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# Biocalcification in porcelaneous foraminifera

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## Abstract

Living organisms control the formation of mineral skeletons and other structures through biomineralization. Major phylogenetic groups usually consistently follow a single biomineralization pathway. Foraminifera, which are very efficient marine calcifiers, making a substantial contribution to global carbonate production and global carbon sequestration, are regarded as the only exception. This phylum has been commonly thought to follow two contrasting models of either "extracellular *in situ* matrix mineralization" attributed to hyaline rotaliid shells, or "intracellular vesicle crystallization" attributed to porcelaneous miliolid shells. Our previous results on rotaliids along with those on miliolids in this paper question such a wide divergence of biomineralization pathways within the same phylum of Foraminifera. We found that both groups produced calcareous shells via the intravesicular formation of unstable mineral precursors (Mg-rich amorphous calcium carbonate) supplied by endocytosed seawater and deposited *in situ* as mesocrystals formed at the site of new wall formation within the organic matrix. We did not observe calcification of the needles within the transported vesicles, which challenges the previous model of miliolid mineralization. Hence, Foraminifera utilize less divergent crystallization pathways, following the recently discovered biomineralization principles. Mesocrystalline chamber walls are therefore created by accumulating and assembling particles of pre-formed liquid amorphous mineral phase within the extracellular organic matrix enclosed in a biologically controlled privileged space by active pseudopodial structures. Both calcification pathways evolved independently in the Paleozoic and are well-conserved in two clades that represent different chamber formation modes.

**eLife assessment**

This manuscript provides **important** information on the calcification process, especially the properties and formation of freshly formed tests (the foraminiferan shells), in the miliolid foraminiferan species Pseudolachlanella eburnea. The evidence from the high-quality SEM images is **solid**, but the evidence is **incomplete** when it comes to the specificity of the auto-fluorescent signals for calcified structures, or the presence of photosynthetic (living) symbionts, which are not verified experimentally. The conclusions based on fluorescent imagery therefore do not have strong support.

## Introduction

Over the past 500 million years, living cells have evolved three major skeleton crystallization pathways represented by: (A) mineralization of extracellular matrix; (B) mineralization within a large vesicle; and (C) mineralization within vesicles located in the intracellular space. Very popular in nature is the mineralization of the extracellular matrix (A), for example, in crustacean cuticles, mollusk shells, vertebrate bones, and teeth composed of dentin and enamel (Weiner and Addadi, 2011; Kahil et al., 2021). Radial foraminifera represented by rotaliids have been traditionally interpreted to make use of this crystallization mode (Weiner and Addadi, 2011). The other two pathways (B, C) are both intravesicular and are characterized by either production of amorphous unstable phase within a large vesicle (B), such as a syncytium, well documented for sea urchin larvae (Beniash et al., 1997) or crystallization of calcite elements within smaller vesicles located in the intracellular space (C), as seen in fish that form guanine crystals and coccolithophores to produce coccoliths. This model has also been attributed to the formation of porcelaneous shells by miliolid foraminifera (Weiner and Addadi, 2011) based on the model proposed by Berthold (1976) and followed by Hemleben et al. (1986).

As such, mineralization of shells in Foraminifera is believed to follow two highly contrasting pathways. The current theory states that Miliolida, characterized by imperforate, opaque milky-white shell walls (porcelaneous) (Angell, 1980; Hemleben et al. 1986; de Nooijer et al., 2009), produce fibrillar crystallites composed of Mg-rich calcite within tiny vesicles enclosed by cytoplasm. Miliolid shells are made of randomly distributed needles that cause light reflection, resulting in opaque (porcelaneous) milky walls (Hohenegger, 2009). Calcite needles are thought to be precipitated completely within these vesicles and then transported to the site of chamber formation to be released via exocytosis (Berthold, 1976; Hemleben et al. 1986; Angell, 1980; de Nooijer et al., 2008, 2009). The pre-formed needles or needle stacks are believed to be continuously embedded in an organic matrix in the shape of the new chamber until the wall is completed. Although this model is commonly accepted, it has never been sufficiently documented *in vivo*, and it does not resolve several conflicting issues. First of all, the question is how pre-formed bundles of parallel calcitic needles are transformed into randomly oriented needles within the shell wall. It is difficult to explain if there is no recrystallization process within the wall structure after discharging the calcite crystallites. This problem was already emphasized by Hemleben et al. (1986). Secondly, why the newly constructed wall is still translucent after deposition of random crystals. We would expect a thin milky opaque layer of the new wall under normal transmitted light, as well as polarized crystals of calcite under crossed nicols. Angell (1980) on his plate 2. presenting chamber formation in *Spiroloculina hyalina* Schulze clearly documented the polarization front being shifted circa a half of the length of the new chamber behind leading edge of the forming chamber. This shift represented more than an hour. Therefore, polarization was missing in the early and middle stage of chamber formation. It means that Angell's (1980) time lapse micrographs of the chamber formation were in conflict with the imaging

under TEM. It seems that Angell (1980) was aware of that problem and stressed that calcification had to be "intense enough to show under crossed nicols lags behind the leading edge of the forming chamber" (p. 93, pl. 2 fig. 12/caption). In fact, all experiments that show the "crystal vacuoles" (sensu Angell, 1980) documented under TEM (Berthold, 1976; Hemleben et al. 1986; Angell, 1980) required fixation of the samples, which was prone to post-fixation artifacts of unwanted calcite precipitation.

Our goal is to test whether the miliolid shell is produced by "agglutination" of premade needle-like calcitic crystallites, and in consequence, whether this large group of calcareous Foraminifera follow the third skeleton biomineralization pathway (C) with crystallization within smaller vesicles located in the intracellular space. Therefore, we re-examined the mineralization pathway in Miliolida based on precisely designed experiments on a living species, *Pseudolachlanella eburnea* (d'Orbigny) (**Fig. 1**). This taxon was selected to facilitate replicated observations of chamber growth under controlled culture conditions.

Our experimental setup excluded TEM imaging to avoid post-fixation artefacts. We included observations of *in vivo* biomineralization using multiphoton and confocal laser scanning microscopy (CLSM) followed by analyses of fixed specimens at different stages of chamber formation by high-resolution field emission scanning electron microscopy (FE-SEM) coupled with energy dispersive X-ray spectrometry (EDS). Our new data challenge of the current understanding of the biomineralization of miliolid foraminifera and such a significant divergence of biomineralization pathways within the Foraminifera.

## Results

All replicated *in vivo* experiments on *Pseudolachlanella eburnea* facilitated by CLSM imaging with the application of membrane-impermeable Calcein and FM1-43 membrane dye (performed in separate experiments) showed intravesicular fluorescence signals from a group of moving vesicles (1–5 µm in size) inside the cytosol (**Fig. 2**, Movies S1 and S2). The fluorescent vesicles inside the cytosol contained seawater, as documented by fluorescence of membrane impermeable Calcein. These vesicles were taken up by endocytosis (FM1-43 staining). The membrane dye (FM1-43) stains the cell membranes and indicates all endocytic vesicles by fluorescence, whereas the other intracellular vesicles remain unstained (Amaral et al., 2011). Both dyes demonstrate the uptake of seawater via the endocytosis of vesicles that are approximately 1–4 µm in diameter and move through the entire cell.

