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Colloid assembly and transformation (CAT): The relationship of PILP to biomineralization

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ABSTRACT

The field of biomineralization has undergone a revolution in the past 25 years, which paralleled the discovery by Gower of a polymer-induced liquid-precursor (PILP) mineralization process. She proposed this *in vitro* model system might be useful for studying the role biopolymers play in biomineralization; however, the ramifications of this pivotal discovery were slow to be recognized. This was presumably because it utilized simple polypeptide additives, and at that time it was not recognized that the charged proteins intimately associated with biominerals are often intrinsically disordered proteins (IDPs). Over the years, many enigmatic biomineral features have been emulated with this model system, too many to be mere coincidence. Yet the PILP system continues to be underacknowledged, probably because of its namesake, which indicates a "liquid precursor", while we now know the phase appears to have viscoelastic character. Another factor is the confusing semantics that arose from the discovery of multiple "non-classical crystallization" pathways. This review suggests a more relevant terminology for the polymer-modulated reactions is "colloid assembly and transformation (CAT)", which we believe more accurately captures the key stages involved in both biomineralization and the PILP process. The PILP model system has helped to decipher the key role that biopolymers, namely the IDPs, play in modulating biomineralization processes, which was not readily accomplished in living biological systems. Some remaining challenges in understanding the organic–inorganic interactions involved in biomineralization are discussed, which further highlight how the PILP model system may prove invaluable for studying the simple, yet complex, CAT crystallization pathway.

1. Introduction

A polymer-induced liquid-precursor (PILP) mineralization process was discovered by Gower ~25 years ago (Gower and Odom, 2000). However, this PILP discovery, even though it was distinctly different from the classical crystallization processes that were summarized at the beginning of every biomineral review, went largely unnoticed until the Gower group gradually demonstrated that this PILP model system could emulate many of the enigmatic features of biominerals that pervaded the 1990s literature (Gower, 2008; Amos et al., 2006). Starting with non-equilibrium morphologies, the hallmark of invertebrate biominerals, to interpenetrating nanostructured composites (Fig. 1); but perhaps even more revealing is the similar defect textures (Fig. 2), because 'mineralogical signatures' point to crystallization mechanisms, which we now know follow a non-classical pathway (De Yoreo et al., 2015). Yet even with this ability to emulate so many features, which surely could not be coincidence, the community still rarely refers to biomineralization as occurring through a PILP-like process. The reason for this, we believe, is because the PILP namesake includes the word "liquid", yet it has become clear in recent years that the amorphous precursor phase of biominerals is not a pure liquid phase given that biominerals ubiquitously have a remnant colloidal or nanogranular texture (Fig. 2B) (De Yoreo et al., 2015). On the other hand, the biomineral precursors are presumably not solid particles given their complete space-filling properties. A recent paper suggests that solid particles attach and then the remaining space is filled in by ion-by-ion growth (Sun et al., 2020). This seems unlikely given that one would expect diffusion-based filling in to occur from outside-inward, which would soon block entry and leave large amounts of porosity in the interior. While entrapped pores are occasionally seen, most biominerals are fully densified. Therefore, this paper is intended to clarify where we believe such incongruities arise, and to propose a description that more adequately captures what is occurring in biomineralization, as derived from our experiences with the PILP model system.

