Biominalization by particle attachment in early animals

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Abstract

Crystallization by particle attachment (CPA) has been demonstrated in modern biomineralized skeletons across a broad phylogenetic range of animals. Precisely the same precursors, hydrated (ACC-H$_2$O) and anhydrous calcium carbonate (ACC), have been observed spectromicroscopically in echinoderms, mollusks, and cnidarians, phyla drawn from the 3 major clades of eumetazoans. Scanning electron microscopy (SEM) here also shows evidence of CPA in tunicate chordates. This is surprising, as species in these clades have no common ancestor that formed a mineralized skeleton and appear to have evolved carbonate biomineralization independently millions of years after their late Neoproterozoic divergence. Here we correlate the occurrence of CPA from ACC precursor particles with nanoparticulate fabric and then use the latter to investigate the antiquity of the former. SEM images of early biominerals from Ediacaran and Cambrian shelly fossils show that these early calcifiers used attachment of ACC particles to form their biominerals. The convergent evolution of biomineral CPA may have been dictated by the same thermodynamics and kinetics as we observe today.

Biomineralization | calcium carbonate | skeleton | particle attachment

Crystalization by particle attachment (CPA) (1) was first observed in synthetic nanocrystallite titanite (2), and, subsequently, in bacterial biominerals (3). A nonclassical crystallization mechanism (4), CPA is now known to occur, as well, during skeletal biomineralization, where amorphous particles attach first and crystallize later. Initially observed in sea urchin teeth (5), CPA of amorphous precursors has been demonstrated in modern biominerals across a broad phylogenetic range of animals, including sea urchin spicules (6), spines (7, 8), and teeth (5, 9); the larval shells (10) and nacre (11) of mollusks; zebrafish bone (12) and mouse enamel (13); and scleractinian coral skeletons (14). Echinoderms, mollusks, and cnidarians, drawn from the 3 major clades of eumetazoans, do not share a common biomineralizing ancestor; nonetheless, spectromicroscopic observations show that they use precisely the same precursors, hydrated (ACC-H$_2$O) and anhydrous calcium carbonate (ACC) (5, 11, 14–16). This is surprising because these groups diverged from one another in the Neoproterozoic Era, but they evolved their characteristic body plans and capacity for skeletal biomineralization only during the latest Ediacaran and Cambrian periods (17, 18). It would be intriguing, therefore, to know whether these mechanistic commonalities already existed in the earliest calcifying animals.

Fig. 1 presents an animal phylogeny, showing the broad but discontinuous phylogenetic distribution of animals with skeletons containing calcite, aragonite, or vaterite (the 3 polymorphs of anhydrous crystalline CaCO$_3$). The earliest known examples of CaCO$_3$ skeletal biomineralization occur in ca. 549 to 541 million year old (Ma) limestones around the world (19, 20), and by 535 to 510 Ma, calcareous skeletons referable to sponges, cnidarians, and extant bilaterian phyla formed globally (21, 22). The observation that skeletal growth by CPA of ACC precursors occurs in cnidarians, mollusks, and echinoderms raises the question of whether this mechanism is general, and if so, given that animals with carbonate skeletons have no common ancestor that was itself calcifying. Here we address this question by probing some of the oldest known examples of calcified skeletons, enabling us to ask whether the CPA–ACC mechanism evolved early in animal history. We focus on skeletons of the problematic, but arguably cnidarian genus Cloudina from terminal Ediacaran (549 to 541 Ma) rocks and lophotrochozoans (halkieriids and hyoliths) of middle Cambrian age (509 to 497 Ma). We examined specimens preserved by early diagenetic phosphatization, because this can preserve skeletal fabric in exquisite detail, revealing nanoscale features commonly obscured by recrystallization in calcareous specimens (23).

Results

CPA of ACC Precursors and Its Proxies. We hypothesize that CaCO$_3$ biomineralization has occurred by the attachment of amorphous particles since skeletal biomineralization first evolved, favored by thermodynamics and kinetics that we can observe today. We

Significance

The mechanisms by which organisms form mineralized skeletons have been a major research focus for the last 50 y and remain so today. Among the most surprising discoveries is the recent observation that different animals use the same mechanisms, and precisely the same amorphous precursors, to form biomineralized structures as diverse as coral skeletons, molluscan shells, and sea urchin spines. In living animals, skeletal biomineralization from amorphous precursors correlates with a distinctive nanoparticulate texture that can be preserved in fossils, enabling us to probe mechanisms of skeletal formation in early animals. We document nanoparticulate texture in some of the oldest known carbonate skeletons, which strongly suggests that skeletons formed from amorphous precursors throughout the recorded history of animals.