LysoGlow84 experiments revealed the presence of numerous acidic vesicles (**Fig. 2**, Movies. S3 and S4) inside the cytosol. Acidic vesicles are accompanied by granules (approximately 1–2 µm in size); these show autofluorescence upon excitation at 405 nm (emission 420–480 nm). This characteristic autofluorescence indicates the carbonate content of the vesicles (Fig. S2), which are considered to be Mg-ACCs (amorphous $MgCaCO_3$) (**Fig. 2**, Movies S4 and S5). Mg-ACC is an unstable, amorphous and hydrated form of $CaCO_3$ with a significantly high concentration of Mg (Raz et al., 2000; Weiner et al., 2003; Bentov and Erez, 2006; Kahil et al., 2021) and is commonly regarded as a resource for most biocalcification processes. Research suggests that a high Mg content not only makes ACC unstable but also facilitates the transport of ACC to the crystallization site, where it is initially transformed into carbonate nanograins (Colfen and Qi, 2001; Addadi and Weiner, 2003; Raz et al., 2003; Dubicka et al., 2023). Carbonate nanograins at the shell construction site were well documented in our SEM-EDS studies (Fig. S6). ACCs have been found in many calcifying marine organisms, such as echinoderms, mollusks, coccolithophorid algae, cyanobacteria, crustaceans, and rotaliid foraminifera, where they are typically interpreted as pre-material phases for the production of calcite skeletons (Hasse, et al., 2000; Weiss et al., 2002; Sviben et al., 2016; Dubicka et al., 2018; Kahil et al., 2021).

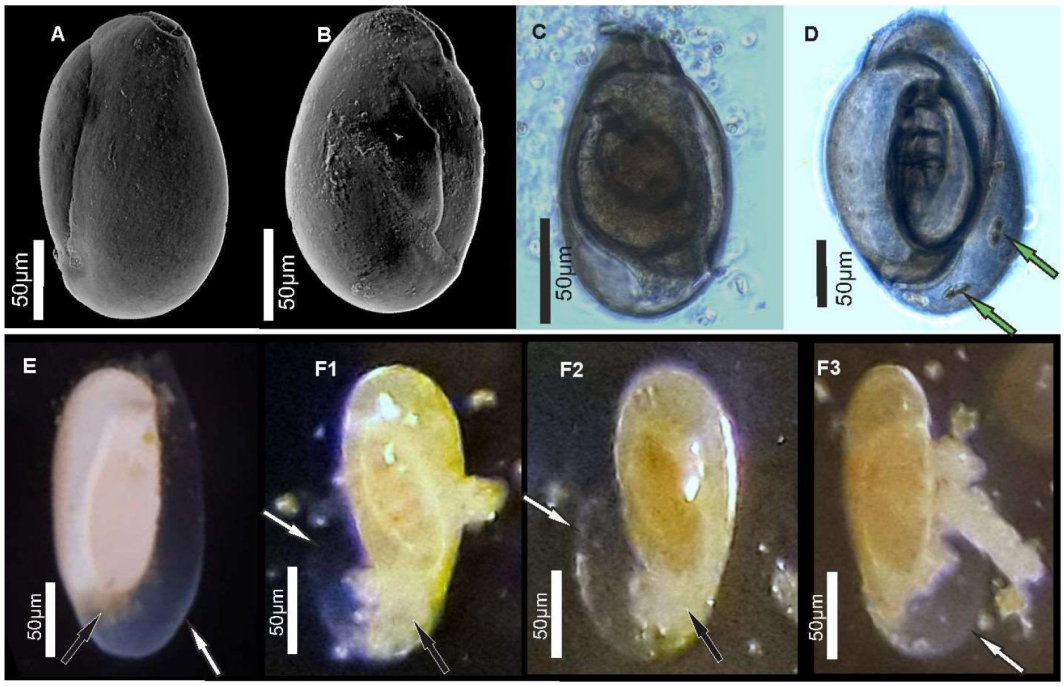


**Figure 1.**

Specimens of miliolid foraminifera, identified as *Pseudolachlanella eburnea* (d'Orbigny), used for experimental studies. (A, B) SEM, (C, D) transmitted light microscope, and (E, F) stereomicroscope images; white arrows show the outer organic sheath of a new chamber during its gradual calcification expressed by its milky, opaque appearance; black arrows show a small mass of cytoplasm extruded from the aperture of exiting the chamber; green arrows show endosymbiotic algae.

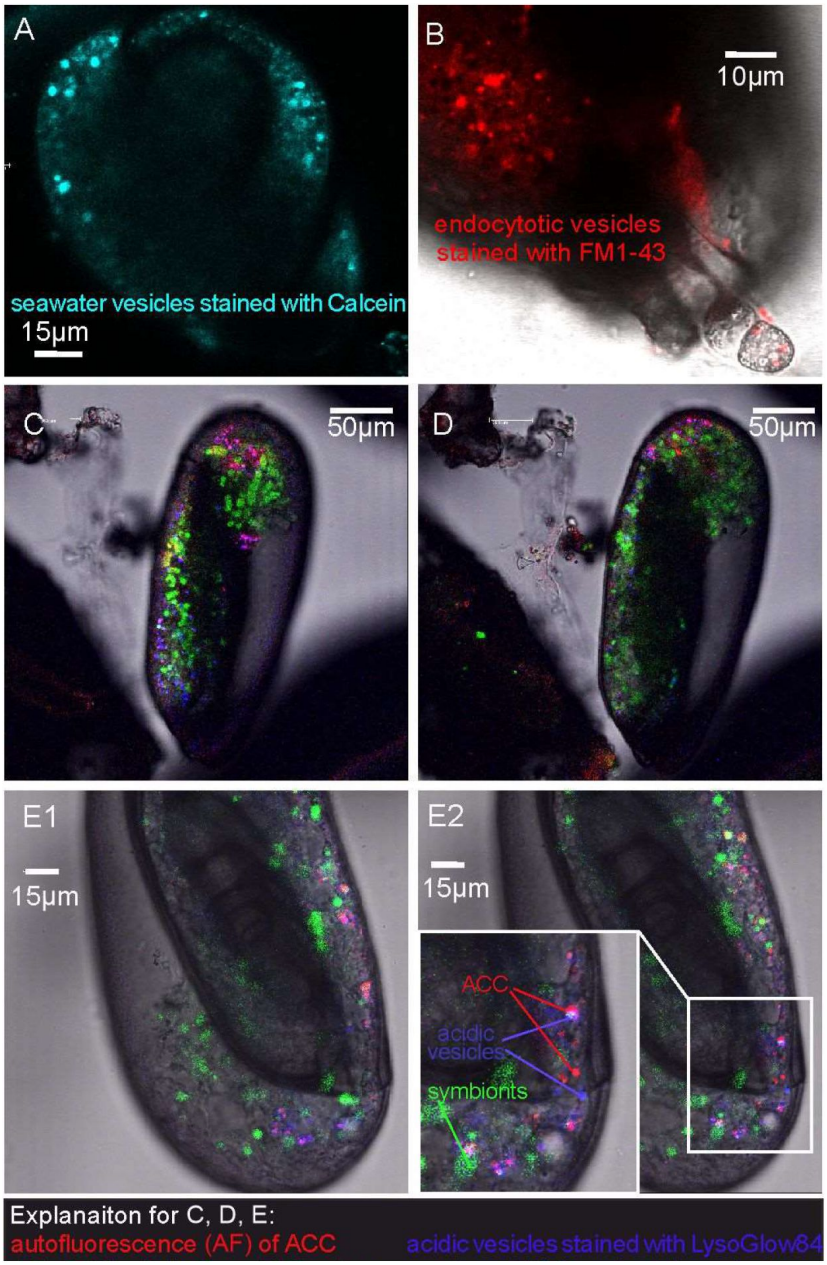


**Figure 2.**

Fluorescence images of living *P. eburnea* conducted by Laser Scanning Microscopy. A-Cell impermeable Calcein (cyan) indicating endocytotic seawater vesicles, see Movie 1. B - FM1-43 membrane dye indicating endocytotic vesicles (red), see Movie 2. C, D, E - LysoGlow84 indicating acidic vesicles (navy blue), autofluorescence of chloroplasts (green), and Mg-ACC pools (red), see Movies 3 and 4, (note the overlap of ACC and acidic vesicles is marked in lilac).

