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Morphological development of *Pleurochysis carterae* coccoliths examined by cryo-electron tomography

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Abstract

Coccolithophores are single-celled marine algae that produce calcified scales called coccoliths. Each scale is composed of anvil-shaped single crystals of calcite that are mechanically interlocked, constituting a remarkable example of the multi-level construction of mineralized structures. Coccolith formation starts with the nucleation of rhombohedral crystals on an organic substrate called base plate. The crystals then grow preferentially along specific directions to generate the mature structure, which is then transported to the outside of the cells. Here, we extracted forming coccoliths from *Pleurochrysis carterae* cells and used cryo-electron tomography to characterize, in their native, hydrated state, the three-dimensional morphology and arrangement of the crystals. Comparing the crystal morphology across three different stages of coccolith formation, we show that competition for space between adjacent crystals contributes significantly to regulation of morphology by constraining growth in certain directions. We further demonstrate that crystals within a coccolith ring develop at different rates and that each crystalline unit rests directly in contact with the base plate and overgrows the rim of the organic substrate during development.

Keywords: coccolithophores, biomineralization, cryoTEM, cryo-electron microscopy; calcite; crystal growth

1. Introduction

Coccolithophores are marine phytoplankton that produce scales of CaCO_3 in the form of calcite called coccoliths. Each coccolith is an assembly of single crystals of calcite that are mechanically interlocked, constituting a remarkable example of the precise level of control organisms exert over the nucleation, size, orientation and assembly of nano-crystalline units into higher-order structures. Calcification by coccolithophores plays an important role in the environment by generating the largest current geological sink for carbon in the oceans and contributing to the transport of organic matter to the deep ocean (Ridgewell and Zeebe, 2005; Taylor et al., 2017; Ziveri et al., 2007). In spite of their importance, the molecular mechanisms by which calcification is controlled are still poorly understood.

Coccolith formation occurs inside an intracellular compartment, the coccolith vesicle (Pienaar, 1971). Ca^{2+} ions are transported from a Ca^{2+} -rich compartment into the vesicle and are deposited around the rim of the base plate, which is a water-insoluble organic scaffold that supports crystal nucleation and growth (Gal et al., 2016; Sviben et al., 2016). The first stage of coccolith development is the formation of the proto-coccolith ring, which is made of a ring-like structure of rhombohedral crystals of calcite on the rim of the base plate (Marsh, 1999). The crystals have alternating crystallographic orientations, with crystals that have the crystallographic c axis oriented vertically with respect to the base plate (V units) alternating with those that have their crystallographic c axis oriented radially with respect to the base plate (R units) (Marsh, 2003). It has been proposed that the alternating oriented nucleation of the calcite units is the result of a ‘folded ribbon’ nucleation template present in the rim of the base plates, however evidence is still needed to confirm this hypothesis (Marsh, 1999; Young et al., 1992). After nucleation, the crystals grow preferentially along specific directions, becoming mechanically interlocked. Once coccolith formation is completed, the structure is extruded to the cell surface.

Acidic polysaccharides are important macromolecules that are present during coccolith biomineralization and assist in controlling mineral formation (Marsh, 1994). While their precise functions are not yet clear, they have been shown to bind to the rim of the base plates in the form of Ca^{2+} -containing complexes, suggesting that they are involved in the transport of ions to the crystal nucleation sites (Gal et al., 2016; Marsh, 1994). *In vitro* experiments demonstrated that they affect crystal morphology and control polymorph type, suggesting similar roles *in vivo* (Borman et al., 1982; Kayano et al., 2011; Lee et al., 2016; Walker et al., 2019). Indeed, a sulfated galacturomannan coccolith-associated polysaccharide (CAP) from *Pleurochrysis cartarae* has been shown to be essential for the growth and morphological development of the calcite crystals beyond the nucleation stage (Marsh et al., 2002). In this species, Golgi derived Ca-rich particles of ~20 nm size termed coccolithosomes facilitate delivery of both the polysaccharides and Ca ions to the coccolith vesicle (Marsh, 1994; Outka and Williams, 1971; Wal et al., 1983).

The baseplate itself has been recently studied by both atomic force microscopy (AFM) and cryo-electron tomography (CryoET), which described the two-sided design of the baseplate, and arrangement of the fibres that make up its structure (Gal et al., 2016; Marzec et al., 2019). These studies also examined the rim of the baseplate, which was shown to have a height of 20 nm. No ‘folded ribbon’ structure was distinguished by either work. In addition to these organic macromolecules, the coccolith vesicle itself has also been suggested to be involved in patterning or controlling the final shape of the coccolith, through the cytoskeleton (Drescher et al., 2012; Langer et al., 2010; Taylor et al., 2007; Westbroek et al., 1973).

