets, the aggregated organic matter within the interlamellar spaces and the vesicles lining the surface membrane. f) Top view of the mineralization chamber with surface membrane removed showing the two most recent interlamellar membranes and nacre towers. After critical point drying, the organic matter that fills the spaces between the interlamellar membranes in the native state, precipitates on the membranes. g) Detail of a tower and an intact interlamellar membrane. h) Detail of the organic material precipitated on the interlamellar membranes.

3.2 Vesicle system

The mantle of gastropods consists of two folds (the inner mantle fold and the outer mantle fold) separated by the periostracal groove (Figure 2a; see also Supplementary Figure S2). The outer shell-facing epithelium (also known as the outer mantle epithelium) is the zone responsible for shell formation (Sud *et al.* 2002). It consists of a single layer of epidermal cells with short microvilli (Figure 2b) and is constituted by three main types of cells: microvillous epidermal cells, ciliated cells and secretory cells (McDougall 2011, Álvarez Nogal *et al.* 2015). The mantle ectodermal cells form an extremely active secretory epithelium, extruding an extraordinary number of vesicles (Figure 2c). The great diversity of vesicle sizes and shapes suggests a complex system controlling the secretion of organic and mineral precursors.

A layer of vesicles invariably appeared on the external side of the surface membrane (Figures 1e, 2d-f). They were mostly round with an average size of ~200 nm (ranging from 50 to 400

nm) and showed the characteristic bilipid membrane (10-15 nm thick) (Figure 2g-i). The cargo was found to be preferentially bound to the inner membrane (Figure 2f, h-i; see also Figure 3b-c). After fusion of the vesicles with the surface membrane, the transported materials diffuse, forming an accumulation of amorphous organic material that mixes with the remaining vesicles (Figure 2h, i).