Symbiont chloroplasts, detected by the typical autofluorescence of chlorophyll (excitation at 405 or 633 nm, emission 650–700, **Fig. 2**, Movies S3 and S4), have been found to move within the cytosol of the observed specimens, forming mobile clusters with Mg-ACCs and acidic vesicles. Interestingly, endosymbionts were detected only during the mineralization process in specimens with carbonate-bearing vesicles detected by *in vivo* CLSM experiments or just below the organic matrix (OM) of the newly created chamber as seen in FE-SEM observations (Figs.S3G, and S3H).

FE-SEM observations of the fully mineralized test walls display the porcelaneous structures (see Parker, 2017; Dubicka et al., 2018), which are made of three mineralized elements: (a) extrados: outer mineralized surface (approximately 200–300 nm in thickness; Figs. S3C and S4C). (b) Porcelain: the main body of the wall constructed from randomly oriented needle-shaped crystals (up to 1–2 µm in length and approximately 200 nm in width). No organic matter was detected between the needles of the porcelain structures (**Figs. 3E**; **3E**; S4C, and S5A). (c) Intrados: inner mineralized surface (approximately 200–300 nm in thickness) made of needle-shaped elements (**Figs. 3E** and S5A).

Growing chambers, captured at the various successive stages of chamber formation in different specimens, have revealed the following morphological features: (a) a solitary, thin organic sheath (approximately 200–300 nm thick) that represents the most distal part of the new chamber and is anchored to the older, underlying solid calcified chamber (**Fig. 3A**); (b) a solitary, outer organic sheath (OOS) filled with spread calcifying nanograins (**Figs. 3B**; S4A, and S4B); (c) a gel-like OM (4–5 µm in thickness), bounded on two sides by intrados and extrados, and containing relatively widely spaced, randomly dispersed carbonate nanograins (**Figs. 3A-B**; **4C**; S3A-D; S5); (d) the test inside was made of carbonate nanograins that are partly transformed to short needles with a small amount of OM in-between (**Figs. 3C**, **2D**, and **4D**); (V) the test inside was composed of needle-shaped crystals with planar faces and without any remaining OM (**Figs. 3E** and **3E**). Both fixation methods (see Material and Methods) yielded highly consistent results. Specimens of *P. eburnea*, which displayed carbonate-bearing vesicles inside the cytosol as determined by CLSM, were fixed using method B, coated with a few nanometers of carbon, and analyzed by SEM-EDS. The main elements detected in the area of the fixed cytoplasm (Fig. S6) were C, O, Na, Mg, P, S, Cl, K, and Ca (of particular interest were the high contents of Mg and Ca), whereas the main elements detected within the area of the new chamber in the form of an organic matrix filled with nanograins (Fig. S6) were C, O, Na, Mg, S, Cl, and Ca. The shell area was strongly enriched with Ca relative to the cytoplasm, which showed a much higher Mg/Ca ratio.

This study shows the lack of premade needle-like crystallites of calcite at the early stages (I-IV) of the wall formation. In contrast, we can clearly infer the *in situ* calcification front with a progressive sequence of crystal growth behind the leading edge of the forming chamber (**Figs 4**, **5**). Therefore, this miliolid species does not produce shells by “agglutination” of premade needle-like crystallites of calcite. In the light of these results, another argument emerges that further confirms in situ calcification of miliolid chambers. It explains the extended transparency of unmineralized walls observed under the light stereomicroscope. The chamber walls under formation tend to gradually change its appearance to milky and opaque aspect during calcification (**Fig. 1 E**). Our results on biomineralization of this miliolid species do not confirm the formation of individual skeletal crystallites within intracellular vesicles. However, in turn, our results do support existence of endocytotic vacuolization of sea water in miliolids that first suggested by Hemleben et al. (1986). We further support Angell’s (1980) interpretation that the calcite crystals are dispersed in the gel-like organic matrix (see **Figs 3A, B**; **4C, D**; **5**). This gel-like fluidal organic matrix most likely include a rich Mg-ACC component as substrate for *in situ* calcification (**Figs 3**-**5**).

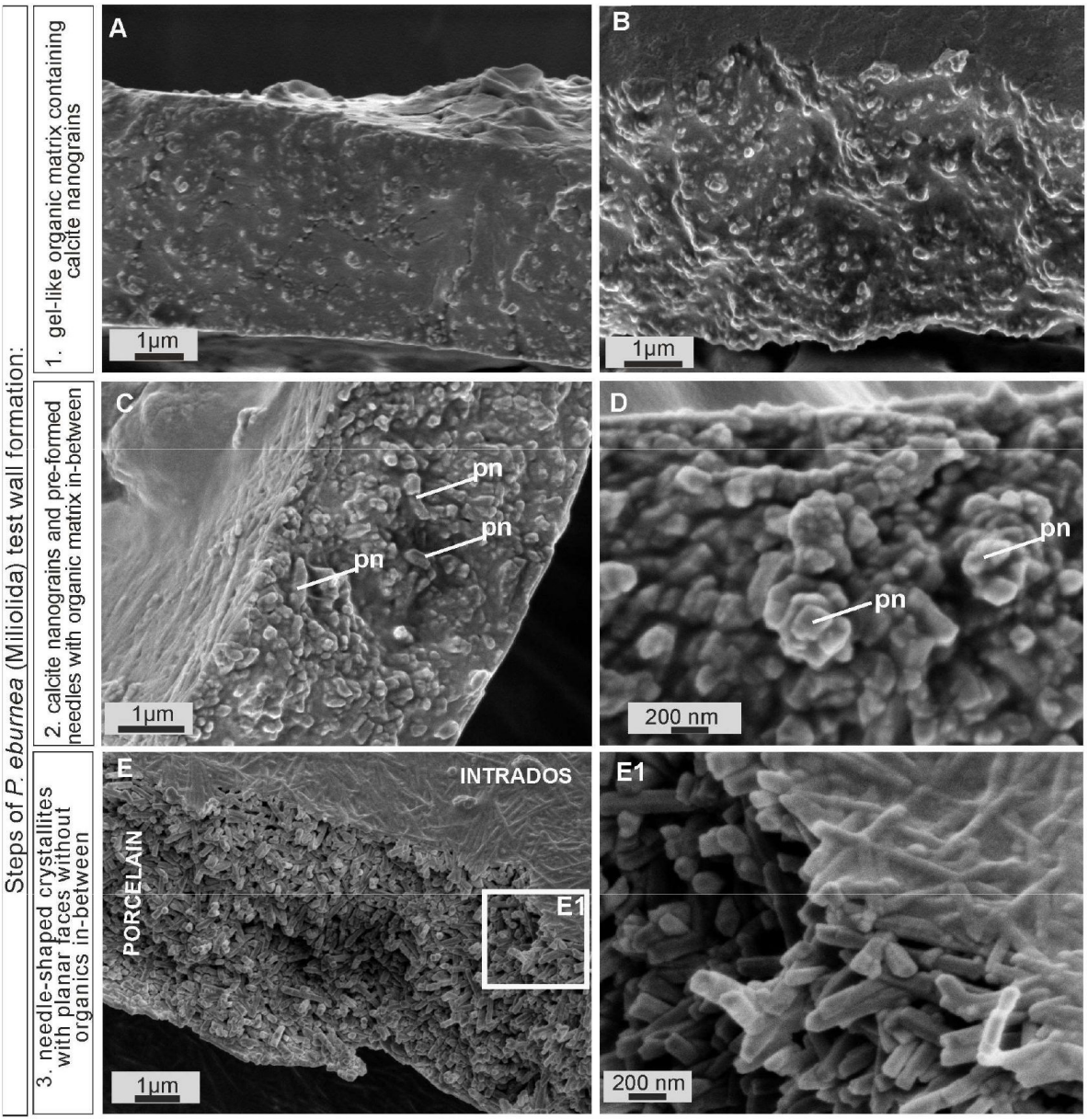


**Figure 3.**

SEM images of the major steps of the formation of *P. eburnea* shell-building components. Test cross-section showing: (A, B) carbonate nanograins within organic matrix, (C, D) nanograins merging into needle-like mesocrystals, (E) fully developed needle-shaped elements; pn –nanograins partly transformed to short needles.