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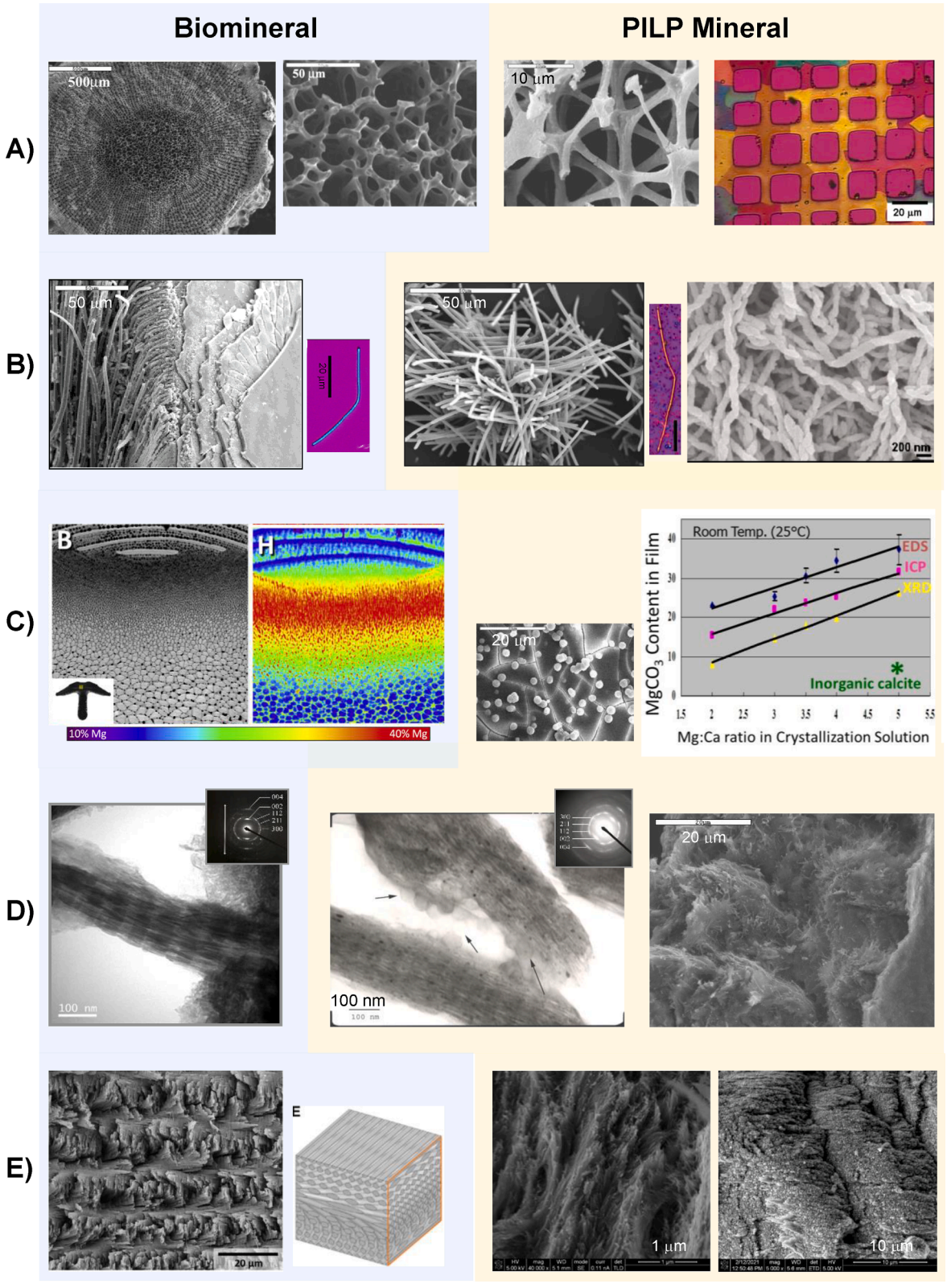

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2. Consistency of precursor phase

One might ask, what is wrong with the terminology that has come into play in recent years, namely "particle attachment" (De Yoreo et al., 2015). Firstly, we have noticed that people seem to interchangeably refer to "particle attachment" as "oriented attachment", and they are not at all the same. Oriented attachment is just one of several particle attachment scenarios, and for ACC precursors, it's not even relevant as