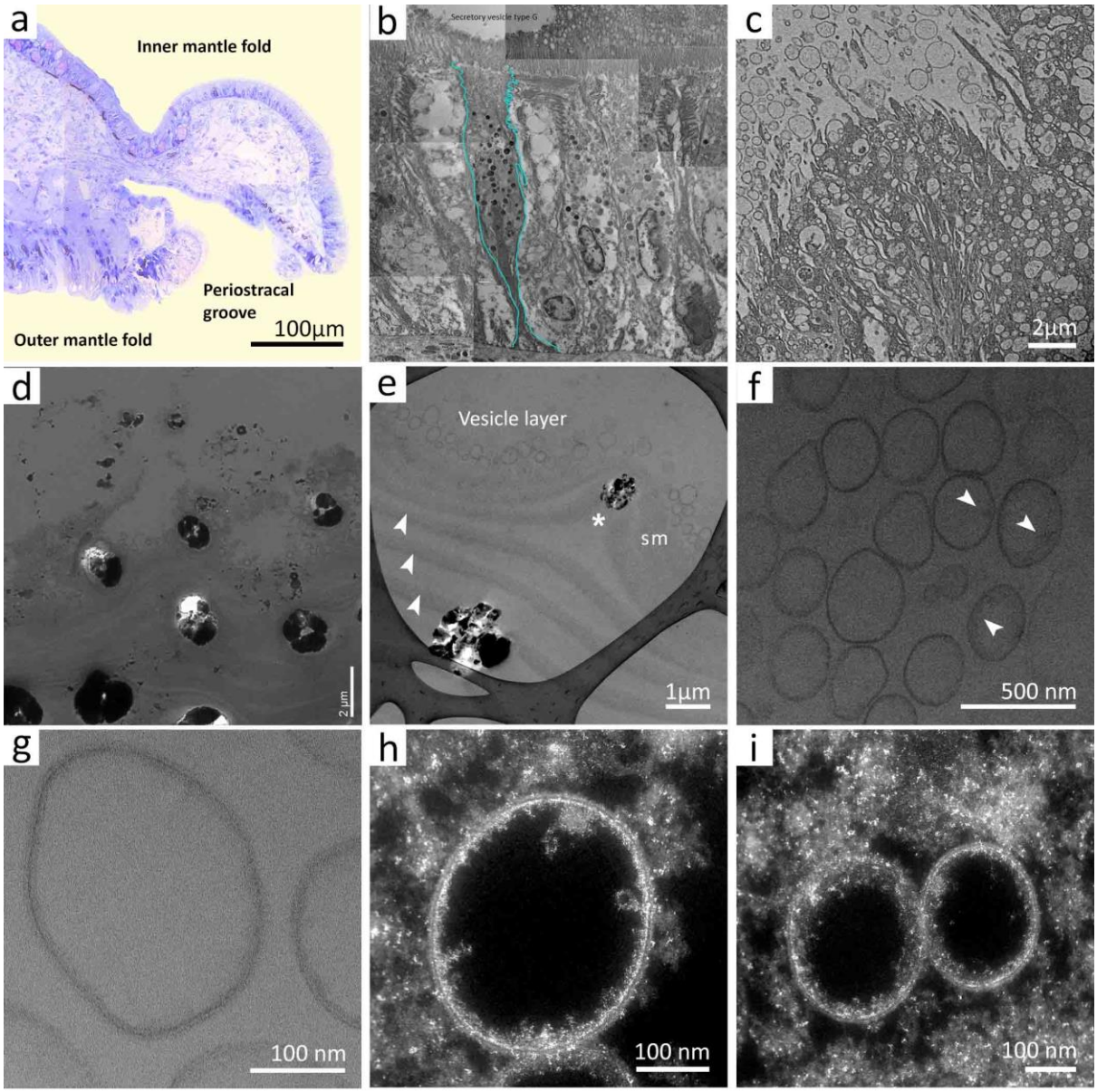

Figure 2. Vesicle system: from the mantle epithelium to the surface membrane. a) Histological slide of resin-embedded mantle epithelium stained with toluidine blue. The inner mantle fold, the outer mantle fold and the periostracal groove are indicated. b) Monolayer of cells in contact with the basal membrane characteristic of the outer mantle fold. A G-type cell is highlighted. c) Microvilli of the cell apical area showing the extremely active exocytosis process. d) On the side of the mineralization compartment, the very tips of the towers appear surrounded by the surface membrane and a large number of vesicles. e) The tips of the first towers (marked with asterisks) are partially immersed in the surface membrane (sm), and the interlamellar membranes (arrowheads) detach from it. f) Cargo attached to the inner membrane

of the vesicles (arrowheads). g) Detail of the lipid bilayer (bright field TEM image). h) HAADF-STEM provides high contrast images based on atomic number differences (Z-contrast). In this case, the osmium tetroxide attached to the lipids double bounds and organic material produces a high contrast against the carbon-based epoxy resin, revealing the bilayer structure of the membrane. i) The vesicle cargo appears adhered to the inner membrane. When the vesicles release the transported material, it aggregates surrounding the remaining vesicles (HAADF-STEM).

3.3 Surface membrane polymerization

Vesicles extruded from the mantle epithelium reach the surface membrane that covers the mineralization compartment. Underneath, the nacre platelets grow in towers within the spaces defined by the interlamellar membranes (Figure 1d; Figure 3a, diagonal section). The formation of the surface membrane results from the fusion of a multitude of vesicles and the release of their cargo (Figure 3b-c). When observed in cross-section, the change in the structure of the surface membrane appears to be the result of a process of polymerization of the transported precursors. Three structurally distinct regions were identified across (indicated in Figure 3c). (1) The outermost region, facing the mantle and next to the vesicular layer, which showed a diffuse and disordered structure (Figure 3b). (2) Towards the mineralization compartment, we found an intermediate region characterized by a *filamentous structure* (Figure 3c). In the innermost region, the surface membrane acquired a *porous structure*, similar to that of the interlamellar membranes (Figure 3c, d). This is also the zone of detachment of the fresh ILMs.

It is important to note that the porous structure is evident in plan-view and in diagonal cross-section (Figure 3d). When cut in transversal cross-section, the interlamellar membranes show the characteristic three-layered structure (Figure 2e, f), as reported previously (Goffinet *et al.* 1977, Nakahara 1979).