Conflict of Interest Statement: P.U.P.A.G., C.-Y.S., and Tali Mass are coauthors on 2 recent papers published in 2017.

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know that the transformations from ACC-H₂O to ACC, and from ACC to calcite or aragonite, are exothermic reactions (24). Synthetic ACC-H₂O dehydrates and crystallizes within seconds, especially in contact with water; however, biogenic ACC-H₂O in animal skeletons takes 20 to 30 h to transform (11, 14, 15). The phases themselves are in energetically downhill sequence, but the longer times between phase transitions imply greater activation barriers between phases in vivo, likely offering the time required for greater physiological control of crystal morphology and orientation. To test whether early biomineralizers used the same mechanism of CPA of ACC precursors, we need to establish a physical proxy that can be preserved in ancient skeletons. Mastropietro et al. (25) argued that growth by amorphous particle attachment leaves a record in the form of a granular fabric observable via nanoscale imaging. Building on this, we correlated the complex and time-consuming synchrotron spectromicroscopy detection of ACC-H₂O and ACC in freshly formed biominerals (11, 14, 15) with observations from far simpler, and more widely available, scanning electron microscopy (SEM), confirming that the biominerals retain a nanoparticulate fabric after their ACC nanoparticles have filled space (26) and fully crystallized. In Figs. 2 and 3 we present evidence of this nanoparticulate fabric in modern biominerals, in which skeletal nanoparticles are 50 to 400 nm, consistent with many previous observations by SEM and coherence lengths measured by X-ray diffraction (27). The images also demonstrate that the nanoparticulate fabric differs from single-crystalline aragonite or calcite formed abiotically. Also in Fig. 2, we show nacre from molluscan shells of 4 different ages: Modern, Miocene (~13 Ma), Cretaceous (~100 Ma), and Late Ordovician (~450 Ma). The Miocene nacre is still aragonitic (28), and exhibits a nanoparticulate fabric comparable to that of modern nacre where tablets are fractured (arrows in Fig. 2 A–E). The Ordovician nacre was phosphatized during early diagenesis; as already observed by Mutvei (29) (cf. figures 2 and 3 in ref. 29 with Fig. 2F and SI Appendix, Fig. S1F2), it shows space between tablet layers, although tablets appear similar to modern nacre tablets from Nautilus after the latter are etched (29, 30), showing elongated nanocrystals oriented parallel to the tablet layers and radially distributed from the center of each tablet (cf. figure 4b in ref. 29 and figure 1b in ref. 30 with Fig. 2F and SI Appendix, S1 F1 and F2).

In modern biominerals, nacre and sea urchin spines in particular, nanoparticles are cemented together and space filling (26); thus, it can be difficult to image them even after cryo-fracturing. However, it is clear that their cryo-fracture figures are neither cleavage planes nor conchoidal, as crystals or glass, respectively, typically break. Cryo-fractured biominerals exhibit irregular, rough fracture figures, as in fractured cement, sandstone, or any other granular materials. In order to more clearly reveal the nanoparticulate fabric of these modern biominerals, we partially etched them after cryo-fracturing and bleaching them. The results are shown in Fig. 2 A–D and B and Fig. 2C for Halicost and Nautilus nacre, respectively, and in Fig. 3A for the sea urchin spine.

Importantly, the nanoparticulate fabric was clearly retained in the phosphatized nacre, even after fossil preparation and maceration. We note that Coronado et al. (31) documented a comparable granular nanofabric in (nonphosphatized) Devonian coral skeletons and interpreted it as evidence for skeletal growth by CPA of ACC. In contrast, nonbiogenic crystals of calcite, aragonite, and apatite do not exhibit the nanoparticulate texture observed in biominerals (Fig. 3 C–F and SI Appendix, Figs. S2 C–F and S6 B2 and C2). We stress that the nanoparticles observed in modern biominerals are irregularly shaped—that is, noneuhedral—even though they become crystalline.