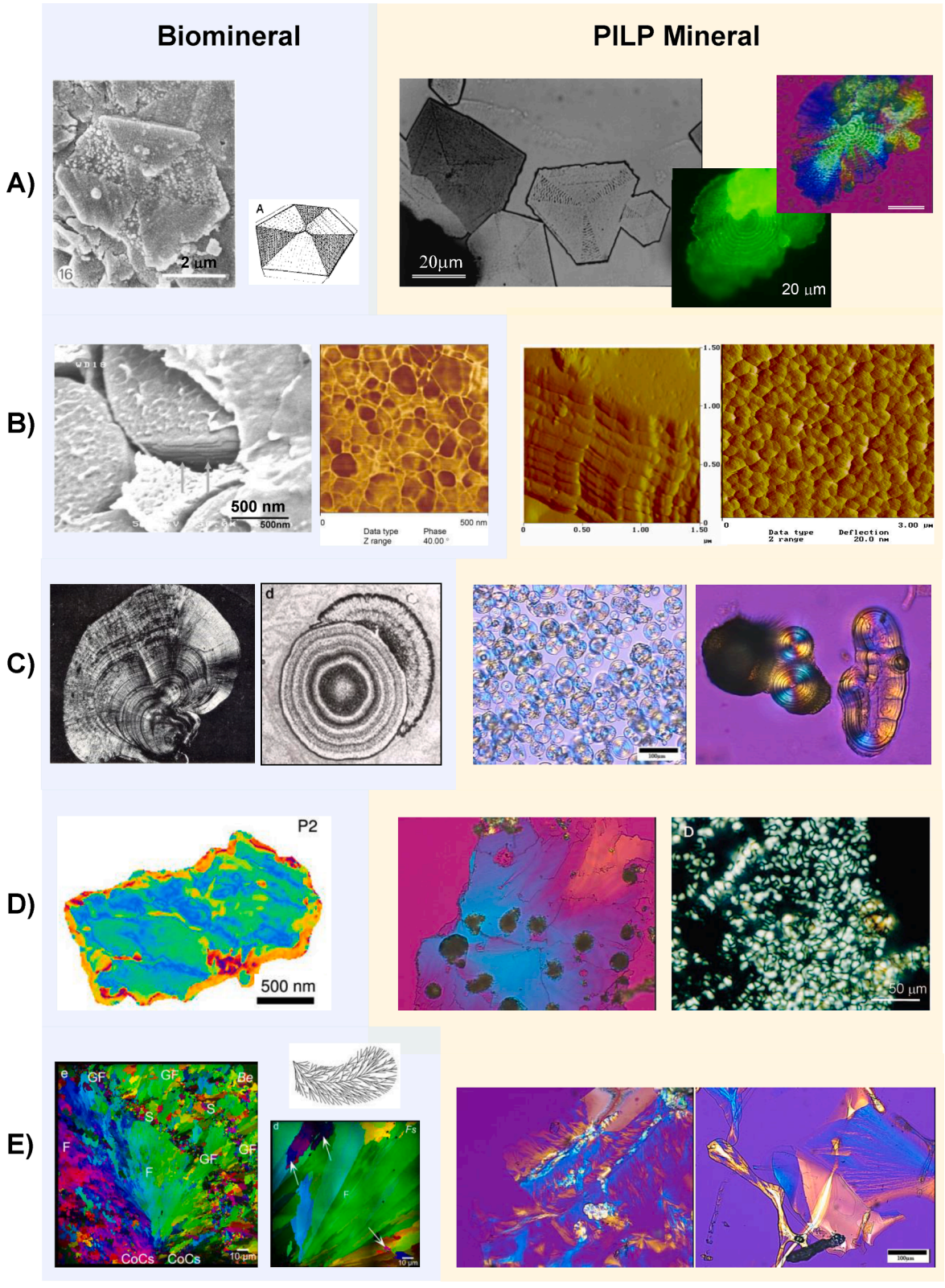
Fig. 1. Comparison of various morphological and microstructural features of biominerals (blue background) versus PILP minerals (yellow background). **(A)** (Left) Complex non-equilibrium morphologies are the hallmark of invertebrate biominerals, with a classic example being the convoluted morphology of sea urchin spines. These types of biominerals are usually "molded" by deposition of amorphous phase in a confined compartment such as a mineral deposition vesicle or a multicellular syncytium. (Right) To demonstrate 'molding' of crystals via the PILP process, an inverse replica technique was used to replicate the convoluted morphology of the interior portion of the urchin spine. In 2D, a PILP film was 'molded' by templated deposition of droplets onto patterned SAMs (polarized light micrograph using gypsum λ-plate). **(B)** Another non-equilibrium morphology is the rods of calcite found in urchin teeth. In the PILP system, rods of calcite were found to serendipitously grow off rhomb seed crystals. Although the PILP fibers are not organized as they are in the biomineral, both biogenic and PILP fibers retain single-crystalline orientation across a bend in the fiber, suggesting the fiber morphology was initially ACP prior to a pseudomorphic amorphous-to-crystalline transformation. On the far right, squiggly $SrCO_3$ fibers (as well as $BaCO_3$ not shown) can be produced with PAA additive. **(C)** High-magnesium calcite is found in some biominerals (up to 40 mol% Mg), which is illustrated here with Raman mapping of an urchin tooth. Note- inorganically grown calcite only incorporates about 8% Mg before converting to the aragonite polymorph. Addition of Mg-ion enhances PILP formation, as seen in this thick film with 'mud-cracks'. High magnesium content is entrapped in these PILP-formed calcite films, but the lower levels measured by XRD relative to ICP and EDS suggest some gets excluded to grain boundaries. **(D)** Some biominerals limit the reaction space with a preformed, dense organic matrix. In bone, the collagen matrix is mineralized with both intrafibrillar and interfibrillar hydroxyapatite nanocrystals which are [0 0 1] aligned parallel to the collagen fibrils. The PILP system was the first ever to reproduce this intrafibrillar mineralization. Once the fibrils are infiltrated with amorphous phase, the A-to-C transformation naturally leads to the [0 0 1] crystallographic orientation, which led to the hypothesis that the crystal orientation is simply governed by crystal growth in confinement (and not epitaxial relationships between mineral and organics). This system also enabled very high levels of mineral incorporation to be achieved, matching that of bone (65 wt%), and even dense organized matrices can be mineralized to a couple hundred microns in depth. On the right, a "molecular crowding" technique was used to create the twisted cholesteric-like structure found in lamellar bone, from which the collagen fibrils could then template the shifting orientation of HAp that forms within the fibrils. **(E)** In invertebrates, such as crustaceans, the organic matrix is chitin, which becomes mineralized with calcite (and some ACC and CaP). Chitin also assembles into the interesting cholesteric-like microstructures, but at a larger scale, as represented by the schematic. Many groups, including ours, have then mineralized such reconstituted matrices using polymer additive to promote infiltration into these dense chitinous matrices. 1A - Left – Reprinted with permission from Wiley (Cheng and Gower, 2006) Copyright © 2006 John Wiley & Sons, Ltd. Right - Reprinted from ref. 8, Copyright 2007, with permission from the American Chemical Society. 1B – Left - Reprinted with permission from ACS (Olszta et al., 2004) Copyright 2004 American Chemical Society. Far Right - Reprinted with permission from ACS (Homeijer et al., 2021) Copyright 2010 American Chemical Society. 1C – Left - Reprinted from (Masic and Weaver, 2015) Copyright 2015, with permission from Elsevier. Right - Reprinted from (Cheng et al., 2007) Copyright 2015, with permission from Elsevier. 1D – Left - Reprinted from (Olszta et al., 2007) Copyright 2007, with permission from Elsevier. Right - Reprinted from (Wingender et al., 2016) Copyright 2007, with permission from Elsevier. 1E - Reprinted from (Grunenfelder et al., 2014) Copyright 2014, with permission from Elsevier. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