While the morphologies of the crystals both at the proto-coccolith ring and the mature stages are well described, the sequence in which they grow and interlock are less well known. Transmission electron microscopy (TEM) studies on forming coccoliths extracted from *P. carterae* cells have shown that at the earliest stage observed, both V and R units have parallelepiped-like shapes (Marsh, 1999; Marsh, 2003). Growth of the V units occurs by expansion of the (1014) faces, leading to the development of the outer-tube element. This is followed by the growth of the inner-tube and proximal shield elements from the R units to generate an interlocked ring. Using scanning electron microscopy (SEM) and backscattered electron diffraction (EBSD), Saruwatari *et al.* (2011) investigated the development of crystal morphology and the crystallographic orientation of the single crystalline units at different stages of coccolith formation. They showed that at the proto-coccolith stage, the crystals were only loosely attached to the base plate. Analysis of crystal growth showed a progression through a trapezoid morphology to reach the final anvil shape of mature units. The studies carried out by Marsh (1999) and Saruwatari *et al.* (2011) had a few limitations: both of them employed harsh extraction conditions, involving treatment of the cells with a detergent. Cell lysis was followed by purification steps involving centrifugation and dehydration for electron microscopy. This harsh treatment was likely to cause artefacts, namely damage to the integrity of the coccolith structure. Indeed, in both studies several of the coccolith rings appear deformed and often broken or with missing units. Additionally, the TEM imaging, as shown by Marsh (1999), produces 2D projections of complex 3D objects. Based on those projections, it is difficult to infer the 3D shape of the crystals, how they are organized in the coccolith and the overall architecture of the structure at different stages of formation.

Here, we used cryo-transmission electron microscopy and tomography (cryoTEM and cryoET) to investigate the development of coccolith morphology in *P. carterae*. Cells were treated with Triton X-100 to rupture the membranes, and the lysate was immediately plunge-frozen in liquid ethane for cryoET analysis (Nudelman *et al.*, 2011). We characterized in 3D the structure and architecture of forming coccoliths in their native, hydrated state. Our data shows how crystal morphology develops during coccolith formation, and how the crystalline building blocks interface with the base plate scaffold.

2. Materials and Methods

Cultures of *Pleurochrysis carterae* were obtained from the Culture Collection of Algae and Protozoa (CCAP), Oban, Scotland, strain 961/2, and incubated at 15 °C on a 12:12 light-dark cycle. K/2 medium, adapted from Keller *et al.*, 1987, was prepared using natural seawater provided by Scottish Association of Marine Science (SAMS), which was filter sterilised before use. Cultures were maintained by subculturing 1 mL of culture into 30 mL of fresh media every 2- 3 weeks. Cell growth was measured using a Leica optical microscope and an improved Neubauer brightline haemocytometer.

To prepare cryoTEM samples, cultures in the exponential phase of growth were centrifuged and the pellet redispersed in 10 mM CaCl₂.H₂O before 10 % Triton X-100 was added and the whole solution left to rock for 30 minutes at 57 opm (oscillations per minute). Au nanoparticles (10 nm, Sigma Aldrich) were added prior to vitrification. An aliquot (3 µL) of the solution was applied to a cryoTEM grid and plunge frozen in liquid ethane at liquid nitrogen temperatures using a vitrification robot (FEI Vitrobot Mark VI). The sample chamber was maintained at 21 °C and 100 % humidity. CryoTEM grids (Au/C, Quantifoil Micro Tools GmbH) were plasma treated for 45s prior to freezing using a Quorumtech Glow Discharge system. Tilt series were collected both at the University of Edinburgh Electron Microscopy Facility and the Electron

Bio-Imaging Centre (eBIC) at Diamond Light Source, Harwell, Oxford. At eBIC, an FEI Titan electron microscope equipped with autoloading capacity, 300 eV emission field gun and K2 direct detection camera with a phase plate was used for imaging for tomography. At the University of Edinburgh, a FEI F20 Technai electron microscope with 200 keV field emission gun, equipped with a Gatan cryoholder operating at *ca.* -170 °C was used for imaging. Images were recorded on an 8k x 8k CMOS TVIPS F816 camera.

Each baseplate was imaged every 1.5 ° for tilt angles between -65 ° and +65 °. Tilt series were analysed and reconstructed using eTomo and Avizo 9.4.0 software.