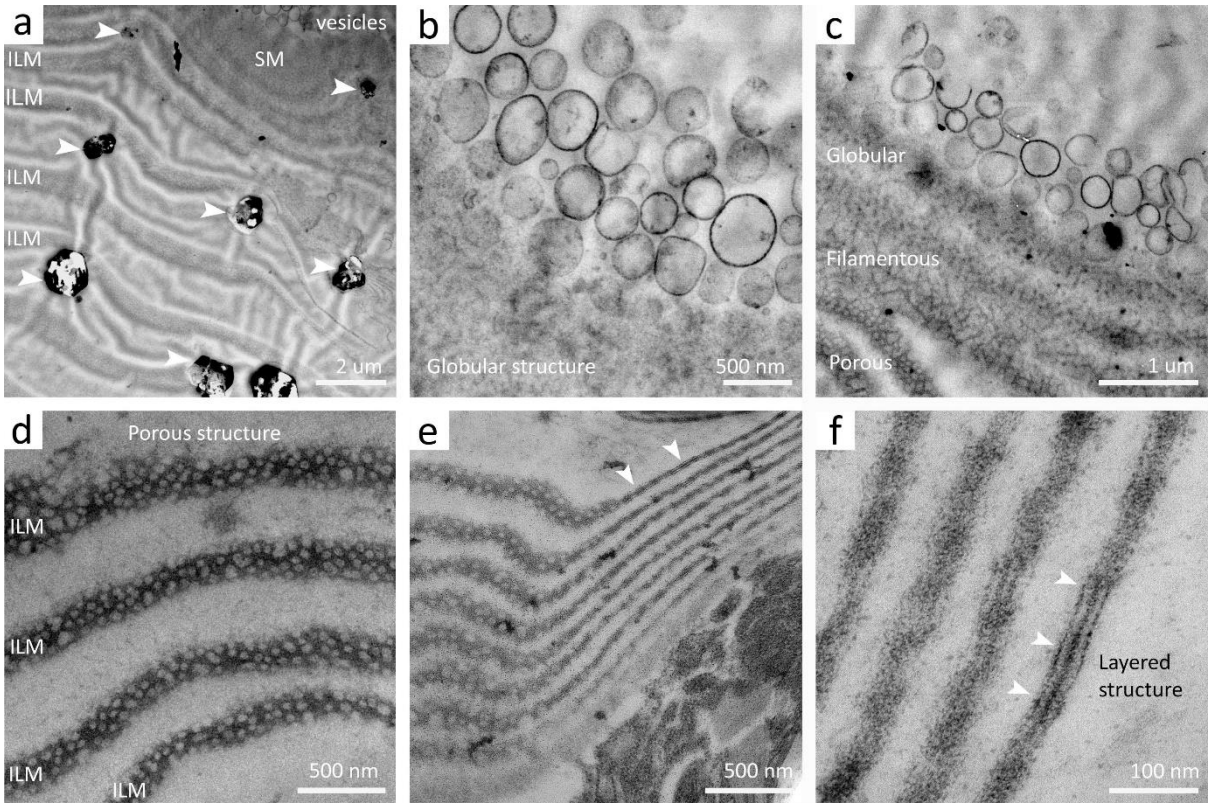

Figure 3. Polymerization of the surface membrane. a) Overview of the system showing the sequential organization from the vesicle layer and the surface membrane (SM) to the interlamellar membranes (ILM). Incipient platelets are indicated by arrowheads. b) Vesicle fusion and cargo release generates a zone of disorganized globular material. c) Transition from a disorganized into a filamentous and, ultimately, a porous structure. d) Porous structure characteristic of gastropod interlamellar membranes (diagonal section). e) Twisting of the interlamellar membranes allows their different projections to be observed in the same image: on the left, the characteristic porous structure visible in diagonal section; on the right, the characteristic three-layer structure observed in transversal cross-section. f) Detail of the three-layer structure. a-c) Inverse HAADF STEM; d-f) BF TEM, post-stained with uranyl acetate and lead citrate.

3.4 Calcium transport

Only flash-frozen/freeze-dried samples retained the calcium content, the signal intensity being higher when OsO_4 was used. Samples prepared by traditional chemical fixation retrieved no calcium signal, probably due to the washout of ionic species and mineral precursors during the different aqueous steps of sample preparation (Hayat 1989, Hayat 2000, Steinbrecht *et al.* 2012).

We conducted a pre-screening using STEM-EDS to determine the spatial distribution of calcium in the organic osmium-stained system. EDS mapping revealed the presence of calcium

in the vesicles (membrane and cargo), the surface membrane and downstream ILMs (Figure 4a-b). These areas were subsequently used to perform systematic EELS measurements.

The epoxy-only regions showed a C K-edge constituted by an initial peak at 285 eV and a second broad feature at 290 eV, characteristic of amorphous carbon. Small features at the N K-edge (400 eV) and O K-edge (532 eV) indicate a minor amount of these elements, as expected from the resin chemical composition (Supplementary Figure S4). No calcium signal was measured. Considering that a significant contribution to the carbon peak comes from the resin, we avoided further analysis of this edge. Vesicle cargo retrieved a significant calcium signal (Ca $L_{2,3}$ edge, 349.3 eV) (Figure 4c-f), while the vesicle membranes also reported a calcium signal, although of lower intensity (Figure 4c, g).