there is no crystallographic order that would require orienting to match with neighboring particles. Secondly, the word "particles" implies a solid phase, which does not appear to be the case. In the PILP model system, as well as the particle accretion process in biominerals, whose mineralogical signatures are equivalent (Fig. 2), the densified textures suggest the accumulating precursor phase probably has a ***viscoelastic*** consistency at the time it is being molded. In other words, a viscous consistency that can flow over time could provide the gradual coalescence that leads to densified, space filling properties, but while also leaving a remnant colloidal texture from the particles/droplets being coated with polymer. Recent cryo-TEM studies of the PILP phase show what appears to be nanogranular fluid (Xu et al., 2018), where the non-homogeneous phase was described as consisting of ~2 nm-sized ACC clusters (Fig. 3A). Other groups have considered these subunits to be pre-nucleation clusters (PNCs), a subject of extensive debate (Gebauer et al., 2014). Regardless of the terminology, one could envision that a "fluid" comprised of PNC nanogranules intercalated with polymer could explain the unusual flow behavior of the PILP phase, such as why streams of PILP phase only slowly coalesce over time (Fig. 3B&C). Likewise, a viscoelastic consistency could explain the gradual densification of "particles" that attach during biomineralization. Even the original PILP papers described the liquid-like character of the PILP phase as being short-lived (Gower and Odom, 2000; Kim et al., 2007), where in minutes it densified to where droplets only partially coalesced, and deposited films became solids with various consistencies before ultimately undergoing the amorphous-to-crystalline transformation (Fig. 3 D-I). These observations suggest the viscoelastic tendency of the phase arises from some type of non-covalent crosslinking. Reversible crosslinking could be ion-based, such as calcium-mediated crosslinks with the intercalated polymer chains; or possibly even a hydrogen-bonding network could be created in these densified phases from interactions between polymer and hydrogenated carbonates (or phosphates in the calcium phosphate system).

3. Biomineral textures

In both the PILP system and biominerals, there is a remnant nanoscale texture created by the accretion of precursor colloids (Fig. 2B) (Gower, 2008; De Yoreo et al., 2015; Kim et al., 2007; Sethmann et al., 2005; Gal et al., 2014; Gilbert et al., 2019). This nanogranular texture would be expected to be enhanced by the exclusion of the polymeric impurities during crystallization, which indeed has been shown to be the case in the nanogranules in mollusk nacre (Fig. 2B) (Rousseau et al., 2005). Not only should one expect polymer be enriched at the surface of the colloids, given that it is involved in creating/stabilizing the phase, one would also expect polymer (and Mg and other impurities) to be excluded during crystallization. Indeed, when the PILP precursor undergoes the amorphous-to-crystalline transformation, exclusion becomes spatially limited, leading to "transition bars' in the PILP forming tablets (Gower and Odom, 2000; Dai et al., 2008), which coincidentally match the etching patterns seen in nacre (Fig. 2A) (Weedon and Taylor, 1995). This leads to occlusion of organics to form mesocrystals (i.e. mesoscopically structured crystal composed of numerous crystallographically aligned nanocrystals that are spatially separated), or polymer might be excluded to "grain" boundaries (e.g. edges of nacre tablets and interlamellar sheets) (Fig. 2A-C), *etc,* leading to 'fuzzy' interfaces with interesting mechanical properties.