3. Results

Early stage coccoliths were extracted from the *P. carterae* coccolithophore cells in the exponential phase of growth by application of Triton X-100 for 30 min to break up the cell membrane. The resulting lysate was immediately plunge-frozen in liquid ethane and imaged using cryo-transmission electron microscopy (cryoTEM), without any further purification steps. We identified coccoliths at three different stages of formation (Fig. 1): the first stage (Fig. 1a, henceforth referred to as Stage 1) was composed of a ring of crystals with irregular shapes measuring 1.2 µm x 860 nm. A further stage of coccolith development, referred to as Stage 2 is shown in Fig. 1b. The coccolith ring was larger, measuring 1.8 µm x 1.2 µm and the crystals had regular and uniform rectangular-like morphologies that started resemble mature crystals. Finally, we observed a coccolith appearing to be in the mature stage (Stage 3, Fig. 1c), with the crystals mechanically interlocked and displaying the morphologies characteristic of V and R units. This coccolith measured 1.5 µm x 1 µm.

Figure 1d shows a schematic representation of a mature coccolith from *P. carterae*, depicting the morphologies of fully formed V and R units and how they interlock. Following the terminology by Young et al (1997), the V crystals are composed of two parts: the distal shield and the outer tube elements. The R units are composed of the inner tube element, which, together with the outer tube element of the V units contributes to the inner contour of the coccoliths, and the proximal shield, which, together with the distal shield element of the V crystals, forms the outer contour of the scales.

To characterize the morphological development of the crystals and their structural organization in the coccoliths at different stages of formation, each of the specimens in Fig. 1 was imaged in 3D using cryo-electron tomography (cryoET). The tilt series and reconstruction movies for each stage can be seen in the Supplementary Information, Movie S1-S6. The resulting 3D structures will be discussed below.

3.1. Stage 1

Figs. 2a-d show different slices from the 3D reconstructed volume of the coccolith at Stage 1 (Fig. 1a). The computer-generated 3D visualization is depicted in Figs. 2e-f, where the crystals are coloured in different shades of green and the base plate in yellow. CryoET confirmed that the individual crystals did not have their fully developed morphology. While the crystals are in contact with each other, the majority are not yet mechanically interlocked (discussed more in detail below), with one crystal dislodged from the structure (Figs. 2e-f, red arrow). The reconstructed tilt-series shows that some crystals appear to have voids inside (Fig. 2b, white circles). Also visible in the 3D reconstruction are the body scales beneath the developing coccolith ring (Fig. 2a, black arrows, and in high magnification in Fig. 2d); these were omitted from the segmentation in Figs. 2e-f and are shown in Fig. S1. The internal radial structure of the base plate (Franke and Brown, 1971; Marzec et al., 2019; Pienaar, 1969) can

be distinguished and could only be partially segmented (2e-f, in yellow) due to low contrast and the similarity in structure with the body scales. Nevertheless, it can be seen that the crystals are overgrowing the rim of the base plates (rim shown in purple and indicated by the white arrows in Fig. 2f).

Most crystals at this stage had rhombohedral-like shapes, with sizes ranging between 50-90 nm in length and *ca.* 50 nm in thickness and height. Few units with irregular morphologies that were not reflective of either a nascent R or V unit were also present, most notably a plate-like crystal located between two larger rhombohedral-like crystals (Figs. 2b and 2e, white arrow). In some cases, the development of the crystals into their mature morphologies could be distinguished. This is illustrated by a 'T'-shaped crystal (Fig. 3a, red box, and Figs. 3b-d, dark green), attributed to an incomplete R unit, located between two units with V unit-like morphology. These V-like units also displayed immature crystal morphologies, with the left-hand crystal displaying a flat edge (Fig. 3b) whereas the right-hand crystal shows extensions on either side (Fig. 3b, black arrows) going towards the adjacent units. Finally, across the ring, crystals size was irregular, ranging from 50 to 100 nm in length.

3.2. Stage 2

Figs. 4 and 5 show the 3D reconstruction of the forming coccolith at Stage 2, depicted in Fig. 1b. The computer-generated 3D visualization is shown in Figs. 4a-d, where different crystals are depicted in different colours and the base plate, partially segmented, is in red. Different slices from the reconstructed volume are shown in Figs. 4e-h. It is clear from the 3D reconstruction that the base plate, distinguishable by its radial structure, covered the bottom surface of each crystal (Figs. 4e and f, insets), indicating that each unit in the ring is growing in the inner part of the organic scaffold. At this stage, the crystals had rectangular-like shapes, however morphologies resembling those of V and R units already started to emerge. Most notably, one R unit displaying the proximal shield element was already present (Fig. 4g, inset 1). Few crystals contained voids, as depicted in Fig. 4g, inset 2 and 4h, white circles. Two clusters of spherical electron-dense particles each *ca.* 25 nm in diameter were present associated with the external surface of the crystals (Figs. 4h, insets, and 5a-b, red arrows). These particles are consistent with the size and shape of coccolithosomes, which are complexes of Ca^{2+} and anionic CAPs that transport Ca^{2+} ions to the mineralization site (Outka and Williams, 1971). Given that the Triton-X treatment is likely to remove the coccolithosomes, these particles must be attached to or in the process of fusing with the crystals.