**CPA in Vaterite Spicules from Tunicates.** The comparison extends even to the third polymorph of CaCO₃, vaterite. It has been suggested (32) that outer spines of vaterite spicules from the ascidian tunicate Herdmania momus do not form via CPA because they cryo-fracture smoothly, akin to nonbiogenic single crystals. Here we reproduce this result with precisely the same magnification and conditions of all other experiments (Fig. 4A), but also show that at the forming end of the spicule the outer spines appear nanoparticulate and irregularly shaped (Fig. 4B). At a
later stage, their morphology is euhedral, and specifically hexagonal pyramidal (Fig. 4), possibly because of a dissolution and (re)crystallization process (33). The inner core of the vaterite spicule remains nanoparticulate and cryofractures accordingly at all stages (Fig. 4C). Thus, tunicates—which evolved biomineralization independently of other clades discussed here and diverged from their closest biomineralizing relatives, the vertebrates, no later than the early Cambrian Period (Fig. 1 and references therein)—show evidence of skeletal mineralization by particle attachment.

**Evidence of CPA in Ediacaran and Cambrian Phosphatized Fossils.** Armed with the observations that CPA of ACC-H₂O and ACC precursors in modern biominerals correlate with nanoparticulate fabric at the SEM level, and that diagenetic phosphatization does not alter this fabric (Fig. 2F and SI Appendix, Fig. S1F), we can use it as a proxy for CPA of ACC-H₂O and ACC in skeletal biominerals from Ediacaran and Cambrian animals.

Fig. 5 and SI Appendix, Fig. S5 show nanoparticulate fabrics in a phosphatized Cloudina specimen (Cloudina 111–2) from the terminal Ediacaran (551 to 541 Ma) Dengying Formation in South China (34). The specimen was imaged using SEM and X-ray microcomputed tomography (SI Appendix, Fig. S3 and Cloudina mov, a movie also in SI Appendix), and was then fractured (SI Appendix, Fig. S4) and imaged again using high-resolution SEM. The fractured cross-section of the shell exhibits irregularly shaped 50- to 400-nm nanoparticles (Fig. 5 and SI Appendix, Fig. S5). This nanoparticulate fabric is distinct from the smooth appearance of a nonbiogenic apatite crystal (Fig. 3D and SI Appendix, Fig. S2C–F) and is confirmed in multiple cross-section regions within 2 specimens from the same population (SI Appendix, Figs. S3 and S4), although one fossil shows localized recrystallization (e.g., SI Appendix, Fig. S6A). Indeed, recrystallization is prevalent in nonphosphatized Cloudina skeletons (e.g., those from Namibia; SI Appendix, Fig. S6B), showing the advantages of phosphatized specimens. We note that, although the size range is the same in modern skeletons and
Cloudina specimens, the average size is slightly larger in Cloudina. This could have been the original size or reflect diagenetic coarsening.

In Fig. 6 we present a hyolith conch (35) and a halkieriid sclerite (36), from the middle Cambrian Gowers and Monastery Creek formations, Georgina Basin, Australia, respectively, both phosphatized during early diagenesis. SI Appendix, Fig. S7 shows these small shelly fossils before and after fracturing. Note in Fig. 6 and SI Appendix, Fig. S7 the 50- to 400-nm nanofilaments in the hyolith and 50- to 400-nm rounded nanoparticles in the halkieriid cross section. SI Appendix, Fig. S8 A–D shows a variety of elongated spherulitic or euhedral crystals in the internal mold or the wall cross-section of other sclerites, whereas SI Appendix, Fig. S8E shows the globular nanoparticulate phosphatized outer layer of a halkieriid sclerite, interpreted as an originally organic layer akin to the periostracum that lines the outer surface of modern molluscan shells (36). As in Cloudina, small shelly fossils occasionally show recrystallization, forming nano- or microscale euhedral crystals (SI Appendix, Fig. S8). Recrystallization is sporadically observed in the shells of middle Cambrian mollusks and the sclerites of echechinoderms (SI Appendix, Figs. S8A and S9 B and C). When well-preserved, phosphatized fossils lack any euhedral crystals, at any magnification (e.g., Fig. 6 and SI Appendix, Fig. S7) exhibiting only irregularly shaped nanoparticulate texture.

Since the internal mold in SI Appendix, Fig. S8D and the outer layer akin to a periostracum in SI Appendix, Fig. S8E both appear nanoparticulate in cross-section, we speculate that both minerals precipitated in the presence of abundant organic molecules, from the decaying halkieriid or its periostracum, and this made them precipitate as nanoparticles. Since these nanoparticles were not deposited by the animal as ACC, they differ morphologically: they are globular where the periostracum was, and euhedral hexagonal prisms in the internal mold. This tentative interpretation is also supported by the observation that most of the recrystallized fossils and their internal molds shown here are euhedral nanoparticulate (halkieriid in SI Appendix, Fig. S8A–C; nacre in SI Appendix, Fig. S9A; and chancelloriid in SI Appendix, Fig. S9B). In these cases, the presence of organics may have madeapatite crystals nanoscaled, but more regularly shaped: either euhedral or spherical.