Layering is also ubiquitous in pathological deposits, such as the concentric laminations in kidney stones and Randall's plaques (Evan et al., 2006) (Fig. 2C). *Ex vivo* observations of biominerals have often led to the conclusion that organics at phase boundaries were indicative of their function as crystal growth modifiers, or that they provided a preformed compartment, or they were daily growth rings, etc., whereas in reality, many occluded organics might simply be excluded impurities (Gower et al., 2010; Amos et al., 2009). Nearly all biominerals have mesoscale layering (Cuif et al., 2012). This is not only important with respect to mineralogical 'signatures' of formation mechanism but is also of great interest toward understanding biomineral's remarkable mechanical properties. Much literature has focused on the enhanced



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fracture toughness that such layering provides, but the layering is usually assumed to result from cellular control. Evolutionary selection would include both mesoscale textures created by this formation mechanism as well as deliberate cell-controlled microstructures.

Gower's *Chem. Review* paper in 2008 provided an extensive discussion on these features (Gower, 2008). Notably, that paper is 13 years old, and yet as the field continues to employ more advanced characterization tools, the findings always parallel what has already been observed with the PILP model system.

Fig. 2. Crystalline defect textures in biominerals and PILP products suggest analogous formation mechanisms. **(A)** (Left) The thin tablet morphology, a few hundred nanometers thick, of both aragonite nacre and calcitic seminacre (shown here); although being different mineral phases, both exhibit a similar defect texture consisting of alternating sectors prone to preferential etching. Even linear striations can be seen in the non-etched regions that match transition bars. (Right) The PILP tablets contain wavy transition bars in alternate sectors, thought to be created by shrinkage stresses, and thus would be expected to correlate with the higher solubility seen in etched sectors of nacreous tablets. Fluorescently-tagged polymer shows transition bars are created by exclusion of polymeric impurity, which in this case occurred during spherulitic growth of PILP film (right image via crossed polars with gypsum λ-plate). **(B)** (Left) SEM of a nacre tablet shows nanolayering within the single-crystalline tablet. (Left Middle) Nacreous tablets (and nearly all biominerals) exhibit a remnant colloidal nanotexture created by their formation mechanism of "particle attachment". This phase contrast AFM image demonstrates occluded polymer at the periphery of the colloids. (Right) Nanolayering and a remnant colloidal nanotexture can be seen in a PILP film grown under a Langmuir monolayer. **(C)** (Left) Layered microstructures are universally seen in most biominerals, including pathological (non-biologically controlled) biominerals, such as the CaOx kidney stone shown here. (Left Middle) Even at the nanoscale, concentric layers are seen in the micron-sized CaP spherules of Randall's plaque (x 70,000), the precursor to idiopathic CaOx stones. (Right middle) In PILP formed spherules of CaP, concentric layers are seen in the early stages, such as these not-fully-densified spherules. (Far Right) Upon densification, such large (~50 μm) spherules become dark brown, whether they undergo dissolution–recrystallization (seen as needles of HAp on the left) or pseudomorphic transformation to form solid spherulites. **(D)** (Left) Ptychography PIC map of a single crystal of aragonite within a coral skeleton showing "orientational diversity" with narrow angular spread. (Right Middle) PILP formed tablets and films often exhibit a gradual shifting of crystallographic orientation, as seen in this in PILP-formed $SrCO_3$ film; although it seems to have one nucleation event, the "single crystalline" patch shows a gradual shifting of retardation color (under cross-polars with gypsum wave-plate) as it experiences greater lattice strain the further it traverses across the constrained film. (Far Right) Even melded droplets of PILP show unusual shrinkage strains, such as the shifting line that resembles disclination defects in liquid crystals. The viscoelastic nature of the precursor phase often leads to mineralogical signatures that resemble liquid crystalline or 'molten' defect textures. **(E)** (Left) PIC map of coral skeleton showing "plumose" spherulitic texture of crystals radiating from linear center of calcification, or to the right, a classic spherulitic texture of neighboring crystals. (Right Middle) Crystallographic "splay" is seen in PILP films where nucleation initiates in the thicker centers of streams of accumulated PILP phase. (Far Right) Classic spherulitic textures are common in PILP films, which in this case is neighboring some single-crystalline patches. 2A – Left- Republished with permission of University of Chicago Press - Journals, from (Weedon and Taylor, 1995); permission conveyed through Copyright Clearance Center, Inc. Middle - Reprinted with permission from ACS (Dai et al., 2008) Copyright 2008 American Chemical Society. Right - Reprinted with permission from ACS (Gower, 2008) Copyright 2008 American Chemical Society. 2B – Left - Reprinted from (Rousseau et al., 2005) Copyright 2005, with permission from Elsevier. Left Middle - Reprinted with permission from ACS (Gower, 2008) Copyright 2008 American Chemical Society. Right - Reprinted with permission from ACS (Kim et al., 2007) Copyright 2007 American Chemical Society. 2C – Left - Reprinted with permission from Wiley (Amos et al., 2006) Copyright © 2006 John Wiley & Sons, Ltd. Left Middle - Reprinted with permission from ACS (Gower, 2008) Copyright 2008 American Chemical Society. Far Right - Reprinted from (Evan et al., 2006) Copyright 2006, with permission from Elsevier 2D – Left - Reprinted with permission from National Academy of Sciences (Lo et al., 2021) Copyright 2021. Far Right - Reprinted from (Gower and Odom, 2000) Copyright 2007, with permission from Elsevier. 2E – Left - Reprinted with permission from ACS (Sun et al., 2021) Copyright 2021. Right middle - Reprinted with permission from ACS (Gower, 2008) Copyright 2008 American Chemical Society. Far Right - Reprinted with permission from Wiley (Amos et al., 2006) Copyright © 2006 John Wiley & Sons, Ltd.