A more detailed examination of the crystals in the coccolith ring gave insights into their morphologies, sequence of growth and organization prior reaching the mature stage. The V units had rectangular-like shapes and measured *ca.* 280 – 350 nm in length and 170 – 210 nm in height (Figs. 5c-f). While most crystals still lacked the distal shield (Figs. 5c-d), in some cases this element had already started to develop (Figs. 5f, red arrow). The thickness of the V units at the base (where it interfaces with the base plate) was similar in all crystals, *ca.* 150 nm. Their thickness at the top, on the other hand, had higher variability, ranging from 30-60 nm in some cases to 100 – 150 nm in others. The R units already displayed a recognizable inner tube element (Figs. 5g-h, white arrow), but only one crystal containing the proximal shield was present, as described above (Fig. 4g, inset 1 and Fig. 5g, red arrow). All other R units lacked the proximal shield. One example of such crystal is displayed in Fig. 5h.

The interlocking of V and R units is depicted at an early stage in Figs. 6a-c. The two V crystals are extending over the inner tube of the R crystal in the middle. Furthermore, at this stage the proximal shield of the R crystal has only started to grow around the back of the

adjacent V units. Finally, mismatches in the VR sequence were present in this coccolith. Figs. 6d-e show two adjacent V units. They exhibit flat lateral surfaces where they interface rather than the typical lateral indentation when they interlock with an R unit. Two adjacent R crystals, slanted towards the center of the coccolith, are shown in Fig. 6f.

3.3. Stage 3

Fig. 7 shows the 3D reconstruction of the mature coccolith depicted in Fig. 1c. Different slices from the reconstructed 3D volume are shown in Figs. 7c-e. The arrangement of the crystals and the base plate underneath are visible in the computer-generated 3D visualization, where different crystals are depicted in different colours (Figs. 7a-b). The underlying base plate is partially segmented in yellow, and the rim (in purple) is visible running through the bottom of the crystals as in the less mature coccolith (Fig. 7b, red arrows). One crystal is incomplete, which could be due to damage during sample preparation, or it may not yet be fully developed (Fig. 7a, red arrow).

Overall, the morphology of the crystals and of the coccolith as a whole is reminiscent of mature structures. Fully formed R units were present (Figs. 8a-b), displaying the characteristic anvil shapes with the inner tube (white arrows) and proximal shield elements (red arrows). The thickness of the crystals (from the inner tube to the proximal shield) measured ~ 180 nm, while their length was *ca.* 200 nm. Likewise, the coccolith contained well developed V units of similar anvil-like morphology (Figs. 8c-d) and displaying the outer tube (Fig. 8c, white arrow) and distal shield (Fig. 8d, red arrow) elements. These crystals were *ca.* 400 nm in length, 170 - 180 nm in thickness (measured at the distal shield), and ~ 240 nm in height. The base of these crystals was resting directly on the base plate, and measured *ca.* 300 nm in length and *ca.* 70 nm in thickness. Figs. 8e-h show interlocking V and R units and the spatial organization between them: the inner tube of the R unit is partially overgrowing the outer tube of the V crystal, and the distal shield of the V crystal growing over the proximal shield of the adjacent R unit. Looking at the base, shows that the purple and brown V crystals are resting on the base plate and are found underneath the blue and partly under the green R units (Fig. 8i).

Not all the crystals had their characteristic V and R morphologies, generally where the VR sequence was disrupted. Fig. 9a shows three crystals that were identified as V units due their morphology. The purple and dark blue crystals on the left and right sides, respectively, have lateral concave indentations where they interlock with adjacent R units (Figs. 9b-c) and one flat lateral side where they interface with the light blue crystal in the middle. These latter observations are similar to those by Marsh (1999) on neighboring VV units at earlier stages of coccolith development. Similarly, the light blue crystal has flat lateral surfaces where it makes contacts with its neighbors (Figs. 9a and 9d). As a result, the thickness of this crystal was uniform from the bottom, where it lies on the base plate, to the top. Another interesting feature of the purple and light blue crystals was the absence of a distal shield extending to the back over a narrow outer tube. Instead, their thickness was also uniform as if they had both proximal and distal shields (Figs. 9e and f). The dark blue crystal, on the other hand, did have a distal shield element (Fig. 9g). However, the back of this crystal was overgrown at the bottom by the light blue crystal and by its neighboring R unit (Figs. 9h). In spite of the different morphology, the dimensions of the three crystals were broadly consistent with those in the typical VR sequence reported above: ~ 250 nm in height and ~ 140 nm in thickness.