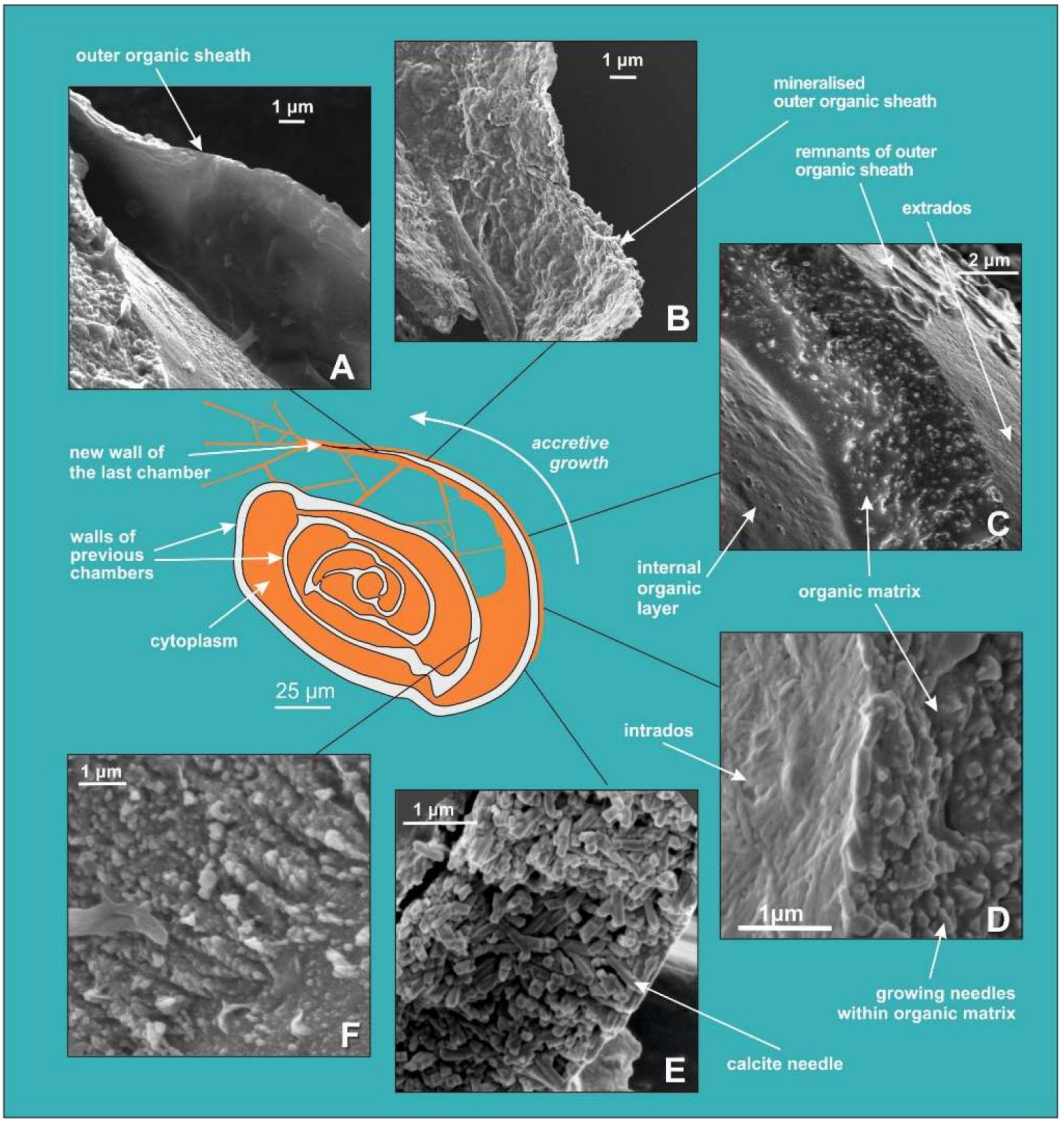


**Figure 4.**

SEM images showing successive stages of new chamber formation in *P. eburnea*. (A) outer organic sheath, (B) mineralized outer organic sheath, (C) calcite nanograins within a gel-like organic matrix, (D) needle-shaped mesocrystal growth, (E) needle-like calcite building elements,(F) nanogranular intrados.

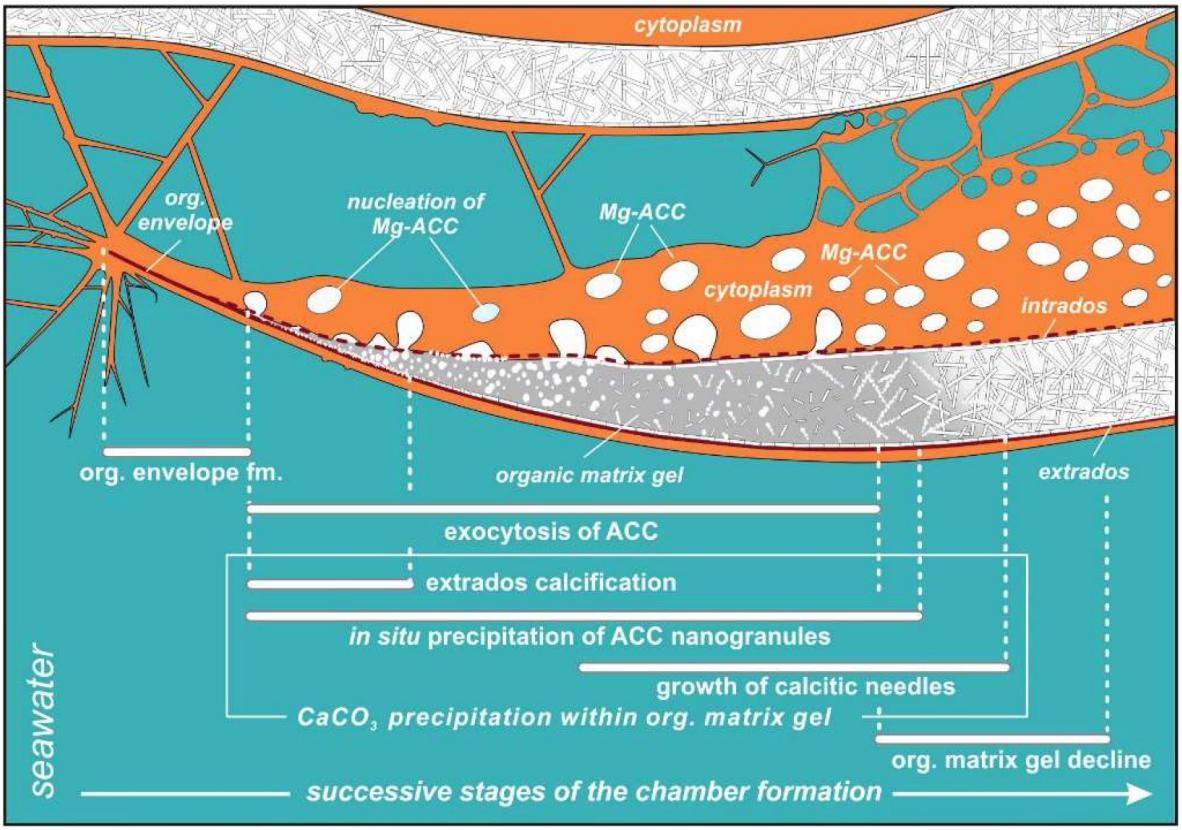


**Figure 5.**

Simplified model of porcelaneous wall construction based on foraminifer *P. eburnea*.