**Discussion**

Figs. 2–6 demonstrate that the nanoparticulate fabric associated with CPA of ACC particles in modern calcifying animals extends back to the earliest record of skeletal biomineralization in Ediacaran and Cambrian fossils. Ion exchange, which substitutes one ion at a time, has been extensively characterized at the macroscale (37), and more recently at the nano- (38–40) and microscales (41). Both nano- and microscale structures (41) retain...
Stylophora pistillata and Cloudina and from Bodega Marine Laboratory, Bodega Desmoceras and | skeletons at the inception of biomineralization and PNAS Latest Articles (42). The nanoparticles observed here in all biominerals, μD Cambrian phosphatized small shelly fossils after fracturing. Fig. S2.

crystallization kinetics suggest that ACC confined to a volume of 0.3 μm³ is stable, not crystallizing to calcite on timescales of up to 10⁶ y (42). The nanoparticles observed here in all biominerals, ranging in size between 50 and 400 nm, occupy volumes 0.0005 to 0.3 μm³; thus, they should be comparably stable, assuming that the nanoparticles were originally ACC and were confined to similar volumes within cells. This suggests that the ACC precursor phase was selected multiple times in the history of biomineralization, at least in part because of its stability when confined and its capacity to crystallize once released from confinement. For a biomineralizing animal, crystallization to the wrong polymorph, or at the wrong time or location would be detrimental; thus, a stable precursor phase is necessary for biomineralization. Also, stabilization by confinement, rather than by proteins, is fully reversible, providing additional benefits to biomineralizing animals. As yet, there is no consensus on why animal biomineralization proceeds by attachment of amorphous particles, but the kinetic advantage of confinement provides one promising hypothesis; other, potentially complementary, avenues to explore include both growth rate and fine control on morphogenesis.

Materials and Methods

Modern Bimineral and Fossil Samples. In order of appearance in the main text, all samples are as follows: Fig. 2A and SI Appendix, Fig. S1A: Modern red abalone (Haliotis rufescens) from Monterey Abalone Company, Monterey, CA, cryofractured in liquid nitrogen (LN₂), thermalized in ethanol, air dried, and Pt-coated.

Fig. 28 and SI Appendix, Fig. S1B: Modern red abalone (Haliotis rufescens) from Monterey Abalone Company, Monterey, CA, 100 mm in length, was notched, cryofractured in LN₂, thermalized in ethanol, bleached for 1 h in 6% NaClO, rinsed twice in DD H₂O at pH 8, etched for 2 s in 1% acetic acid, rinsed twice in DD H₂O at pH 8, rinsed in ethanol, air dried, and Pt-coated.

Fig. 2C and SI Appendix, Fig. S1C: Modern Nautilus pompilius shell (141.8 mm length) originated off the coast of Jolo Island, Philippines. It was notched, cryofractured in LN₂, thermalized in ethanol, bleached for 1 h in 6% NaClO, rinsed twice in DD H₂O at pH 8, etched for 2 s in 1% acetic acid, rinsed twice in DD H₂O at pH 8, rinsed in ethanol, air dried, and Pt-coated.

Fig. 2D and SI Appendix, Fig. S1D: Miocene (~13 Ma) nacre from Atrina harrisii, Choptank Fm, Calvert, MD.

Fig. 2E and SI Appendix, Fig. S1E: Cryofractured modern coral skeleton from Haliotis rubra, Cryofractured modern coral skeleton from Strongylocentrotus purpuratus, from Sodega Marine Laboratory, Bodega Bay, CA. The spine was notched, cryofractured in LN₂, thermalized in ethanol, bleached for 1 h in 6% NaClO, rinsed twice in DD H₂O at pH 8, etched for 1 s in 1% acetic acid, rinsed twice in DD H₂O at pH 8, rinsed in ethanol, air dried, and Pt-coated.

Fig. 2F and SI Appendix, Fig. S1F: Upper Ordovician (~450 Ma) nacre from Isothecoceras sociale from Maquoketa Formation, Graf, IA. This nacre is completely phosphatized, and extracted by maceration in 5 to 10% acetic acid for a few days, thus any residual aragonite was removed.