Disruption of the alternating pattern was not limited to V units, as adjacent R units were present which also displayed different morphologies when compared to R units that grow between V units (Figs. 10a-b). Both crystals were wider than typical R units and did not display

concave lateral indentations between the proximal shield and inner tube. Furthermore, the coccolith contained two adjacent crystals separated by a gap (Figs. 10c-d). The beige crystal (Fig. 10c, left) is a V unit with a concave indentation on its left side where an interlocking R unit is present (Fig. 7a) and a distal shield element (Fig. 10d). The right side, which lacks an interlocking crystal, developed a flat lateral surface. The purple crystal (Fig. 10c, right), on the other hand, has a proximal shield suggesting that it is an R unit (Fig. 10d). This crystal developed a high inner tube element, as opposed to characteristic R units (Figs. 8a-b), and while it displays a concave indentation on its right side, where it interlocks with another R unit (Fig. 7a), its left side has a flat surface.

Finally, we identified incomplete units, including one R unit with the inner tube but lacking the proximal shield (Fig. 10e); a second R unit with a small inner tube and proximal shield (Fig. 10f) where it is not known if the crystal is incomplete or reached its maximum dimensions given the spatial constraints from its neighboring crystals; and an incomplete V unit with the distal shield still growing (Figs. 10g-h). The base of the beige and pink V units, as well as that of the growing blue R unit in the middle can be seen resting on the base plate and overgrowing the rim (Fig. 10i).

4. Discussion

Using cryoTEM and cryoET, we have analyzed three different stages of coccolith development. Comparison of these stages showed how crystal morphology, and hence coccolith structure, developed as mineralization progressed.

Analysis of coccolith morphology *in vivo* using conventional or cryo-TEM is challenging due to specimen thickness. *P. carterae* cells are typically *ca.* 10 μm in diameter, while samples for TEM must be up to 250 – 300 nm thick. We circumvented this limitation by using a detergent to lyse the cells and release the intracellular coccoliths. We then plunge-froze the cell lysates on cryoTEM grids in order to avoid additional purification steps, such as centrifugation, which can disrupt the coccolith structure. This sample preparation method ensured the preservation of the coccoliths in their native, hydrated state while maintaining their structural integrity and organizational structure. This is in contrast to previous reports involving harsher purification methods that resulted in broken coccoliths (Marsh, 1999; Saruwatari et al., 2011). A consequence of our approach, however, was that developing coccoliths were studied outside the cellular context. Thus, we could not determine how other factors such as the coccolith membrane, cytoskeleton and transport of Ca^{2+} ions help to control the biomineralization process.

The use of cryo-electron tomography was fundamental for the structural characterization of the coccoliths. Transmission electron microscopy images are 2D projections of 3D objects, such that the resulting images are made of the superposition of multiple overlapping features. In the case of coccoliths, one cannot determine, based on the 2D projection, the 3D morphology of the crystals or their organization and morphological orientation with respect to the base plate. We overcame this limitation by using electron tomography, in which angular projections of the object were taken at different tilt angles, typically between -60° and $+60^\circ$, and the resulting images were used to reconstruct the object in 3D (Nudelman et al., 2011). The resulting reconstructions were then segmented to produce a computer-generated 3D visualization. We could image the main structural components of coccoliths and resolve their organization in 3D: the morphology of individual crystals; how they interlock at different stages; the base plate and rim; and the structural relationship between the base plate and rim and the crystals. The significance of our data to coccolith biomineralization will be discussed below.

4.1. Crystal growth

To date, little is known about how the development of the crystal shape is controlled. The picture that emerges by analyzing the morphology of the crystals in the coccoliths at Stages 2 and 3 is as follows: the V units grow preferentially in the vertical direction to develop the outer tube element expressing the (104) face (Saruwatari et al., 2011) while lateral growth is constrained due to the presence of a neighbouring R crystals. The R units will grow preferentially first radially inwards to the base plate to develop the inner tube. Lateral growth is also constrained by the neighbouring V crystals. Once the V crystals have grown higher than the adjacent R units, lateral growth will resume so that the former will grow on top of the latter. The V crystals will then develop the distal shield element. As for the R unit, once the inner tube is formed the crystal will grow radially outwards to form the proximal shield, which will extend around the back of the adjacent V units. This sequence of growth is consistent with that described by Saruwatari (2011).

It is interesting to note that coccoliths often contain defects, either in the form of mismatches in the VR sequence (as observed here and reported by Marsh, 1999), or with crystals apparently missing (Fig. 7d, arrow). In those cases, crystal morphology was significantly altered: neighbouring V units displayed flat lateral surfaces instead of lateral indentations and their width and thickness were uniform along the base of the crystal, the outer tube and the distal shield, spanning both the inner and outer parts of the base plate. This resulted in mature crystals with cuboid morphologies. R units in an RR sequence displayed altered morphologies as well: while they still developed the inner tube and proximal shields, they also had flat lateral surfaces where they interfaced with each. Additionally, the absence of a crystal in the coccolith ring enabled crystalline units to grow laterally unrestricted. This is particularly evident in the coccolith at Stage 3 (Fig. 7).