4. Colloid assembly and transformation (CAT)

A second issue related to semantics is based on the highly cited ***Science*** paper on "Crystallization by particle attachment" (De Yoreo et al., 2015). Although their schematic nicely illustrates the variety of non-classical species present in the reaction media, it does not adequately address what can be accomplished by a liquid phase precursor, at least if the precursor is stabilized by polymer, as in the PILP process (Fig. 4) (Gower and Odom, 2000; Gower and Tirrell, 1998). The original diagram shows all the pathways ending up at the same euhedral crystal, but the hallmark of biominerals is their non-equilibrium (non-faceted) morphologies. Thus, we argue that the polymer is critical, not only for sequestering the precursor colloids, but also in stabilizing the molded phase to undergo a pseudomorphic transformation, which is vital for creating the unique non-equilibrium morphologies. One could call it a Polymer-Induced Liquid-Gel-Solid-Pseudomorphic Transformation Non-Classical Crystallization (PI-LGS-PT-NCC) Process, but this seems a bit cumbersome.  Therefore, Wolf and Gower (Wolf et al., 2017) came up with a simple representative terminology– Colloid Assembly and Transformation (CAT)– which we believe captures the most pertinent aspects of biomineralization, without the various confounding semantics issues described above. Namely, Colloid defines size rather than consistency, avoiding the term 'particle', which implies a solid. And the remainder- Assembly and Transformation- puts the focus on how such non-equilibrium morphologies are generated, as illustrated in Fig. 5.

5. Intrinsically disordered proteins (IDPs) as process-directing agents

It has become clear over the years that one reason the PILP model system didn't gather the attention it deserved was because it was an exceedingly simple *in vitro* model system that used polymer additives that were not real proteins. Back in the 1990s, one simply did not expect a repetitive polypeptide such as polyaspartic acid to be able to mimic the functionality of a real protein. At that time, people considered proteins to be fibrous or globular, whose form and function were based on the classic "lock-n-key" mechanism of molecular recognition. Therefore, the biomineralization hypotheses were focused on demonstrating a high degree of specificity between the functional groups on a protein surface that might have an epitaxial or stereospecific relationship with the crystal lattice (Addadi et al., 1989). But it is now recognized that many proteins, especially those that are highly charged (as are the biomineral proteins!), are intrinsically disordered proteins (IDPs) (Wojtas et al., 2012). Thus, they take the form of a random coil polyelectrolyte, as does polyaspartic acid, etc. One can now see why this overly simple model system was capable of emulating many of the morphological features of biominerals. That's not to say that IDPs do not have domains with specific interactions with ion clusters or crystal surfaces, and certainly they do with cell integrins, but the role IDPs play in morphological control of biominerals clearly falls in the realm of materials science – polyelectrolytes sequester ion clusters that phase separate in solution. Thus, from this perspective, the role of these charged biopolymers, namely the IDPs, seems to be as a process-directing agent (as Gower proposed years ago) (Gower and Odom, 2000; Thula et al., 2011), in a process that the community now defines as a non-classical crystallization process. But with the growing number of non-classical pathways suggested in that Science paper (De Yoreo et al., 2015), we propose the CAT mechanism may a better terminology for describing the key role such IDP biopolymers play in biomineralization, as illustrated in Fig. 5.