Fig. 3A and SI Appendix, Fig. S2A: Modern sea urchin spine from Strongylocentrotus purpuratus, from Sodega Marine Laboratory, Bodega Bay, CA. The spine was notched, cryofractured in LN₂, thermalized in ethanol, bleached for 1 h in 6% NaClO, rinsed twice in DD H₂O at pH 8, etched for 10 min in 1% acetic acid, rinsed twice in DD H₂O at pH 8, rinsed in ethanol, air dried, and Pt-coated.

Fig. 3B and SI Appendix, Fig. S2B: Cryofractured nacre from Strongylocentrotus purpuratus, from Sodega Marine Laboratory, Bodega Bay, CA. The spine was notched, cryofractured in LN₂, thermalized in ethanol, bleached for 1 h in 6% NaClO, rinsed twice in DD H₂O at pH 8, etched for 10 min in 1% acetic acid, rinsed twice in DD H₂O at pH 8, rinsed in ethanol, air dried, and Pt-coated.

Fig. 5. Irregularly shaped nanoparticles in the fractured walls of phosphatized Ediacaran (~550 Ma) Cloudina skeletons. Lower-magnification, cocentered, colabeled images from these locations are presented in SI Appendix, Fig. S5.

Fig. 6. Cambrian phosphatized small shelly fossils after fracturing. (A and B) Halkierid sclerite. (C and D) Hyolith conch. All 4 cross-sections show irregularly shaped, noneuhedral nanoparticles. Lower-magnification, cocentered, colabeled images from these locations and others are presented in SI Appendix, Fig. S7.
All fossil biominerals were fractured at room temperature (RT), and only cross-sections fractured by us were analyzed, to avoid secondary overgrowth or contaminants that precipitated or were deposited on the shell surfaces during geologic time or during sample preparation.

All geologic minerals and modern biominerals were cryofractured to minimize cleavage and prevent smearing. To cryofracture them at predictable locations, and at the desired size compatible with SEM mounting, all samples were notched on 3 sides using a diamond saw, with water as a lubricant and coolant. They were then rinsed in DD water, then ethanol, and air-dried overnight. They were then immersed in liquid nitrogen, let thermalize, and fractured with a microscalpel at the notches, to reveal a cryofractured surface. They were then immediately removed from liquid nitrogen and im- mersed in DD-H2O at pH 8.0. All samples were cryofractured as described above, bleached for 1 h in 6% NaOCl, rinsed twice in DD-H2O at pH 8.0. The Haliotis rufescens, Nautilus pompilus, and ammonite Desmoceras sp. and Miocene Atrina harrisii, and for coral skeletons, as well as nonbiogenic aragonite. Etching was done for 1 to 20 min with Strongylocentrotus purpuratus spines. Before etching, these samples were cryofractured as described above, bleached for 1 h in 6% NaOCl, rinsed twice in DD-H2O at pH 8.0. The Haliotis rufescens, Nautilus pompilus, and ammonite Desmoceras sp. samples in Figs. 2 B, C, and E were then etched for 2 s. The Strongylocentrotus purpuratus spine sample selected for Fig. 3A was etched for 10 min. The coral sample is presented unetched, as its nanoparticles are clearly visible even before etching.

All samples were magnetron sputter-coated with 20 nm Pt while rotating and tilting the sample, using a Cressington 208 HR sputter coater (Ted Pella). This thicker than normal coating, combined with tilting and rotation during coating greatly reduced charging artifacts, which otherwise would have made the experiments impossible in fractured samples with great topographic relief as those analyzed here. We note that such thick coating could in principle hide nanoparticles smaller than 20 nm, but such particles, when they protrude on the surface of fractured biominerals, are still clearly visible in Figs. 2, 3, 5, and 6.

All samples were analyzed using a Hitachi S5000 SEM at the Electron Microscopy Laboratory at University of California, Berkeley, CA. The microscope was operated at 10 kV, in secondary electron mode. All samples were focused at 450,000×, then imaged at 100,000×, 50,000×, 10,000×, 1,000×, and 250×.

The images in SI Appendix, Fig. 54 E, H, and I were acquired using a Hitachi TM1000 SEM, also at EML. This instrument operates at 15 kV, in back-scattered electron mode. All figures were assembled in Adobe Photoshop CC 2017. The only image manipulation done was “auto-level” in Photoshop, to adjust brightness and contrast. Arrows and labels were also added in Photoshop.

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