Based on these observations, we propose that the arrangement of the crystals in the coccolith ring play an important role in controlling crystal morphology. Under this scenario, neighbouring units limit the directions in which each crystal can grow as they compete for space. The presence of the proximal shield of R units prevents the lower part of the V crystals from growing outwards from the coccolith, so that only the upper part of the latter grows and gives rise to the distal shield. Additionally, two adjacent V units will grow vertically at similar rates and reach similar heights, so that one will not be able to grow over the top of the other, as a V unit extends over the top of an R crystal. Likewise, lateral growth of the individual crystals is limited by the presence of a neighbouring unit. In its absence, crystals will grow unrestricted along the rim of the base plate and become longer, as seen with the crystals depicted in Fig. 10c. As for the R units, our observations show that the height of the inner tube and the width of the proximal shield are also restricted by the presence of adjacent crystals. Taken together, we propose a mechanism in which the arrangement of the V and R units in the coccolith, together with their preferred directions of growth, will provide a physical constrain that will limit the morphological development of the neighbouring crystals. This mechanism will, in turn, contribute to the control over the morphology of the calcite crystals in the coccolith, leading to units with the typical “anvil”-like shapes.

Competition between crystals for space has been observed in geological and synthetic growth of spherulites (Grigor'ev, 1965; Shtukenberg et al., 2012). It has also been suggested to occur in biomineralizing systems as a mechanism for design control, working alone or in concert with organic structures to produce the wide variety of morphologies observed (Checa, 2018; Sommerdijk and Cusack, 2014), Examples of this include the calcitic shell of female

Argonauts (Stevens et al., 2017), columnar and non-columnar bivalve calcite (Bayerlein et al., 2014; Harper et al., 2009) and aragonite in molluscs (Checa et al., 2009).

It is very likely that additional mechanisms are also in place to control crystal morphology in coccoliths. In particular, the vesicle membrane has also been suggested to play a role in this process (Durak et al., 2017; Langer et al., 2010; Outka and Williams, 1971). Cross-sections of *P. carterae* cells show the vesicle membrane following the contour, and in several cases juxtaposed, to the crystals (Marsh, 1999; Outka and Williams, 1971). This suggests that the membrane of the coccolith vesicle limits the crystal growth area in order to regulate the dimensions of the crystals, and the height and the thickness of the coccolith ring. In this way, the confined space provided by the coccolith vesicle would act in concert with constraints over crystal growth caused by the organization of the crystalline units themselves in order to control crystal morphology. The end result is the directional control over the growth of the crystals, leading, for instance, to V units with platy outer tubes expressing the (104) faces in the direction of growth.

Other mechanisms such as the role of CAPs, the baseplate structure and spatial control over mineral deposition may also work in concert to regulate the direction of crystal growth. *In vitro* CaCO_3 precipitation experiments have shown that the sulfated galacturonomannan CAP extracted from *Emiliania huxleyi* inhibits calcite growth at the acute steps, resulting in crystals elongated along their *c*-axis (Henriksen et al., 2004). This composition of *E. huxleyi* CAP is similar to one of the three CAPs present in *P. carterae*, known as PS3 (Marsh, 2003). Furthermore, coccoliths from mutant *P. carterae* cells lacking PS3 do not develop beyond the proto-coccolith stage suggesting that this polysaccharide also plays a role in controlling crystal morphology (Marsh et al., 2002). This suggests CAPs are involved in regulating growth and shaping of crystals in both species (Fichtinger-Schepman et al., 1981). Controlled *in vitro* recrystallization of extracted baseplates has also proved challenging, suggesting that a combination of mechanisms leads to the advanced morphologies observed in the final coccolith (Gal et al., 2017).

The three reconstructions also show that crystal growth in a single stage is not synchronous. Comparing examples of R units from within both Stage 2 (e.g. the purple and pink crystals in Fig. 6a and c respectively) and 3 (the green crystals in Fig. 7), it is evident that the interior morphologies are not equally developed. As crystal growth is dependent on the rate of deposition of Ca^{2+} and CO_3^{2-} on the crystals, the presence of crystals at different growth stages in the same coccolith suggests that ion influx is not uniform across the vesicles. In other words, at a given time, some regions in the vesicle, and hence some crystals, may receive a higher influx of Ca^{2+} and CO_3^{2-} ions than others and grow at a faster rate. Little is known, however, about how the transport of mineral to the coccolith vesicle is regulated by the cells. *P. carterae* contain a calcium-storage compartment that is part of the pathway supplying the coccolith vesicle with calcium ions (Gal et al., 2018). Although it is not known how calcium ions are transported from the storage compartment to the mineralization site, it is generally postulated that coccolithosomes play an important role in the calcium transport pathway (Gal et al., 2018; Outka and Williams, 1971). Therefore, to understand how the rate of crystal growth is controlled by the cells, it is necessary to characterize the transport of the coccolithosomes to the mineralization vesicle within the context of the cells.

