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Biomineralization pathways in a foraminifer revealed using a novel correlative cryo-fluorescence–SEM–EDS technique

Gal Mor Khalifa [a], David Kirchenbuechler [b], Naama Koifman [c], Olga Kleinerman [c], Yeshayahu Talmon [c], Michael Elbaum [b], Lia Addadi [a], Steve Weiner [a,*], Jonathan Erez [d]

[a] Department of Structural Biology, Weizmann Institute of Science, 234 Herzl Street, Rehovot 7610001, Israel
[b] Department of Materials and Interfaces, Weizmann Institute of Science, 234 Herzl Street, Rehovot 7610001, Israel
[c] Department of Chemical Engineering and the Russell Berrie Nanotechnology Institute (RBNI), Technion-Israel Institute of Technology, Haifa 3200003, Israel
[d] Institute of Earth Sciences, The Hebrew University of Jerusalem, Jerusalem 91904, Israel

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ABSTRACT

Foraminifera are marine protozoans that are widespread in oceans throughout the world. Understanding biomineralization pathways in foraminifera is particularly important because their calcitic shells are major components of global calcium carbonate production. We introduce here a novel correlative approach combining cryo-SEM, cryo-fluorescence imaging and cryo-EDS. This approach is applied to the study of ion transport processes in the benthic foraminifer genus *Amphistegina*. We confirm the presence of large sea water vacuoles previously identified in intact and partially decalcified *Amphistegina lobifera* specimens. We observed relatively small vesicles that were labelled strongly with calcein, and also identified magnesium (Mg)-rich mineral particles in the cytoplasm, as well as in the large sea water vacuoles. The combination of cryo-microscopy with elemental microanalysis and fluorescence imaging reveals new aspects of the biomineralization pathway in foraminifera which are, to date, unique in the world of biomineralization. This approach is equally applicable to the study of biomineralization pathways in other organisms.

1. Introduction

Mineral formation in biology involves the uptake of ions from the environment and/or from food, followed by their transport in both dissolved and solid forms to the final site of deposition usually in a mineralized tissue. The challenge is therefore to follow the pathways of both the anions and the cations from uptake until final deposition. Here we report a novel correlative approach to investigate aspects of biomineralization pathways, and apply it to the study of the biomineralization pathways in benthic foraminifera. We use the membrane impermeable dye calcein and energy dispersive X-ray spectroscopy (EDS) to provide information on elemental distributions within the complex cellular milieu revealed by cryo-SEM inside the cytosol.

Foraminifera are unicellular marine protozoans that produce mainly calcitic shells (Lee and Anderson, 1991). Foraminifera are widespread in oceans throughout the world (Hemleben et al., 1989; Murray, 1991), and are major contributors to calcium carbonate global production (Erez, 2003). Their shells accumulate on the ocean floor and are eventually fossilized into chalks and limestones. The chemical and stable isotopic compositions of their calcitic shells are used to reconstruct past ocean conditions (Broecker and Peng, 1982; Emiliani, 1955; Katz et al., 2010; Wefer et al., 1999). Relatively little is known, however, about the mechanisms by which foraminifera produce their shells. This information is necessary not only for understanding a widespread biomineralization process, but also for understanding the connections between shell chemistry and environmental parameters used for paleoclimate reconstruction (Erez, 2003).

Foraminifera form their shells by the addition of consecutive chambers. The chambers are interconnected and the cytoplasm streams between them (Cushman, 1947). Shell mineralization in foraminifera is, therefore, not a continuous process but occurs in pulses each time a successive chamber is added to the shell. The calcitic radial (also known as perforated) foraminifera form their new chambers by first producing an organic template (anlage) and further mineralizing it with calcite crystals from both the inner and outer sides of a thin organic matrix often called the primary organic sheet (Hottinger, 1986). Every time a new chamber is

* Corresponding author.
E-mail address: Steve.weiner@weizmann.ac.il (S. Weiner).

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formed, the foraminifer adds an additional shell layer to its entire outer surface in a process termed lamination (Hottinger, 1986) (Fig. 1, Movie in SI1). In addition it adds an internal layer of calcium carbonate called the inner lining (Hansen and Reiss, 1971).

Studies of various benthic foraminifera have shown that a major mode of ion uptake is via the incorporation (endocytosis) of sea water into vacuoles that enter into the cytoplasm (Bentov et al., 2009; Erez, 2003). These vacuoles provide the ions to the fluid that is in intimate contact with the site of mineralization and is most likely the source of calcium and most of the carbonate (Bentov et al., 2009; Erez, 2003).

Some perforated foraminifera store the calcium (Ca^{2+}) and inorganic carbon extracted from the sea water during the intervals between chamber construction (Anderson and Faber, 1984; Angell, 1979; Erez, 2003; Lea et al., 1995; Nehrke et al., 2013; ter Kuile and Erez, 1987, 1988; ter Kuile et al., 1989; Toyofuku et al., 2008). The fraction of ions destined for mineralization and derived from the intracellular Ca^{2+} pools varies between different species. Some species have no internal pools, some have small pools (Angell, 1979; Lea et al., 1995; Nehrke et al., 2013), and some have large pools which store significant amounts (up to 90%) of the Ca^{2+} needed for shell mineralization (Anderson and Faber, 1984; Erez, 2003; Toyofuku et al., 2008). The calculated concentrations of Ca^{2+} and carbonate in the intracellular pools suggest that these ions may be stored in the cytoplasm, possibly as an amorphous calcium carbonate (ACC) phase (Bentov et al., 2009; Erez, 2003), but no such mineral phase has been identified to date. Other studies obtained during proxy calibration experiments and based on shell chemistry are interpreted to suggest that Ca^{2+} and carbonate are stored in separate pools in the cytoplasm (Duenas-Bohorquez et al., 2011; Raitzsch et al., 2010). It has also been proposed that part of the Ca^{2+} ions are transported by Ca^{2+} pumps directly from sea water, with no lag time between incorporation into the cytoplasm and deposition in the shell wall, and/or part of the ions are transferred by sea water vacuolization (Mewes et al., 2015; Nehrke et al., 2013). Differences between various proposed biomineralization mechanisms may be attributed to different species being used in the studies. Clearly much remains to be understood about the biomineralization pathways.

