Coccolithophore biomineralization: New questions, new answers

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Summary

Coccolithophores are unicellular phytoplankton that are characterised by the presence intricately formed calcite scales (coccoliths) on their surfaces. Coccolith formation is an entirely intracellular process – crystal growth is confined within a Golgi-derived vesicle. A wide range of coccolith morphologies can be found amongst the different coccolithophore groups. This review discusses the cellular factors that regulate coccolith production, from the roles of organic components, endomembrane organisation and cytoskeleton to the mechanisms of delivery of substrates to the calcifying compartment. New findings are also providing important information on how the delivery of substrates to the calcification site is co-ordinated with the removal of H^+ that are a bi-product of the calcification reaction. While there appear to be a number of species-specific features of the structural and biochemical components underlying coccolith formation, the fluxes of Ca^{2+} and a HCO_3^- required to support coccolith formation appear to involve spatially organised recruitment of conserved transport processes.

1. Introduction

Coccolithophores are single celled marine photosynthetic protists belonging to the Haptophyte division of the chromalveolate eukaryotes. They are significant components of the marine phytoplankton with certain species, such as the cosmopolitan *Emiliana huxleyi* able to form massive blooms in temperate and sub-polar waters. Because of this their ecology, physiology and palaeontology have been well-studied. Coccolithophores also present a paradigm for the study of calcification mechanisms. The ease with which certain species can be cultured, the relative tractability of a unicellular calcification system that produces intricate calcium carbonate structures (coccoliths) allows questions relating to the biological control of crystal formation and morphology to be addressed.

Coccolithophore calcification has received considerable attention in recent years with many studies directed to the potential impacts of ocean acidification – the decrease in ocean pH associated with the dissolution of anthropogenically-derived CO₂ into the surface ocean. While these studies have generally not directly addressed questions relating to better mechanistic understanding of coccolithophore calcification, they have revealed a number of features of coccolithophore biology (e.g. strain variability, plasticity of calcification response, genetic adaptation, species differences) that are pertinent to the calcification mechanism [e.g. 1-3]. Nevertheless, important questions remain to be answered in order to fully elucidate the cellular drivers and regulators of calcification that are essential for understanding the roles of coccolithophores in the ecology of the oceans, predicting responses to changing ocean chemistry and realising the potential of coccolithophores for biotechnological applications.

2. The essentials of coccolithophore calcification

Well preserved coccoliths can be found well preserved in sedimentary records 220 Ma and molecular clock studies estimate that the first calcifying haptophytes (calcihaptophytes)

originated ~330 Ma [4]. This suggests that coccolithophores evolved under ocean carbonate chemistry conditions that were significantly different from those of the present day. Most studies of coccolithophore calcification mechanisms have focussed on the “model” species *E. huxleyi* which is easily isolated and cultured, with a large body of physiological data derived from culture experiments. These advantages, together with a fully sequenced genome [5] and an array of additional genomic resources have led to significant advances in understanding the biology and physiology of coccolithophores. The calcite coccoliths of diploid *E. huxleyi* cells are exquisitely sculpted complex multi-crystalline plates that are formed via crystal growth, uniquely, in an intracellular compartment, the coccolith vesicle (CV). Mature coccoliths are secreted to the cell surface where they form an outer coat (coccosphere) (Figure 1). In many species (with the exception of *E. huxleyi*) the haploid phase produces simpler holococcoliths, formed from rhombohedral crystalline units most likely, in an extracellular space [6]. Nevertheless, the diploid heterococcolith producing life cycle stage represents the diploid calcifying stage that is predominantly found in natural populations and is the dominates production of particulate inorganic carbon in the oceans.

3. The determinants of coccolith morphology.

The wide range of coccolith shapes and sizes produced by different species suggests a range of functional roles as well as species-specific cellular factors that determine coccolith morphology. In order to understand the regulation of coccolith morphology it is necessary to understand the cell structures and physiology that are brought into play during coccolith development. Ultrastructural studies of *E. huxleyi* show the CV to be derived from Golgi cisternae [7]. Coccolith growth proceeds as the CV matures and completed coccoliths are secreted to the cell surface in a single exocytotic event [8]. Coccolith growth begins with the nucleation of calcite crystals with alternating orientations (V and R units) in a circular arrangement known as the protococcolith ring [7]. The coccolith matures into a distal (upper) shield and outer tube formed of V-units. The lower proximal shield, inner tube and central area elements are derived from R-units. The two unit types alternate with each other