# Discussion

## Porcelaneous shell formation

Comparative analysis of the nanostructures of the newly built chambers combined with the elemental composition obtained from SEM-EDS, as well as data from CLSM, allowed us to identify important steps in the formation of *P. eburnea* shells. The formation of a new chamber begins with the construction of a thin outer organic sheath that pre-shapes the new chamber (**Figs. 4**, **5**). The outer organic sheath is made by pseudopodial structures supported by the cytoskeleton immediately after the extrusion of a small mass of cytoplasm from the aperture (**Figs. 1E**, and **1F**). Once the OOS is constructed, the first calcium carbonate accumulation takes place inside in the form of carbonate nanograins (Figs. S4A and S4B), creating the extrados. The extrados stabilizes the final chamber morphology relatively quickly. Subsequently, the OM gradually thickens and forms the primary accumulation site for the next batches of Mg-rich $CaCO_3$ (hydrated and amorphous). The existence of intracellular, vesicular intermediate amorphous phase (Mg-ACC pools), which supply successive doses of carbonate material to shell production, was supported by autofluorescence (excitation at 405 nm; **Fig. 2**; Movies S3 and S4; see Dubicka et al., 2023) and a high content of Ca and Mg quantified from the area of cytoplasm by SEM-EDS analysis (Fig. S6). More precise elemental measurements with higher resolution are needed in the future. However, the small size of carbonate-bearing vesicles (approximately 1–2 µm) may make this difficult. $Mg^{2+}$ and $Ca^{2+}$ ions for intracellular production of Mg-ACCs are obtained from seawater and taken up by endocytosis, as independently indicated by membrane impermeable Calcein, as well as by the probe FM1-43 selectively labelling membranes of endocytic vesicles (**Figs. 1A** and **1B**, Movies S1 and S2). We hypothesized that vesicles are carried along cytoskeletal structures to the OM, as observed in Rotaliid foraminifera (Dubicka et al., 2023), where they dock and release their contents (**Fig. 4**). The nanograins then precipitated within the gelatinous OM, following the release of carbonate from the vesicles (**Figs. 3A-C**, **4C**, and **5**). Nanograins filled with OM gradually merge into needle-shaped elements, precipitating *in situ* within the final wall structure (**Figs. 3C, 3D**, and **4D**). The gel-like OM appears to be involved in needle formation; however, the OM seems to disappear (**Figs. 3E** and **4E**) when the needle-shaped crystals are created. We suspect that the OM was removed from the test wall and recycled by the cell itself. The calcification of extrados and intrados occurs before the interior of the wall crystallizes, providing stability to the new chamber at both edges of the wall (**Fig. 3D**).

The protruding cytoplasm appears to immediately form a chamber wall by secreting OM and crystals from the vesicles. As calcite secretion continues along the leading edge, the newly formed segment remains covered by a thin, moving sheet of cytoplasm that is called by Angell (1980) the “active sheet”. This thin active sheet of cytoplasm may represent a lamellipodium, that is, a pseudopodial structure involved in the biomineralization of Rotaliida (Tyszka et al., 2019). It is also likely that reticulopodial structures (that do not coat the whole calcification site) are responsible for the distribution and shape of the internal surface of the chamber wall. This occurs by successive accumulation of ACC and OM as identified on TEM images by Angell (1980; p.97). Results suggest that crystallization of calcite needles is “limited to a confined space controlled by active cytoplasmic structures” and strictly separated by membranes from the cytosol.

## Formation of shell crystallites a paradigm shift

Miliolids were thought to share the same crystallization pathway as coccolith formation in coccolithophorids (Weiner and Addadi, 2011) that evolved in the Triassic, that is c. 210 Myr ago (Gardin et al., 2012). Coccoliths are produced within intracellular Golgi-derived vesicles and then exported to the inner surface of the extracellular coccosphere (de Vrind-de Jong et al., 1986).Miliolids, with their unique fibrillar calcitic microstructures, evolved much earlier, that is ca. 300 Myr ago in the late Paleozoic (Fig. S7). It is generally considered that miliolid crystals also

precipitate within vesicles in the cytoplasm and are then transported to the location of the wall construction, where they are released by exocytosis (Berthold, 1976; Angell, 1980; de Noojier et al., 2009; Hemleben et al., 1986; Weiner and Addadi, 2011).

Our results show that needle-shaped crystallites are secreted *in situ* at the area of shell formation within the organic matrix (**Figs. 4** and **5**), in contrast to the traditional miliolid calcification model (Berthold, 1976; Angell, 1980; Hemleben et al., 1986), in which needle-shaped crystals are initially performed inside the vesicles. Interestingly, the previous studies by Angell (1980) did not support crystal formation within vacuoles either. Precipitation of calcite nanograins, which then merge and transform into crystallites, occurs within the organic matter after the release of $Mg^{2+}$ from Mg-ACC. The organic matter provides an appropriate physiochemical microenvironment for initiating and maintaining the crystallization process by manipulating many essential factors including pH, and kinetics of the system (Kahil et al., 2021). According to Tyszka et al. (Tyszka et al., 2021), the organic matrix involved in the biomineralization of foraminiferal shells may contain collagen-like networks.

Our *in vivo* CLSM observations show a miliolid cytoplasm containing intracellular carbonate-bearing vesicles. Such vesicles have been well-documented by Angell (1980), who stressed their crucial role in the biomineralization process. However, rather than transporting pre-formed solid needles, the vesicles likely carry liquid or quasi-liquid calcification substrates. The liquid phase of the ACC apparently was maintained by a relatively high concentration of Mg (Figs. S2 and S6), which was much higher than that in the shell, as detected by SEM-EDS.

Recently, an independent study was performed on another miliolid species - *Sorites orbiculus* (Nagai et al., 2023). The researchers reported highly complementary results that indicate the lack of crystal-like structures within the intracellular vesicles. Their results suggested that calcification of this miliolid species did not follow Hemleben's et al (1986) model because intracellular vesicles did not produce needle-like crystals to construct the shell wall. They also proposed the need to describe a model of biomineralization in foraminifera.

Because, miliolid wall texture originated together with the appearance of miliolid foraminifera as it has also been recorded within Paleozoic taxa (Fig. S5) thus the calcification mode of miliolids is apparently evolved in the late Paleozoic (≥ 350 Mya) and is well conserved in this clade till today. It should be emphasized that our recent understanding of all calcification pathways in Foraminifera implies their independent evolution within main phylogenetic groups, besides miliolids and rotaliids, also including spirillinids, nodosariids, and robertinids (Mikhalevich, 2004; Pawlowski et al., 2013; Mikhalevich, 2014; Dubicka et al., 2018; Dubicka, 2019; Mikhalevich, 2021; Sierra et al., 2022; de Nooijer et al., 2023). In fact, most of these biomineralization evolutionary transitions from agglutination to calcification originated in the mid and late Paleozoic.

Mg-ACC has also recently been documented in rotaliid foraminifera (Dubicka et al., 2023). Therefore, the biocalcification processes in Rotaliida and Miliolida, which belong to the two main foraminiferal classes Globothalamea and Tubothalamea, respectively (Pawlowski et al., 2013), are more similar than previously thought (Weiner and Addadi, 2011). Their mesocrystalline chamber walls are created by accumulating and assembling particles of pre-formed liquid amorphous mineral phase. Their calcification occurs within the extracellular organic matrix enclosed in a biologically controlled privileged space by active pseudopodial structures. However, we are aware that this process must also vary to some extent as the chemical composition of the calcite, as well as primary crystallite geometries differ between the groups. Seawater provides the relevant Ca and Mg ions for calcification, which are taken up in both groups by endocytosis. In *Amphistegina* (Rotaliida), this process is performed by shell pores (Dubicka et al., 2023), as well as apertures and foramina; in non-porous Miliolida, it is done by granuloreticulopodia emanating from the aperture (**Fig. 2**, Movie S2). In both the rotaliid *Amphistegina* and the miliolid *P. eburnea* carbonate-bearing vesicles are surrounded by moving acidic vesicles (**Fig. 2**, Movies S3

and S4),which likely facilitate pH regulation at the mineralization front (see 28). It is very likely that pH is controlled by active outward proton pumping by a V-type H+ ATPase that is responsible for the proton flux and related calcification (Toyofuku et al., 2017). We suspect much higher pH values within vesicles transporting MgACC to the site of calcification. Such alkaline vesicles were detected by the HPTS fluorescent labelling and reported by several previous studies (de Nooijer et al., 2008; 2009).