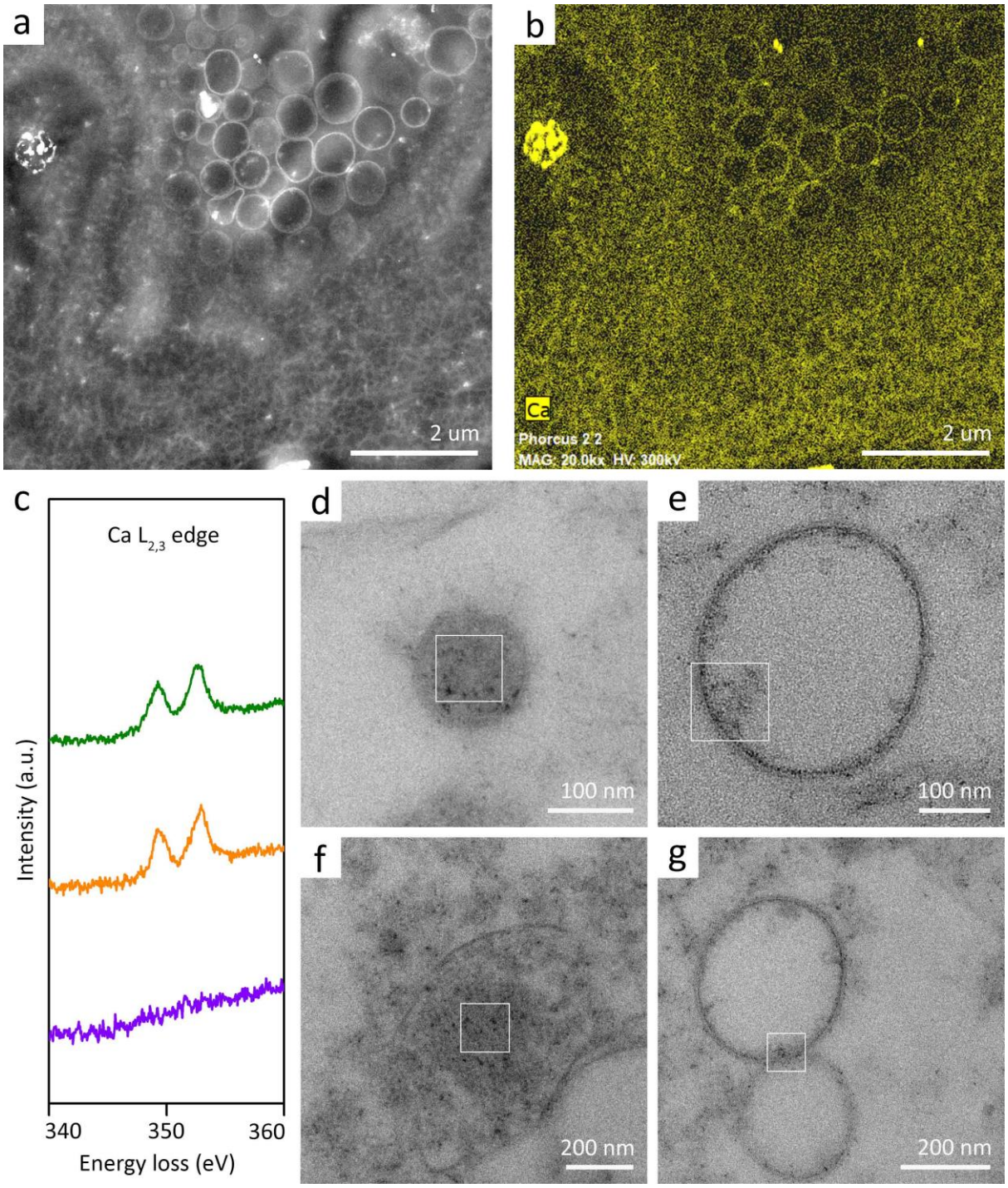

Figure 4. The organic content of the vesicles incorporates a certain amount of calcium. a) HAADF-STEM image of the vesicle layer, the surface membrane and the interlamellar membranes. b) EDS-STEM elemental mapping from the same area showing the calcium distribution in the system. c) Comparative Ca $L_{3,2}$-edge of three different areas: vesicle cargo (green), vesicle membrane (orange) and resin (purple). d-g) TEM images showing the vesicle content. The squares indicate the measured area.