4.2. Transition from Stages 1 to 3

Analysis of the morphology of the crystals in the coccolith at Stage 1 showed only very few crystals displaying nascent V and R-like morphologies. The majority had rhombohedral-like

shapes that did not indicate the development of the mature morphologies. The coccolith in Stage 2 contained rectangular-like crystals that were longer, thicker and taller than the ones in Stage 1 and were clearly developing the characteristic morphologies of the V and R units: the V units were overgrowing neighbouring R crystals and had started developing the distal shield. R units were distinguishable by the inner tube and in one case already contained the proximal shield. The coccolith in Stage 3 was nearly mature, with most crystals displaying the typical morphologies of V and R units.

Examining the overall sequence of development presented here, the evolution from the second to the third stage is clear, but the change from the first stage observed to the second stage is less evident. As previously mentioned, neighbouring crystals limit lateral growth. As all crystals nucleate and rest on the baseplate which appears to remain constant in size, it is difficult to identify how the crystals we observe in Stage 1, where the ring is complete, will develop into the rectangular morphologies shown in Stage 2. One explanation is that this particular ring is malformed, and will not grow further than this stage. Comparatively, the next stage of development is easy to see. Additionally, in the first two stages, regions of low electron density within the crystal or voids were observed. Whilst it is likely that these are defects, but their origin is currently unknown.

4.3. Interface of crystals with the base plate

The current picture of crystal nucleation during coccolith formation in *P. carterae* involves the accumulation of polysaccharide-Ca²⁺ complexes on the rim of the base plates, followed by the appearance of rectangular parallelepiped-shaped crystals with alternating crystallographic orientations (Marsh, 1999). The V units will grow first vertically to develop the outer tube element expressing the (104) face, while the R units will grow radially both inwards and outwards to the base plate to develop, respectively, the inner tube and proximal shield elements. It has been proposed that the base of the V unit is inclined to the base plate by an angle due to the suggested folded ribbon structure of the base plate rim, (Marsh, 1999; Saruwatari et al., 2011; Young et al., 1992) and only the outer edge of the crystals are in contact with the substrate. Our results show a different picture: the base of the V units rests directly in contact with the base plate. This observation is consistent with rectangular rhombohedral-shaped crystals resting on the base plate at the initial stage, and then growing normal to the substrate to give rise to the inner tube element of V crystals. As for the R units, while they were all in contact with the rim, whether they were resting on the base plate or not depended on their adjacent crystals. All crystals had a notch at the base, where they overgrew the rim. This is consistent with the observations of Gal et al., (2016) and Marzec et al. (2019) who describe the attraction of Ca-polysaccharide complexes to the inner rim of the baseplate, which may be the location of nucleation sites. Neither study identified a ribbon-like structure at the rim of the base plate, but discerning such a structure is likely to prove challenging. In order to reconcile the various theories surrounding the role and structure of the base plate rim, further information at the onset of crystal nucleation is required.

5. Conclusions

We have used cryoET to characterize three stages in the formation of *P. carterae* coccoliths in their native, hydrated state in three dimensions. Looking across the stages, we demonstrate that during development, crystals across the coccolith ring do not develop synchronously. Based on the development of the V and R units, we propose a model in which the arrangement of the crystals provides constraints in the directions in which the neighboring units can grow,

and thus regulating crystal morphology. Finally, we show that all crystals interface with the baseplate in the early stages of growth. Overall, examining coccolith development by cryoET can provide both morphological and mechanistic information to shed light on the complex process of biomineralization that occurs in coccolithophorid cells.

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Figure 1. Cryo-transmission electron microscopy images of coccoliths at different stages of development extracted from *Pleurochrysis carterae*. **a.** Stage 1 (earliest stage observed). **b.** Stage 2. **c.** Stage 3 (most mature observed). **d.** Schematic representation of a coccolith from *P. carterae*, showing the organization of the V and R units (left); the morphology of the crystals (middle); and how the alternating V and R crystals interlock in a coccolith (right).

Figure 2. Cryo-electron tomography of the coccolith in stage 1 depicted in figure 1. **a-c**. Three different z-slices from the tomographic reconstruction. White circles in **b**: voids inside the calcite crystals. **d**. Higher magnification of the area marked by the red square in **a**. Black arrows in **a** and **d**: body scale that was underneath the coccolith. **e** and **f**. Computer-generated 3D visualization of the tomogram showing a top (**e**) and a bottom (**f**) view. Individual crystals are colored in shades of green, making up the periphery oval shape. The strands that are visible of the base plate (centre of the oval) are coloured yellow, and the rim of the base plate in purple. The red arrow in **e** and **f** indicates a dislodged crystal. White arrows in **e** indicate a plate-shaped crystal in the coccolith. White arrow in **f** indicate the rim of the base plate.