Our findings are in line with recent work in biomineralization, supporting that "biominerals grow by the accretion of amorphous particles, which are later transformed into the corresponding mineral phase" (Macías-Sánchez et al., 2011, p. 1; see also Meldun and Cölfen, 2008). Miliolid needles, assembled with calcite nanoparticles, are unique examples of biogenic mesocrystals (see Cölfen and Antonietti, 2005), forming distinct geometric shapes limited by planar crystalline faces. Mesocrystals are constructed from highly ordered individual nanoparticles (Cölfen and Mann, 2003; Strum and Cölfen, 2016; 2017) that form hierarchically structured solid materials in the crystallographic register. These result from the aggregation, self-assembly, and mesoscopic transformation of amorphous precursor nanoparticles. Mesocrystals are common biogenic components in the skeletons of marine organisms, such as corals, echinoderms, bivalves, sea urchins, and rotaliid foraminifera (e.g., Macías-Sánchez et al., 2011; Benzerara et al., 2011; Seto et al., 2012; Evans et al., 2019; Dubicka et al., 2023).

Our biomineralization model further explains the random orientation pattern of the calcite needles within the shell wall. The miliolid intertwined calcitic structure cannot be explained by the models proposed by Berthold (1976) and followed by Hemleben (1986), that is, by the successive deposition of vesicles with ready bundles of solid calcitic fibers (needles) without additional recrystallization processes. In our proposed *in situ* calcification model, calcite crystallites have sufficient space to grow within the flexible gelatinous organic matrix. In addition, our model explains the need for a light and dark phase for the algae that are present inside *P. eburnea* during the biomineralization processes, as these algae possibly play an important role. Small miliolid coiling foraminifera has been regarded as a non-symbiotic taxon because their shells are not transparent, however, this is not true for red and infrared light. Fully developed miliolid shells are made of randomly distributed needles that cause light reflection, resulting in opaque (porcelaneous) walls that possibly protect the foraminifera from UV irradiation and allow them to live in extremely illuminated shallow seas (Hohenegger, 2009). These walls are permeable to red and infrared light, as we observed using multiphoton laser. Red light is commonly believed to be the most efficient waveband for photosynthesis however green light may achieve higher quantum yield of $CO_2$ assimilation and net $CO_2$ assimilation rate (Liu and Lerser, 2021). *Pseudolachlanella eburnea* may acquire its facultative symbionts only for the duration of the biomineralization process. The late stage of needle formation in the shell production process ensures that the wall remains transparent by the time the needles are completed. Similar patterns of the gradual change from transparent to opaque whitish walls were also observed in larger symbiotic miliolids by Marshalek (1969), Wetmore (1999), and Tremblin et al. (2022). The latter authors (Tremblin et al., 2022) documented chamber formation of miliolid *Vertebralina striata* with cytoplasm enveloped by a transparent sheath decorated with striate already present in the transparent wall before calcification. They also interpret white areas on the sheath, indicating incipient concentrations of minute calcite crystallites that represent the mineralised wall. The biomineralization process is likely aided by their dark respiratory activity (see Hallock, 1999), as they could supply calcification substrates such as $HCO_{3-}$ through respiration or by increasing pH at the calcification site during the light phase. Similarly, representatives of miliolid large benthic foraminifera (Archaiasidae, Soritidae, and Peneroplidae) host endosymbiotic algae (Lee, 2006; Prazes and Renema, 2019). Therefore, they have developed additional morphological and textural features such as pits, grooves/striate, or windows, which enable light penetration into the places where symbionts are positioned (see Hohenegger, 2009, Parker 2017).

## Materials and Methods

Living foraminifera, collected from the coral reef aquarium in the Burgers Zoo (Arnhem, Netherlands), were cultured in a 10 L aquarium containing seawater with a salinity of 32‰, pH of 8.2, and a temperature of 24 °C. *Pseudolachlanella eburnea* (d'Orbigny) was placed in 4 mL petri dishes and observed under a Zeiss Stemi SV8 Stereomikroskope.

Selected individuals were studied *in vivo* using a Leica SP5 Confocal Laser Scanning Microscope equipped with an argon, helium-neon, neon, diode, and multiphoton Mai Tai laser (Spectra-Physics) at the Alfred-Wegener-Institut, Bremerhaven, Germany. In *vivo* experiments were performed by labeling samples with different fluorescent dyes (**Table 1**) just before imaging using, p*H*-sensitive LysoGlow84 (50 µM exc. MP720 nm exc/em. 380–415 nm and 450–470 nm, Marnas Biochemicals Bremerhaven, incubation time: 2 h), FM1-43 membrane stain (1 µM, exc.488 nm em. 580–620 nm, Invitrogen, incubation time: 24 h), and membrane impermeable Calcein (0.7 mg/10 mL, exc. 488 nm, em. 510–555 nm, incubation time: 24 h). The foraminifera were removed from the Petri dish with clean water using a pipette. In addition, the autofluorescence of specific foraminiferal structures at the chosen excitation/emission wavelength was detected. All experiments were replicated with at least several individuals of the same species. All fluorescence probe experiments were performed with appropriate controls.

Additional foraminifera individuals that had been studied by CLSM were fixed for further analysis. The fixation process followed two different methods: (A) 60 individuals were transferred to 3% glutaraldehyde for 5 s and then dehydrated stepwise for a few seconds with an ethanol/distilled water mixture with increasing concentrations (30%, 50%, 70%, and 99%). (B) The seawater was removed from 50 individuals by pipetting and applying a small piece of Kimtech lab wipe (without any rinsing), followed by quick drying in warm air (30–35 °C). This method stops the dissolution of the amorphous mineral phase because there is no contact with other liquids. Fixed foraminifers of both procedures were gently broken using a fine needle to coat the cross-sectional surfaces and tested inside with a few nanometers of either gold or carbon. Foraminifera were then studied using a Zeiss ‰igma variable-pressure field-emission scanning electron microscope (VP-FESEM) equipped with EDS at the Faculty of Geology, University of Warsaw.

## Acknowledgements

This work was supported by the Alexander von Humboldt Foundation Research Fellowship for experienced researchers (to Z.D.) and the Polish National Science Center (UMO-2018/29/B/ST10/01811) to J.T. and Z.D., a grant coordinated by Grzegorz Racki, University of Silesia. We thank Oscar Branson for his comments on an earlier version of the manuscript and the suggested improvements.

## Movie captions

**Movie S1 (separate file)**. Living *P. eburnea* showing cell impermeable Calcein (blue, exc. 488nm em. 505-555) in a series of 107 overlaid images taken during 428 s. Calcein staining indicates the occurrence of seawater vesicles inside the cytosol.

**Movie S2 (separate file)**. FM1-43 membrane probe fluorescent signals (red, exc. 488nm, em. 580-620nm) emitted by intracellular vesicles within cytosol of *P. eburnea*. Because FM1-43 stains only the cell membrane, the observed vesicles must be originated during the process of endocytosis. The movie was taken by overlaid of 84 images during 433 s.

| dye | concentration | excitation nm | emission nm | source | function |
|---|---|---|---|---|---|
| Lysoglow84 | 50µM | Multiphoton 730 | 380-415/450-470 | Marnas Biochemicals | pH, membrane permeable |
| FM1-43 | 1µM | Argon 488 or Multiphoton 1000nm | 580-620 | Thermo Fisher Scientific | Membrane staining |
| Calcein | 0,7mg/10mL | Argon 488 | 510-555 | Thermo Fisher Scientific | Membrane impermeable water soluble dye |
| autofluorescence | | Diode 405 MP 800 | 420-490 | | $CaCO_3$, ACC |
| autofluorescence | | Diode 405/ HeNe 633 | 650-700 | | Chlorophyll of symbionts |

**Table 1.**

**Wavelengths and dyes**

**Movie S3 (separate file)**. Living *P. eburnea* showing fluorescence signal inside the cytosol: autofluorescence of Mg-ACC pools (red, exc. 405nm, em. 420-490nm) and symbiontic chloroplasts (green, exc. 633nm, em. 640-690nm), fluorescent signal of LysoGlow84 pH sensitive dye (exc. MP 720nm, em. 440-470nm) indicating acidic vesicles. The movie was taken by overlaid of 37 images during 555 s.