in a ring on the proximal face of the coccolith and this is interpreted to be the proto-coccolith ring locus, i.e., the location where nucleation occurred. It has been proposed that growth of the protococcolith ring initiates from an organic baseplate of alternating structure that establishes the alternating crystal orientations. In several species this baseplate has been visualised using transmission electron microscopy (TEM) [e.g. 8,9] although its organic composition remains uncharacterised. So far, the only protein known to be intimately associated with coccoliths is the so-called GPA (glutamine, proline, alanine-rich protein) that was initially isolated from a coccolith-associated polysaccharide from *E. huxleyi* [10]. Subsequently Schroeder et al., [12] identified specific sequences of a non-coding region of the *GPA* gene that correlated with specific coccolith strain morphotypes (A and B) characterised by subtle variations in degree of calcification and coccolith element dimensions. However, the GPA protein does not appear to be directly involved in determining coccolith morphology because transcriptomics studies have shown that the expression of GPA is inversely correlated with the rate of calcification [12]. For example, GPA expression was shown to be higher in non-calcifying haploid cells of *E. huxleyi* [12] and in diploid cells grown under regimes where calcification was inhibited. It is possible, therefore, that the GPA protein may have a direct role as a negative regulator of calcification. Alternatively, GPA may act as an inhibitor of calcification-related gene expression or the increased levels of *GPA* transcripts in non-calcifying cells may reflect a translation block of the GPA protein under non-calcifying conditions leading to an accumulation of transcripts. These hypotheses have yet to be tested.

Polysaccharides have also been shown to play a likely key role in the regulation of coccolith formation. A single coccolith-associated polysaccharide isolated from *E. huxleyi* was shown to prevent calcite precipitation in vitro, leading to the suggestion that the polysaccharide may form an inhibitory barrier regulating calcite crystal growth [13-15]. *Pleurochrysis carterae* was subsequently shown to produce three types of coccolith-associated acidic polysaccharide (PS-1,2, and 3) [16,17]. One of these, PS-2, was shown to be likely required

for coccolith nucleation since mutant strains lacking this polysaccharide produced few coccoliths. Cells lacking PS-3, a sulfated galacturonomannan, produced coccoliths that were deficient in calcite [17], suggesting that this was required for coccolith growth and morphology. PS-3 was also shown to localise at the interface between the growing crystal and the membrane of the CV [18]. Moreover, the role of PS-3 was shown to be restricted to the later stages of crystal growth after the protooccolith ring had been formed. This is consistent with later studies in the related species *P. haptanemofera* [19] showing that PS-1 and PS-3 were surface associated while PS-2 was associated with both the surface and interior of the coccolith, suggesting a role in crystal nucleation or maintenance of coccolith structure. The roles of other intra-crystalline organic components on the control of coccolith nucleation and growth remain to be elucidated. Smith et al [20] extracted a number of protein and polysaccharide components from *P. carterae* coccoliths with potential roles in regulating coccolith growth and morphology. Clearly a large potential array of organic components await characterization from a range of coccolithophore species.

Further controls on coccolith morphology are likely to come from the cytoskeleton and the organisation of the endomembrane system associated with the developing CV. It has been proposed by several workers that cytoskeletal connections with the maturing CV could underpin overall morphology of the coccolith. Indeed studies using inhibitors of actin or tubulin polymerisation have been shown to produce abnormal coccolith morphology [21] though whether this results from inhibition of direct cytoskeletal-CV interactions or indirect effects on Golgi vesicle transport and delivery of substrates to the calcification site (see below) remains to be determined. The role of endomembranes in determining coccolith morphology is also apparent from ultrastructural studies of complex endomembrane anastomosing tubules termed the reticular body closely associated with the CV and likely contiguous with the endoplasmic reticulum in the coccolithophore *Scyphosphaera apstenii* [10] (Figure 1). Intrusions of the reticular body appear to form membranous “plugs” that contact the organic baseplate, somehow preventing mineralization. These specific ordered

endomembrane structures were proposed to be critical in the formation of the pattern of central pores of the disk-like coccoliths (muroliths) and the bases of the cup-shaped lapodaliths produced by this species (Figure 1).