Figure 3. Computer-generated 3D visualization of the coccolith in figure 2. **b-d** show in high magnification the three crystals marked by the red square in **a**. **b**. Two crystals with 'V'-like morphology (light green) interlocked with one crystal in the middle resembling a developing 'R' unit (dark green). Black arrows: extensions on the sides of the crystals overgrowing the adjacent units. **c**. Interlocking between one 'V'-like unit and one 'R'-like unit. **d**. Bottom view of the three crystals where the rim of the base plate (in purple) is visible. It is also evident that the 'R'-like unit does not extend to the outer rim of the coccolith. Black arrows: extensions on the sides of the crystals overgrowing the adjacent units.

Figure 4. Cryo-electron tomography of the coccolith in figure 1b. **a-d.** Computer-generated 3D visualization of the tomogram. Each crystal is represented in a different color. **e-h.** Z-slices from the tomographic reconstruction. Insets in **e** show the rim (inset 1, red arrow) and radial structure of the base plate (inset 2). Insets in **f** show the radial structure covering the bottom of the crystals in the coccolith. Insets in **g** show one 'R' unit with a developing proximal shield (inset 1) and voids inside one crystal (inset 2, red arrow). Insets in **h** show clusters of electron-dense particles 25 nm in size associated to the mineral.

Figure 5. Computer-generated 3D visualization of crystals from the tomogram in figure 4. **a** and **b** show in detail the clusters of 25 nm particles associated with the mineral (in blue in **a** and beige in **b**, red arrows). **c-f** show front view (**c** and **e**) and side view (**d** and **f**) of two 'V' units. The red arrow in **f** indicates the developing distal shield. **g** and **h** show two different 'R' units at different stages of development. The red arrow in **g** indicates the proximal shield.

Figure 6. Computer-generated 3D visualization of crystals from the tomogram in figure 4. **a** and **b** show front (**a**) and back (**b**) views of two 'V' crystals (in blue and beige) overgrowing one 'R' crystal (in purple) in the middle. Note that the 25 nm particles that are associated with the purple crystal (in the middle) were omitted from this image. **c**. Front view of two 'V' crystals (leftmost in light yellow and rightmost in dark green) overgrowing one 'R' unit in the middle (in pink). The 'R' unit has not yet developed the proximal shield. The dark green crystal is adjacent to another 'V' unit, shown in **d** (front view) and **e** (back view). **f**. Front view of two adjacent 'R' units.

Figure 7. Cryo-electron tomography of the coccolith in figure 1b. **a-b**. Computer-generated 3D visualization of the tomogram showing the top (**a**) and bottom (**b**) views of the coccolith. Each crystal is represented in a different color, the base plate is in yellow and the rim of the base plate in purple. Red arrow in **a**: incomplete crystal. Red arrow in **b**: rim of the base plate. **c-e**. Z-slices from the tomographic reconstruction. Red arrow in **d** and **e**: gap between two adjacent crystals.

Figure 8. Computer-generated 3D visualization of crystals from the tomogram in figure 7. **a** and **b**. Mature R units. Red arrows indicate the proximal shield and white arrows indicate the inner tube. **c** and **d**. Front (**c**) and side (**d**) views of one 'V' unit. Red arrow: distal shield element. The interlocking between R and V units is shown from the front/top view (**e-g**), back view (**h**) and bottom view (**i**).

Figure 9. Computer-generated 3D visualization of crystals from the tomogram in figure 7. **a**. Three adjacent 'V' units. The dark blue (rightmost) and purple (leftmost) crystals are adjacent to one 'V' unit (light blue in the middle in **a**) and to one 'R' unit (**b** and **c**). **d** and **e**. Front (**d**) and side (**e**) views of the light blue 'V' unit. **f**. Side view of the purple crystal. **g**. Side view of the dark blue crystal. **h**. Back view of the three crystals in **a**, also including one adjacent 'R' unit (in green, bottom left).

Figure 10. Computer-generated 3D visualization of crystals from the tomogram in figure 7. **a** and **b**. Front (**a**) and back (**b**) views of two overgrowing 'R' units. **c**. and **d**. Front (**c**) and top (**d**) views of one 'V' (beige, left side) and one 'R' units separated by a gap. **e** and **f**. Two incomplete 'R' units. **g**. and **h**. Front (**g**) and back (**h**) views of one incomplete 'V' unit in pink. **i**. Bottom view of the beige (**c**) and pink (**g**) 'V' units interlocking with the incomplete light blue 'R' crystal in the middle (**e**).