**Movie S4 (separate file)**. Living *P. eburnea* showing fluorescence signal inside the cytosol: autofluorescence of Mg-ACC pools (red, exc. 405nm, em. 420-490nm) and symbiontic chloroplasts (green, exc. 633nm, em. 640-690nm), fluorescent signal of LysoGlow84 pH sensitive dye (exc. MP 720nm, em. 440-470nm) indicating acidic vesicles. The movie was taken by overlaid of 37 images during 555 s.

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**Reviewer #1 (Public Review):**

Summary:
The manuscript by Dubicka and co-workers on calcification in miliolid foraminifera presents an interesting piece of work. The study uses confocal and electron microscopy to show that the traditional picture of calcification in porcelaneous foraminifera is incorrect.

Strengths:
The authors present high-quality images and an original approach to a relatively solid (so I thought) model of calcification.

Weaknesses:
There are several major shortcomings. Despite the interesting subject and the wonderful images, the conclusions of this manuscript are simply not supported at all by the results. The fluorescent images may not have any relation to the process of calcification and should therefore not be part of this manuscript. The SEM images, however, do point to an outdated idea of miliolid calcification. I think the manuscript would be much stronger with the focus on the SEM images and with the speculation of the physiological processes greatly reduced.

https://doi.org/10.7554/eLife.91568.1.sa1

**Reviewer #2 (Public Review):**

Summary:
Dubicka et al. in their paper entitled " Biocalcification in porcelaneous foraminifera" suggest that in contrast to the traditionally claimed two different modes of test calcification by rotallid and porcelaneous miliolid formaminifera, both groups produce calcareous tests via the intravesicular mineral precursors (Mg-rich amorphous calcium carbonate). These precursors are proposed to be supplied by endocytosed seawater and deposited in situ as mesocrystals formed at the site of new wall formation within the organic matrix. The authors

did not observe the calcification of the needles within the transported vesicles, which challenges the previous model of miliolid mineralization. Although the authors argue that these two groups of foraminifera utilize the same calcification mechanism, they also suggest that these calcification pathways evolved independently in the Paleozoic.

Strengths:
The authors document various unknown aspects of calcification of Pseudolachlanella eburnea and elucidate some poorly explained phenomena (e.g., translucent properties of the freshly formed test) however there are several problematic observations/interpretations which in my opinion should be carefully addressed.

Weaknesses:
1. The authors (line 122) suggest that "characteristic autofluorescence indicates the carbonate content of the vesicles (Fig. S2), which are considered to be Mg-ACCs (amorphous MgCaCO3) (Fig. 2, Movies S4 and S5)". Figure S2 which the authors refer to shows only broken sections of organic sheath at different stages of mineralization. Movie S4 shows that only in a few regions some vesicles exhibit red autofluorescence interpreted as Mg-ACC (S5 is missing but probably the authors were referring to S3). In their previous paper (Dubicka et al 2023: Heliyon), the authors used exactly the same methodology to suggest that these are intracellularly formed Mg-rich amorphous calcium carbonate particles that transform into a stable mineral phase in rotaliid Aphistegina lessonii. However, in Figure 1D (Dubicka et al 2023) the apparently carbonate-loaded vesicles show the same red autofluorescence as the test, whereas in their current paper, no evidence of autofluorescence of Mg-ACC grains accumulated within the "gel-like" organic matrix is given. The S3 and S4 movies show circulation of various fluorescing components, but no initial phase of test formation is observable (numerous mineral grains embedded within the organic matrix - Figures 3A and B - should be clearly observed also as autofluorescence of the whole layer). Thus the crucial argument supporting the calcification model (Figure 5) is missing. There is no support for the following interpretation (lines 199-203) "The existence of intracellular, vesicular intermediate amorphous phase (Mg-ACC pools), which supply successive doses of carbonate material to shell production, was supported by autofluorescence (excitation at 405 nm; Fig. 2; Movies S3 and S4; see Dubicka et al., 2023) and a high content of Ca and Mg quantified from the area of cytoplasm by SEM-EDS analysis (Fig. S6)."

2. The authors suggest that "no organic matter was detected between the needles of the porcelain structures (Figures 3E; 3E; S4C, and S5A)". Such a suggestion, which is highly unusual considering that biogenic minerals almost by definition contain various organic components, was made based only on FE-SEM observation. The authors should either provide clearcut evidence of the lack of organic matter (unlikely) or may suggest that intense calcium carbonate precipitation within organic matrix gel ultimately results in a decrease of the amount of the organic phase (but not its complete elimination), alike the pure calcium carbonate crystals are separated from the remaining liquid with impurities ("mother liquor"). On the other hand, if (249-250) "organic matrix involved in the biomineralization of foraminiferal shells may contain collagen-like networks", such "laminar" organization of the organic matrix may partly explain the arrangement of carbonate fibers parallel to the surface as observed in Fig. 3E1.

3. The author's observations indeed do not show the formation of individual skeletal crystallites within intracellular vesicles, however, do not explain either what is the structure of individual skeletal crystallites and how they are formed. Especially, what are the structures observed in polarized light (and interpreted as calcite crystallites) by De Nooijer et al. 2009? The author's explanation of the process (lines 213-216) is not particularly convincing "we suspect that the OM was removed from the test wall and recycled by the cell itself".

4. The following passage (lines 296-304) which deals with the concept of mesocrystals is not supported by the authors' methodology or observations. The authors state that miliolid

needles "assembled with calcite nanoparticles, are unique examples of biogenic mesocrystals (see Cölfen and Antonietti, 2005), forming distinct geometric shapes limited by planar crystalline faces" (later in the same passage the authors say that "mesocrystals are common biogenic components in the skeletons of marine organisms" (are they thus unique or are they common)? It is my suggestion to completely eliminate this concept here until various crystallographic details of the miliolid test formation are well documented.

https://doi.org/10.7554/eLife.91568.1.sa0

**Author Response**

> ***Reviewer #1 (Public Review):***
>
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> *The manuscript by Dubicka and co-workers on calcification in miliolid foraminifera presents an interesting piece of work. The study uses confocal and electron microscopy to show that the traditional picture of calcification in porcelaneous foraminifera is incorrect.*
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> *There are several major shortcomings. Despite the interesting subject and the wonderful images, the conclusions of this manuscript are simply not supported at all by the results. The fluorescent images may not have any relation to the process of calcification and should therefore not be part of this manuscript. The SEM images, however, do point to an outdated idea of miliolid calcification. I think the manuscript would be much stronger with the focus on the SEM images and with the speculation of the physiological processes greatly reduced.*

Reply: We would like to give thanks for all of the highly valuable comments. Prior to our study, we were also convinced that the calcification model of Miliolid (porcelaneous) foraminifera was relatively solid. Nevertheless, our SEM imaging results surprisingly contradicted the old model. The main difference is the in situ biomineralization of calcitic needles that precipitate within the chamber wall after deposition of ACC-bearing vesicles. We agree that our fluorescence studies presented in the paper are not conclusive evidence for the calcification model used by the studied Miliolid species. However, our fluorescent results show that “the old model” (sensu Hemleben et al., 1986) is not completely outdated. Most of the fluorescent imaging data show a vesicular transport of substrates necessary for calcification. This transport is presented by Calcein labelling experiments (Movie 1 that show a high number of dynamic endocytic vesicles of sea water circulation within the cytoplasm. These very fine Calcein-labelled vesicles are most likely responsible for transport and deposition of $Ca^{2+}$ ions. This is partly consistent with the model presented by Hemleben et al. (1986). We may speculate that calcite nucleation is already occurring within the transported vesicles, but at this stage of research we have no evidence for this phenomenon.