4. Physiology of calcification

4.1. Co-ordination of Ca^{2+} and DIC transport: An issue of compartmentalization?

How are the pathways for Ca^{2+} and DIC organised to prevent unregulated CaCO_3 precipitation and to allow co-ordination of inorganic carbon requirements for photosynthesis and calcification? Coccolith crystal growth requires delivery of Ca^{2+} and dissolved inorganic carbon (DIC) from the external medium to the growing coccolith crystals in the CV. Estimates of Ca^{2+} influx based on measured rates of calcification in an *E. huxleyi* cell that is calcifying at rate comparable to the rate of organic carbon fixation by photosynthesis indicate that the net trans-cellular flux of Ca^{2+} is arguably the largest sustained trans-cellular Ca^{2+} flux of any cell type [22]. This massive net Ca^{2+} flux poses a number of kinetic and energetic problems for the cell's transport machinery considered in several recent studies. Conventional cellular Ca^{2+} uptake involves channel-mediated Ca^{2+} influx across the plasma membrane into the cytosol where it may be transported into endomembrane compartments and organelles such as mitochondria or extruded from the cell by the activities of Ca^{2+} -ATPases and/or $\text{Ca}^{2+}/\text{H}^+$ antiporters. The requirement to maintain very low cytosolic $[\text{Ca}^{2+}]$ (around 100nM) in all eukaryotic cells imposes potential severe kinetic constraints on the trans-cytosolic movement of Ca^{2+} from the inner surface of the plasma membrane to the site of calcification [22,23]. Ca^{2+} almost certainly diffuses across the coccolithophore plasma membrane into the cytosol down an electrochemical gradient through, as yet unidentified, Ca^{2+} -permeable channels. However, a straightforward entry of Ca^{2+} into the cytosol across the plasma membrane, followed by diffusion across the cytosol and uphill transport into the coccolith-forming compartment is unlikely [22,24], particularly if this involves a long diffusion path across the cytosol. An alternative or supplement to trans-cytoplasmic Ca^{2+} diffusion is

the direct uptake of Ca^{2+} via plasma membrane vesicle endocytosis, or fluid phase transport that would result in trans-cellular movement of endocytotic vesicles carrying a seawater cargo containing $\sim 10\text{mM}$ Ca^{2+} . However, studies to date have been unable to demonstrate a fluid phase transport mechanism operating in *E. huxleyi* or *Coccolithus pelagicus* [23]. Moreover, a recent modelling study [25] estimated that unfeasible rates of vesicle turnover would be required to accommodate the net Ca^{2+} flux required for coccolith production if endocytotic vesicles containing 10 mM Ca^{2+} were the principal delivery route. Similarly, direct pumping of Ca^{2+} from the cytosol into the CV [26] via Ca^{2+} -ATPases is unlikely to satisfy the demands of calcification because of the relatively low transport capacity of Ca^{2+} -ATPases would require an unfeasibly high number of transporters on the CV membrane to effect the required fluxes [27]. Higher capacity transporters such as $\text{Ca}^{2+}/\text{H}^{+}$ exchangers or $\text{Ca}^{2+}/\text{Na}^{+}$ exchangers are more likely to transport Ca^{2+} into an endomembrane precursor CV compartment, particularly if the path length for Ca^{2+} across the cytosol was kept to a minimum [27]. Supporting this hypothesis, the involvement of $\text{Ca}^{2+}/\text{H}^{+}$ exchangers is predicted to be kinetically feasible in the modelling study of Holz *et al.*, [25]. In this respect the cortical endoplasmic reticulum could potentially play a role as the primary recipient of Ca^{2+} entering the cell across the plasma membrane. In this scenario the action of V-type H^{+} -ATPases maintains H^{+} electrochemical gradients (inside acidic) in endomembrane compartments, including Golgi-derived compartments. Ca^{2+} accumulation into an acidic endomembrane compartment via $\text{Ca}^{2+}/\text{H}^{+}$ exchangers could occur prior to the biomineralization step. Progressive alkalinization of this Ca^{2+} -charged compartment, possibly resulting from down-regulation of V-type H^{+} ATPase activity would occur as the compartment matures [27], ultimately delivering a high capacity Ca^{2+} source to the CV at appropriate pH for calcification. Gene expression studies support the involvement of $\text{Ca}^{2+}/\text{H}^{+}$ transporters at some stage in the accumulation of Ca^{2+} into the CV or CV precursor compartment [12,28]. The putative $\text{Ca}^{2+}/\text{H}^{+}$ exchanger CAX3, but not CAX4 was shown to be up-regulated in calcifying diploid *E. huxleyi* compared with haploid non-calcifying cells and showed a relation

with the rate of calcification when this was manipulated in different calcifying diploid strains. This suggested that CAX 3 may play a role in calcification-related Ca^{2+} transport while the role of CAX 4 may be more related to general Ca^{2+} homeostasis. Further support for a role of an endomembrane transport pathway for Ca^{2+} comes from ultrastructural studies showing association of ER tubules providing a large membrane surface area in close proximity to the CV (Figure 1) [8,9, 29].