The interlamellar membranes showed an intense calcium signal (Ca $L_{2,3}$ edge, ~349.3 eV) and a significant amount of nitrogen (N K-edge, 400 eV), hallmark of proteins. Interestingly, the electrodense organic aggregates dispersed between the interlamellar membranes also showed both calcium and nitrogen signals, although with lower intensity.

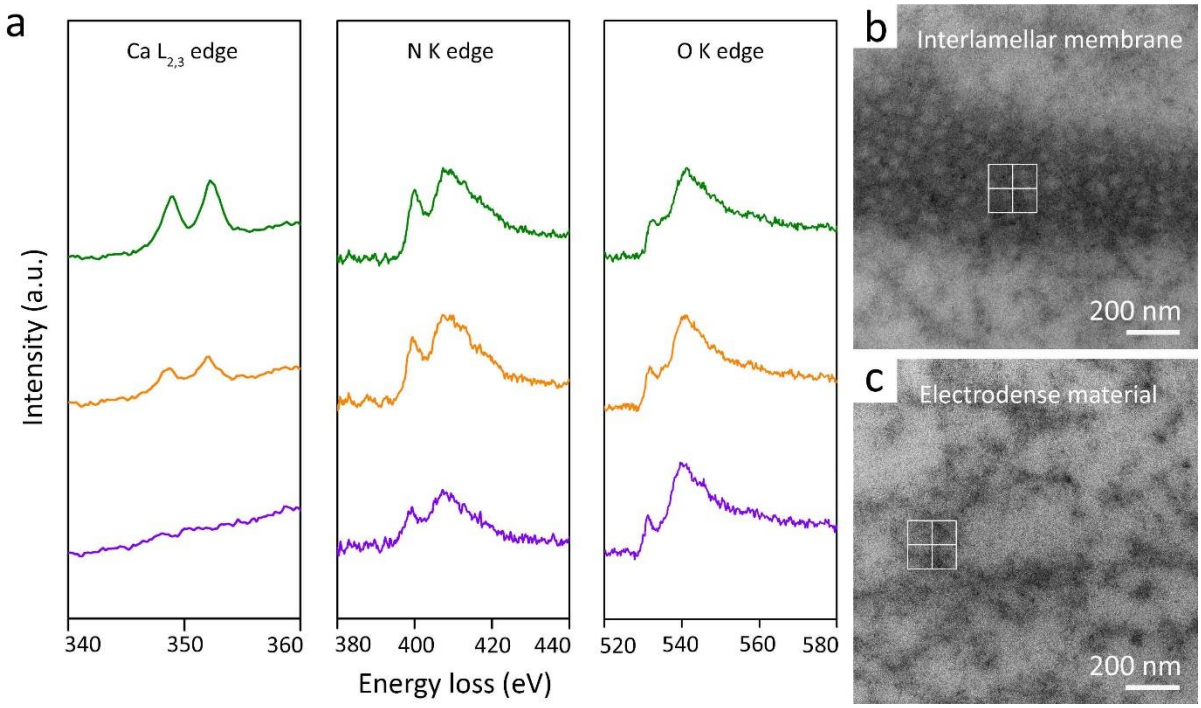

Figure 5. Calcium is bound to protein complexes. c) The three core-loss edges (Ca $L_{2,3}$-edge, N K-edge and O K-edge) of the EEL spectra from three areas: interlamellar membranes (green), electrodense material (orange) and resin (purple). Single spectra, no smoothing was applied. Intensity is normalized and expressed in arbitrary units (a.u.) to facilitate comparison. d-e) TEM images of the sampled areas.

4. Discussion

4.1 Vesicle system

The thickness of the vesicle double membrane and the intense OsO_4 staining (Figure 2e-i), which binds preferentially to double bounds of phospholipids, indicate that they are endosomes constituted of a lipidic bilayer and formed by strangulation of the cell membrane (Figure 2c).

The content of the vesicles was variable in amount, ranging from full to partially empty vesicles, and was mostly attached to the inner membrane (Figure 2e-i; Figure 3b-c; Figure 4a, d-g). Although we could identify calcium within the vesicles, not all of them yielded calcium signal,

supporting previous proposals that different precursors may be secreted independently by specialized mantle cells restricted to discrete areas (Sud *et al.* 2002).

4.2 Surface membrane: chitin self-assembly and formation of the ILMs

Far from being a static structure, the surface membrane is a dynamic entity, assembled by the addition of vesicles and their cargo at the mantle side, and deconstructed at the mineralization chamber side, by the separation of the interlamellar membranes.