Further live imaging fluorescence data show autofluorescence of vesicles upon excitation at 405 nm (emission 420–480 nm) associated with acidic vesicles marked by pH-sensitive LysoGlow84, may be a hint indicating association of ACC-bearing vesicles with acidic vesicles.

Such spatial association of these vesicles may indicate a mechanism of pH elevation in the vesicles transporting Ca2+-rich gel to the calcifying wall of the new chamber.

We will do our best to limit the physiological interpretation presented based on fluorescence studies in the revised version of the manuscript. We are convinced that our fluorescent live imaging experiments provide important observations in biomineralizing Miliolid foraminifera, which are still missing in the existing literature. It should be stressed that all the fluorescent experiments and SEM observations were based on specimens constructing and biomineralizing new chambers. All of them belong to the same species and come from the same culture. Due to the aforementioned reasons, it is worthwhile presenting these complimentary results of our study. In the future they may be helpful in further exploration and understanding of all aspects of calcification in foraminifera.

> ***Reviewer #2 (Public Review):***
>
> *Summary:*
>
> *Dubicka et al. in their paper entitled " Biocalcification in porcelaneous foraminifera" suggest that in contrast to the traditionally claimed two different modes of test calcification by rotallid and porcelaneous miliolid formaminifera, both groups produce calcareous tests via the intravesicular mineral precursors (Mg-rich amorphous calcium carbonate). These precursors are proposed to be supplied by endocytosed seawater and deposited in situ as mesocrystals formed at the site of new wall formation within the organic matrix. The authors did not observe the calcification of the needles within the transported vesicles, which challenges the previous model of miliolid mineralization. Although the authors argue that these two groups of foraminifera utilize the same calcification mechanism, they also suggest that these calcification pathways evolved independently in the Paleozoic.*

Reply: We would like to acknowledge the review and all valuable comments. We do not argue that Miliolida and Rotallida utilise an identical calcification mechanism, but both groups utilize less divergent crystallization pathways, where mesocrystalline chamber walls are created by accumulating and assembling particles of pre-formed liquid amorphous mineral phase.

> *Strengths:*
>
> *The authors document various unknown aspects of calcification of Pseudolachlanella eburnea and elucidate some poorly explained phenomena (e.g., translucent properties of the freshly formed test) however there are several problematic observations/interpretations which in my opinion should be carefully addressed.*
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1. *The authors (line 122) suggest that "characteristic autofluorescence indicates the carbonate content of the vesicles (Fig. S2), which are considered to be Mg-ACCs (amorphous MgCaCO3) (Fig. 2, Movies S4 and S5)". Figure S2 which the authors refer to shows only broken sections of organic sheath at different stages of mineralization. Movie S4 shows that only in a few regions some vesicles exhibit red autofluorescence interpreted as Mg-ACC (S5 is missing but probably the authors were referring to S3). In their previous paper (Dubicka et al 2023: Heliyon), the authors used exactly the same methodology to suggest that these are intracellularly formed Mg-rich amorphous calcium carbonate particles that transform into a stable mineral phase in rotaliid Aphistegina lessonii. However, in Figure 1D (Dubicka et al 2023) the apparently carbonate-loaded vesicles show the same red autofluorescence as the test, whereas in their current paper, no evidence of autofluorescence of Mg-ACC grains accumulated within the "gel-like" organic matrix is given. The S3 and S4 movies show circulation of various fluorescing components, but no initial phase of test formation is observable (numerous mineral grains embedded within the organic matrix - Figures 3A and B - should be clearly observed also as autofluorescence of the whole layer). Thus the crucial argument supporting the calcification model (Figure 5) is missing. There is no support for the following interpretation (lines 199-203) "The existence of intracellular, vesicular intermediate amorphous phase (Mg-ACC pools), which supply successive doses of carbonate material to shell production, was supported by autofluorescence (excitation at 405 nm; Fig. 2; Movies S3 and S4; see Dubicka et al., 2023) and a high content of Ca and Mg quantified from the area of cytoplasm by SEM-EDS analysis (Fig. S6)."*

Reply: We used laser line 405nm and multiphoton excitation to detect ACCs. These wavelengths (partly) permeate the shell to excite ACCs autofluorescence. The autofluorescence of the shells is present as well, but it is not clearly visible in movieS4 as the fluorescence of ACCs is stronger. This may be related to the plane/section of the cell which is shown. The laser permeates the shell above the ACCs (short distance), but to excite the shell CaCO3 around foraminifera in the same three-dimensional section where ACCs are shown, the light must pass a thick CaCO3 area due to the three-dimensional structure of the foraminifera shell. Therefore, the laser light intensity is reduced. In a revised version a movie/image with reduced threshold will be shown.

1. *The authors suggest that "no organic matter was detected between the needles of the porcelain structures (Figures 3E; 3E; S4C, and S5A)". Such a suggestion, which is highly unusual considering that biogenic minerals almost by definition contain various organic components, was made based only on FE-SEM observation. The authors should either provide clearcut evidence of the lack of organic matter (unlikely) or may suggest that intense calcium carbonate precipitation within organic matrix gel ultimately results in a decrease of the amount of the organic phase (but not its complete elimination), alike the pure calcium carbonate crystals are separated from the remaining liquid with impurities ("mother liquor"). On the other hand, if (249-250) "organic matrix involved in the biomineralization of foraminiferal shells may contain collagen-like networks", such "laminar" organization of the organic matrix may partly explain the arrangement of carbonate fibers parallel to the surface as observed in Fig. 3E1.*

Reply: We agree with the reviewer that biogenic minerals should, by definition, contain some organic components. We wrote that "no organic matter was detected between the needles of the porcelain structures" as we did not detect any organic structures based only on our FE-SEM observations. We are convinced that the shell incorporates a limited amount of organic matrix. We will rephrase this part of the text to avoid further confusion.

> 1. *The author's observations indeed do not show the formation of individual skeletal crystallites within intracellular vesicles, however, do not explain either what is the structure of individual skeletal crystallites and how they are formed. Especially, what are the structures observed in polarized light (and interpreted as calcite crystallites) by De Nooijer et al. 2009? The author's explanation of the process (lines 213-216) is not particularly convincing "we suspect that the OM was removed from the test wall and recycled by the cell itself".*

Reply: Thank you for this comment. We will do our best to supplement our explanations. We are aware of the structures observed in polarized light by De Nooijer et al. (2009). However, Goleń et al. (2022, Protist, https://doi.org/10.1016/j.protis.2022.125886) showed that organic polymers may also exhibit light polarization. Additional experimental studies are needed to distinguish these types of polarization. We will aim to investigate this issue in our future research.

> 1. *The following passage (lines 296-304) which deals with the concept of mesocrystals is not supported by the authors' methodology or observations. The authors state that miliolid needles "assembled with calcite nanoparticles, are unique examples of biogenic mesocrystals (see Cölfen and Antonietti, 2005), forming distinct geometric shapes limited by planar crystalline faces" (later in the same passage the authors say that "mesocrystals are common biogenic components in the skeletons of marine organisms" (are they thus unique or are they common)? It is my suggestion to completely eliminate this concept here until various crystallographic details of the miliolid test formation are well documented.*

Reply: Our intention was to express that mesocrystals are common biogenic components in the skeletons of marine organisms, however Miliolid needles that form distinct geometric shapes limited by planar crystalline faces are unique type of mesocrystals.