The accumulation of Ca^{2+} into precursor compartment(s) that is subsequently delivered to the site of calcification in the CV is consistent with other models of calcification where it has been shown that CaCO_3 formation as amorphous calcium carbonate (ACC) occurs prior to calcite crystallization. Delivery of ACC to the calcification site by vesicle transport in has been shown in sea urchin spicules and a range of other systems and is likely to be a widespread mechanism [30-32]. In this way Ca^{2+} can be transported to the calcification site in a highly concentrated form, allowing vesicle delivery of Ca^{2+} at rates necessary to sustain calcification. This raises the key question of whether ACC is involved in the calcification process in coccolithophores. To date no evidence has been provided for the existence of ACC in coccolithophores. However, vesicles containing tiny granules (25 nm) termed coccolithosomes with high Ca^{2+} content have been demonstrated in EM studies of in *Hymenomonas carteri* and *Pleurochrysis carterae*. [16,33]. These have been shown to fuse with the rim of the CV [16,34]. The possibility exists that coccolithosomes represent a highly concentrated form of Ca^{2+} such as ACC. However, Van der Wal *et al.*, [33] showed that while Golgi-derived vesicles containing coccolithosomes fused with CV precursor vesicles, the coccolithosomes disappeared at the onset of calcification. This suggests that either the coccolithosomes were only involved in the early stages of coccolith formation or that the full complement of coccolithosomes required for complete coccolith formation is present from the outset. In invertebrate systems such as the developing sea urchin spicule, a complete structure comprising ACC is first laid down followed by a transition to crystalline CaCO_3 [30-

32]. It has also been proposed that this occurs in a non-aqueous, solid phase environment. In electron microscopy studies to date, however, coccolithophore crystal growth appears to occur without any obvious pre-ACC phase, even at the earliest stages of protococcolith ring formation.

The extracellular source of DIC for calcification in *E. huxleyi* has been shown in a number of studies to be HCO_3^- whereas CO_2 is utilized directly for photosynthesis under CO_2 replete conditions [35-39]. Whether coccolithophores possess a chloroplast-based CCM that allows the accumulation of HCO_3^- via the action of chloroplast carbonic anhydrase (CA) is not clear. However, a number of studies have indicated that coccolithophores may utilise external HCO_3^- for photosynthesis at limiting CO_2 concentrations, especially at high light [37,39]. Since only HCO_3^- can be utilised for calcification the assumption is that cytosolic conversion of HCO_3^- to CO_2 is low. On the basis of a detailed modelling study Holz et al [40] proposed that under conditions of high DIC demand (high light and low CO_2) the conversion of CO_2 within the cytosol would not represent an energy-efficient mechanism. Rather, an alternative pathway whereby HCO_3^- may be taken up directly by the chloroplast may operate under these circumstances. The above argument, along with other experimental observations [39,41-44] indicate that both photosynthesis and calcification may draw on a common cytosolic HCO_3^- pool to supply their requirements. How this pool is maintained has not been elucidated, although gene expression studies have pointed to a putative anion transporter (SLC-4) that is specific to diploid calcifying cells of *E. huxleyi* [45,12]. Most likely this operates as a $\text{HCO}_3^-/\text{Cl}^-$ antiporter at the plasma membrane [12]. The transporters operating to deliver HCO_3^- to the CV or precursor compartment have yet to be identified.

4.2. Dicing with death H^+ ? The use of HCO_3^- as the DIC external source for calcification results in the intracellular production of H^+ in a 1:1 molar ratio with CaCO_3 precipitation (Figure 2). Multifactorial experiments [e.g. 38,39] have shown convincingly that *E. huxleyi*

calcification rates with were dependent on external HCO_3^- and independent of CO_3^{2-} . Assuming that H^+ production occurs at, or close to, the site of CaCO_3 precipitation, H^+ will need to be removed from the CV and ultimately the cytosol to prevent acidosis and cell death. Hypotheses have been proposed around the potential use of this supply of H^+ for CO_2 generation in the cytosol but experimental evidence listed above does not support this hypothesis. H^+ removed from the CV or precursor membrane compartments involved in Ca^{2+} accumulation through the activity of $\text{Ca}^{2+}/\text{H}^+$ antiporters operating with a stoichiometry of at least $2\text{H}^+:1\text{Ca}^{2+}$ can potentially be recycled into the endomembrane compartment through the activity of $\text{V-H}^+\text{ATPases}$ (Figure 2) [12]. The finding that coccolithophores possess voltage-regulated H^+ channels in their plasma membrane that activate upon cytosolic acidification and/or plasma membrane potential depolarization provides a mechanism whereby rapid and high capacity H^+ efflux from the cell can be mediated (Taylor et al, 2011). The presence of plasma membrane H^+ channels is consistent with observations that intracellular pH is strongly influenced by changes in external pH (Taylor et al; Suffrian et al). This in turn is consistent with the evolution of a pH regulatory mechanism that relies on fairly constant external seawater pH for its effective operation. Recent studies have shown that coccolithophores, along with diatoms and likely other marine protists, have electrically excitable plasma membranes, able to generate plasma membrane depolarizations that are identical to action potentials of metazoan nerve and muscle [46,47]. These provide a potentially very effective mechanism for control of voltage-regulated H^+ channel activity and intracellular pH homeostasis.