The cellular environment of intact foraminifera cannot be well characterized by conventional optical imaging techniques due to scattering of light from the shell, which blurs the images of deeper levels. Observation by Cryo Scanning Electron Microscopy (cryo-SEM) of vitrified, freeze fractured biological samples allows access to the morphology of the internal environment at high resolution without requiring any modification of the specimen composition. However, the micrographs alone cannot distinguish the ion and mineral fluxes that underlie the biomineralization process. This information is provided by cryo energy-dispersive X-ray spectroscopy (EDS) of X-rays emitted from the specimen for local elemental identification, and by fluorescence imaging of calcein, a cell impermeable fluorescent dye that labels endocytosed seawater vacuoles and vesicles, binding to calcium ions. Images from the same sample in the same location are merged in a correlative manner using native landmarks, primarily the foraminifera shell and photosynthetic symbiont organisms. Use of vitrified materials across the analysis preserves in place any highly unstable phases, or even ions in solution. Imaging and analysis are therefore carried out on vitrified specimens.

We apply this approach to the study of aspects of the biomineralization ion pathway from sea water to the shell in the benthic symbiont-bearing foraminifera *Amphistegina lobifera* and *Amphistegina lessoni*. *Amphistegina* belongs to the calcitic radial-perforated foraminifera group. *A. lobifera* deposits a calcitic shell with a Mg^{2+} content of 4–5 mol% (Bentov and Erez, 2006). In this species, a new chamber is formed once every 1–2 days in the juvenile life stage (0–30 days old), and roughly once every 1–2 weeks during the adult life stage (Erez, 2003). Adult *A. lobifera* possess the largest cytoplasmic Ca^{2+} pool documented to date, storing up to 90% of the Ca^{2+} used for mineralization of a new chamber, whereas internal Ca^{2+} reservoirs are small in the juveniles (Erez, 2003; ter Kuile and Erez, 1987,1988; ter Kuile et al., 1989). Sea water endocytosis plays an important role in the biomineralization pathway of *A. lobifera* (Bentov et al., 2009). This incorporation process was demonstrated using in vivo labeling with different cell impermeable fluorescent dyes (calcein and FITC-dextran). In pulse-chase experiments, the newly formed shell mineral is labelled during the chase, showing that the sea water vacuoles are involved in shell mineralization. Many of these seminal studies were carried out on *A. lobifera* specimens that were re-mineralizing after their shells were partially dissolved using EDTA. This was done in order to be able to visualize the biomineralization process directly using light and confocal microscopy without the interference of the thick shell (Bentov et al., 2009). By contrast, the present study focuses on the Ca^{2+} and Mg^{2+} ion interplay during mineralization in intact *A. lobifera* specimens.

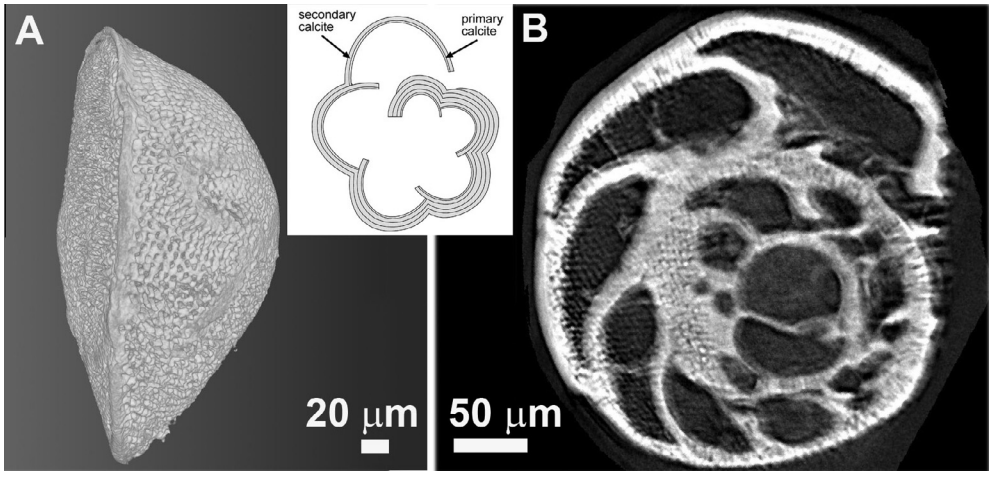

Fig. 1. micro-CT of an *Amphistegina lobifera* shell. (A) 3D reconstruction, lateral view; (B) ventral view of the shell in a semi-horizontal cross-section showing the chambers successively built from the first smaller internal chambers to the most recent larger external chambers. Insert: schematic representation of the lamination process, whereby a new shell layer is added to the entire outer surface with the building of each new chamber (scheme taken from Erez (2003)).

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