It is well established that the interlamellar membranes are mainly composed of proteins and β-chitin (Goffinet 1969), with a small amount of lipids (0.54% by weight) (Rousseau *et al.* 2006), which, in the case of gastropods, must derive from the vesicles involved in the transport system. We can assume the composition of the surface membrane to be similar since it constitutes a preliminary stage of the interlamellar membranes. In fact, the surface membrane represents the *medium* where the chitin self-assembly and the interaction with the proteins takes place. The optimal pH range for the formation of chitin microfibers ranges from 7 to 8.5, which includes not only the pH of physiological conditions but also that of seawater (Montroni *et al.* 2019). The pH of the extrapallial fluid in marine bivalve species ranges from 7.2 to 7.4 (Misogianes *et al.* 1979) and up to 8.3 in freshwater species (Wilbur *et al.* 1983). It is important to note that in gastropods there is no permanent extrapallial space, and in response to external stimuli, the animal retracts inside the shell leaving the growing nacre compartment exposed to seawater (Supplementary Figure S3). This behavior does not seem to alter the formation process, so the surface membrane should be impermeable enough to keep the necessary concentrations of organic and mineral precursors for the formation of the interlamellar membranes and the subsequent mineralization of nacre platelets. SEM images showed the outer surface of the surface membrane as a fibrous structure embedded in a dense protein matrix, with no apparent pores (Figure 1d-e), although we cannot rule out if this appearance is an effect of the critical point drying.

Moving in depth, the structure changes acquiring the characteristic fibrous and porous structure, probably driven by a reorganization of the protein layer. Chitin-protein interaction occurs through hydrogen bonds, once chitin polymerization has concluded (Blackwell *et al.* 1980) and appears to protect the nanofibrils against disassembly and biodegradation (Montroni *et al.* 2019). This chitin-protein network is in turn embedded in an additional protein matrix (Osuna-Mascaró *et al.* 2015).

It should be noted that the tips of the towers are always embedded within the surface membrane (Checa *et al.* 2009). In addition, previous work has highlighted that the nuclei of gastropods tablets are enriched with organic material (Nakahara 1983, Mutvei 1978) and constitute a continuous central axis across superposed tablets (Checa *et al.* 2009). Consequently, the organic material that composes the central core of the plates should be absorbed from the surface membrane. In other groups (*Atrina*, Bivalvia; *Nautilus*, Cephalopoda) the core mainly consists of acidic proteins (Crenshaw 1975) that appear to be concentrically distributed (Nudelman *et al.* 2006).

4.3 Calcium transport

The osmium-stained organic material retrieved a clear calcium signal throughout the entire transport system, indicating that a substantial proportion of the calcium was bound to organic macromolecules. Osmium tetroxide preferentially binds to the C=C double bonds, i.e. phospholipids, but also to some proteins containing certain amino acids (tryptophane, cysteine, histidine) and unoxidized sulfur (e.g. sulfhydryl groups) (Bahr 1954, Hayat 2000), which may relate with the presence of sulfated glycoproteins. Furthermore, the C K edge of the EELS spectra did not yield the characteristic carbonate peak at 290 eV, but only amorphous carbon, indicating that the calcium content bound to organic matter is in organic form.

Of particular interest is the presence of calcium in the organic threads scattered between the interlamellar membranes (Figure 1e, g, h; Figure 5c). Nakahara (1979) first noted the presence of organic aggregates between the interlamellar membranes, indicating that they form a network associated with the organic sheets covering the sides of the growing crystals. According to their nitrogen signature, our results show that they are proteins extruded by the mantle and transported by vesicles. These results are in line to previous reports which indicated that 74.3% of the calcium in the extrapallial space of *Mytilus edulis* was bound to small chelates, and 9.2% was tightly bound to insoluble sulphated carbohydrates (Misogianes *et al.* 1979).

Our data demonstrated that fraction that forms the surface membrane and interlamellar membranes bound a significant amount of calcium in the form of calcium-protein complexes prior to mineralization. The preparation techniques used in this study did not allow for the detection of amorphous calcium carbonate (ACC), and in fact, we could not completely exclude the loss of soluble calcium. However, it is noteworthy that hydrated ACC was found distributed along the interlamellar membranes (DeVol *et al.* 2015, Figure S3, S6), suggesting a cooperative mechanism of calcium transport involving proteins.

Declaration of competing interest

The authors declare no competing interests.

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