6. Paleontology simplified

Was it any surprise that Gilbert's group found evidence of "particle attachment" across many phyla in the paleontological record (Gilbert et al., 2019)? As Gower suggested some years ago (Gower and Odom, 2000; Gower and Tirrell, 1998), there's a reason that nacre in mollusks and semi-nacre in bryozoan, which are in organisms that evolved down very different evolutionary paths, both exhibit a similar thin tablet morphology (Gower, 2008). That same morphology was readily reproduced with the PILP process, and such a thickness was simply a result of

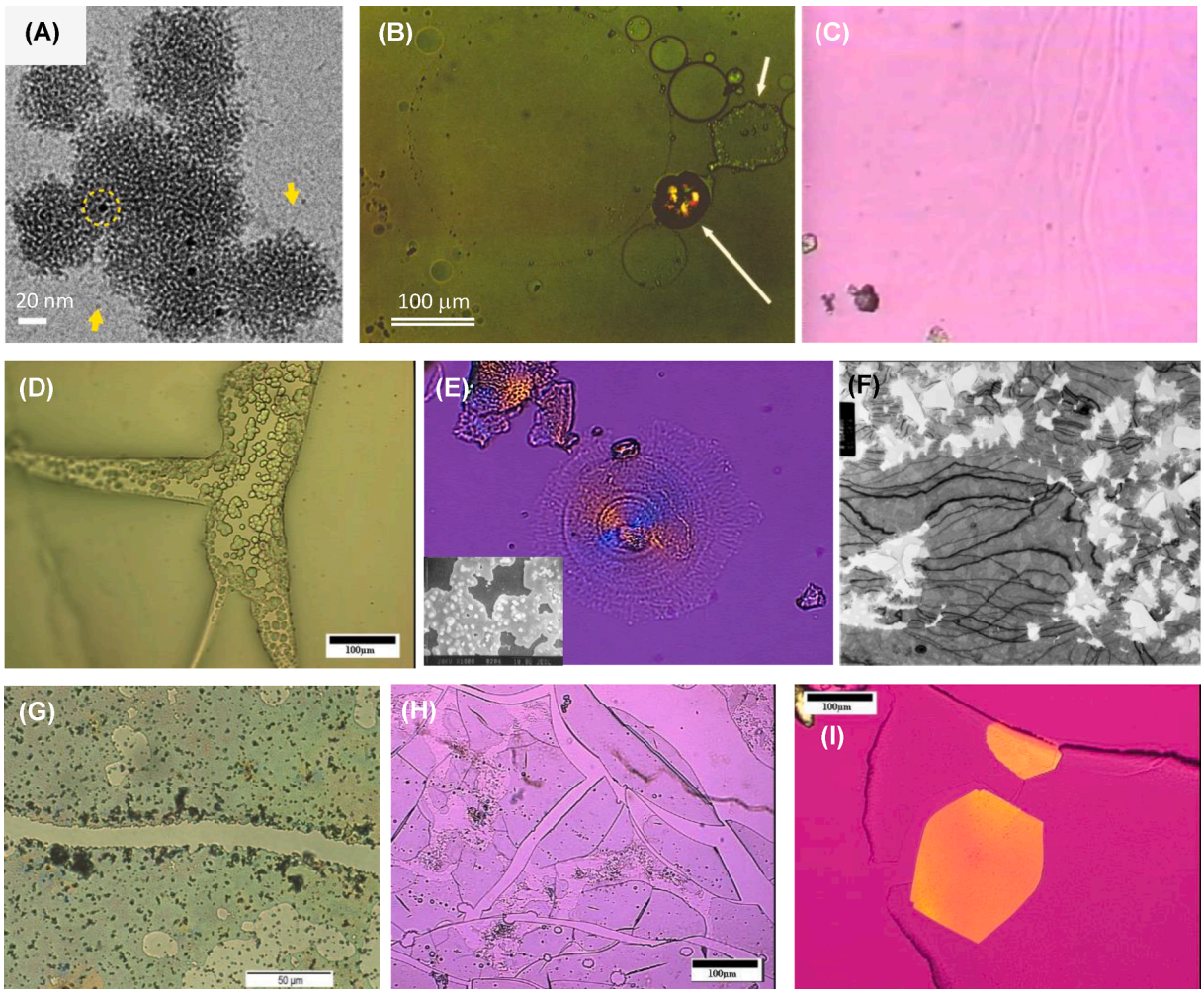


Fig. 3. Consistency and textures of PILP precursor phase(s). **(A)** Cryo-TEM study of the PILP system found the coalescing PILP droplets have a nanogranular subunit texture, presumably comprised of ~ 2 nm prenucleation clusters. **(B)** The submicron PILP droplets accumulated into a macroscale drop under an air bubble. However, some drops did not immediately coalesce into one another or the macrodrop. **(C)** This is a snapshot of a video clip which showed that when the bubble was perturbed, the PILP phase flowed like a slightly viscous liquid, but interestingly, there were neighboring streams of phase that did not readily coalesce into a singular phase. **(D)** This thick accumulation of PILP droplets under a Langmuir monolayer appeared to have a foamy texture. Some partially melded droplets are seen in between the thick foam. **(E)** The calcium phosphate spherules described in Fig. 2 C were not dense and appeared to be like a hydrogel (one would expect 100 µm solid particles to be dark brown under an optical microscope). This one weakly birefringent (partially crystalline) spherule collapsed onto the substrate into a film-like gel. The debris to the top left appears more solidified, but still not glassy or rigid. The SEM in the inset is what led to the original discovery that the mineral films were comprised of fused PILP droplets. **(F)** TEM of a single-crystalline patch of PILP film shows that there was an underlayer that does not have the Bragg contours, and was apparently not yet crystallized. The stretched appearance of the underlayer is suggestive of a viscoelastic nature. Other scenarios, such as extrusion of fibers from globules, and films dewetting into disconnected crystals, etc., also seem suggestive of some type of