5. Concluding remarks: Do all coccolithophores calcify by the same mechanism?

All calcifying coccolithophores have the ability to calcify in an intracellular Golgi-derived vesicle, suggesting the existence of common underlying mechanisms. The basic machinery of calcification appears to be conserved across the heterococcolith producing

coccolithophores, for example the recruitment pathways and mechanisms that are conserved across the eukaryotes for transport of Ca^{2+} , HCO_3^- and H^+ . However certain caveats need to be considered when extrapolating mechanistic information obtained largely from a single model species, *E. huxleyi*.

A number of examples illustrate this. Firstly, as described above, different coccolithophore species appear to produce different polysaccharides associated with calcification. Secondly, a study of carbon isotope fractionation in two different coccolithophore species, *Gephyrocapsa oceanica*, a close relative of *E. huxleyi* and *Coccolithus pelagicus* ssp. *braarudii* revealed some intriguing differences in their isotope fractionation properties in relation to DIC supply [48]. The authors proposed that *C. braarudii* may utilise H^+ produced from the intracellular production of CO_3^{2-} from HCO_3^- for CO_2 production for photosynthesis through the action of cytosolic carbonic anhydrase. In contrast, they proposed that the smaller cells of *G. oceanica* may utilise H^+ removed from the cell to facilitate external CO_2 generation for photosynthesis through the action of external carbonic anhydrase. Note that both of these possibilities contrast with the conclusions from experiments with *E. huxleyi* in which calcification was inhibited by removal of external Ca^{2+} [41,42] that indicated no mechanistic dependence of calcification on photosynthesis in that species. Thirdly, the *GPA* gene, proposed to be involved in the regulation of coccolith morphology does not appear in the transcriptomes of coccolithophores other than *E. huxleyi* and *G. oceanica*. Given the likely fundamental role of the GPA protein in regulation of coccolith growth and morphology, it seems likely that the organic regulatory components may vary considerably among coccolithophore species. Finally, ultrastructural studies reveal subtle differences in the organisation of the endomembranes involved in calcification. Coccolithosomes, for example have only been observed in two species to date. Further work is clearly needed reveal the variety of mechanistic specializations that give rise to the current array of coccolith morphologies.

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Figure Legends

Figure 1. (A-D) SEM images of examples of the four groups of calcihaptophytes. (E) Detail of placoliths from *S. apstenii* (B). (F) TEM section through a coccolith vesicle (CV) of *S. apstenii* showing associated endoplasmic reticulum (ER) layers surrounding the vesicle, the CV-associated reticular body (RB) and inter- and intracrystalline organic matrix (OM). (G) Ordered association of reticular body projections (*) with a base plate scale of *S. apstenii* reflecting the pattern of distribution of pores in the central region of the mature coccolith (see text for details). Scale bar 10mm in (A,B); 3 mm in (C,D), 1mm in (E,F), 0,25 mm in (G) (E-F reproduced with permission from [9]).

Figure 2. Hypothetical representation of Ca^{2+} and H^+ pathways associated with coccolithophore calcification (see text for details). Top: Representative cell showing the hypothesized distribution of V-type ATPases and $\text{Ca}^{2+}/\text{H}^+$ antiporters (CAX) underlying the accumulation of Ca^{2+} in the CV precursor endomembranes. CAX-mediated Ca^{2+} accumulation in precursor Golgi vesicles (G) is driven by V-type H^+ -ATPase activity and inside acid lumen. Conditions for CaCO_3 precipitation are reached as vesicles mature associated with declining H^+ -ATPase activity (right). Alternatively Ca^{2+} may accumulate in the ER through the activity of CAX transporters (left). Inset (below): Ca^{2+} may move from the ER into the CV via Ca^{2+} channels in the ER membrane and close proximity CAX transporters in the CV membrane.

COCCOLITHALES
Coccolithus pelagicus
ssp. braarudii

ZYGODISCALES
Scyphosphaera apstenii

SYRACOSPHAERALES
Syracosphaera borealis

ISOCHRYSIDALES
Emiliana huxleyi

Figure 2