partial elasticity of the precursor phase. **(G)** A needle scratch through the freshly deposited amorphous PILP film shows a sandy granular texture of the pile-up debris. When zooming in on the image, one can see a remnant colloidal texture within the smooth film because the scattering was enhanced by narrowing the field-stop aperture. Such a scratch often nucleates the amorphous-to-crystalline transformation, leading to something like the splay seen in Fig. 2E. **(H)** This amorphous PILP film deposited under a Langmuir monolayer was initially smooth and continuous, but exhibited a brittle glassy texture when it was scooped onto a glass slide. **(I)** A single-crystalline aragonite tablet undergoing a pseudomorphic transformation within an amorphous PILP film that was formed under a Langmuir monolayer. Note the slight shift in crystallographic orientation (lighter orange retardation color) toward the periphery of the forming tablet, which was apparently caused by strain created by dehydration shrinkage during the A-to-C transformation. Some have argued that such a transformation is not truly pseudomorphic since there is a small loss in volume from water loss, but it clearly differs from dissolution-reprecipitation, so perhaps should be called a pseudo-solid-state transformation. 3A - Reprinted from (Xu et al., 2018) Copyright 2018, with permission from Springer. 3B - Reprinted from (Gower and Odom, 2000) Copyright 2000, with permission from Elsevier. 3D - Reprinted with permission from Wiley (Amos et al., 2006) Copyright © 2006 John Wiley & Sons, Ltd. 3E - Reprinted with permission from Wiley (Amos et al., 2006) Copyright © 2006 John Wiley & Sons, Ltd. Inset - Reprinted from (Gower and Odom, 2000) Copyright 2007, with permission from Elsevier. 3I - Reprinted with permission from ACS (Amos et al., 2007) Copyright 2007 American Chemical Society. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

a given supersaturation of calcium carbonate that can be sequestered and stabilized by polymer in the media without crashing out in an uncontrolled fashion. Certainly, the first step in the evolution of biominerals had to be in biopolymer sequestration of calcium to avoid cell toxicity, and a more soluble phase provides ease of ion mobility for homeostasis. The IDPs likely evolved from sequestering ions away to

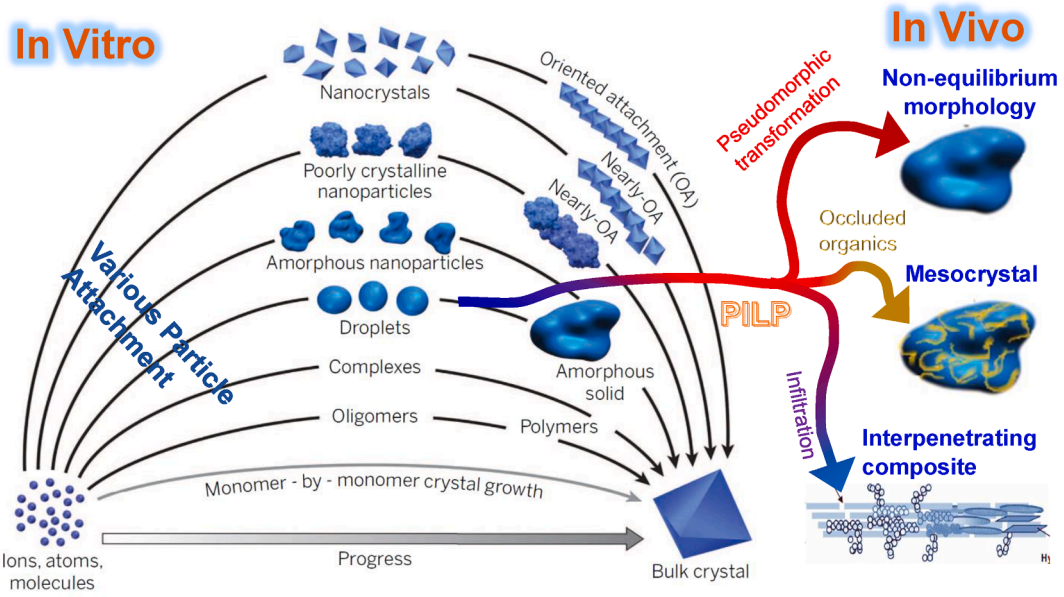


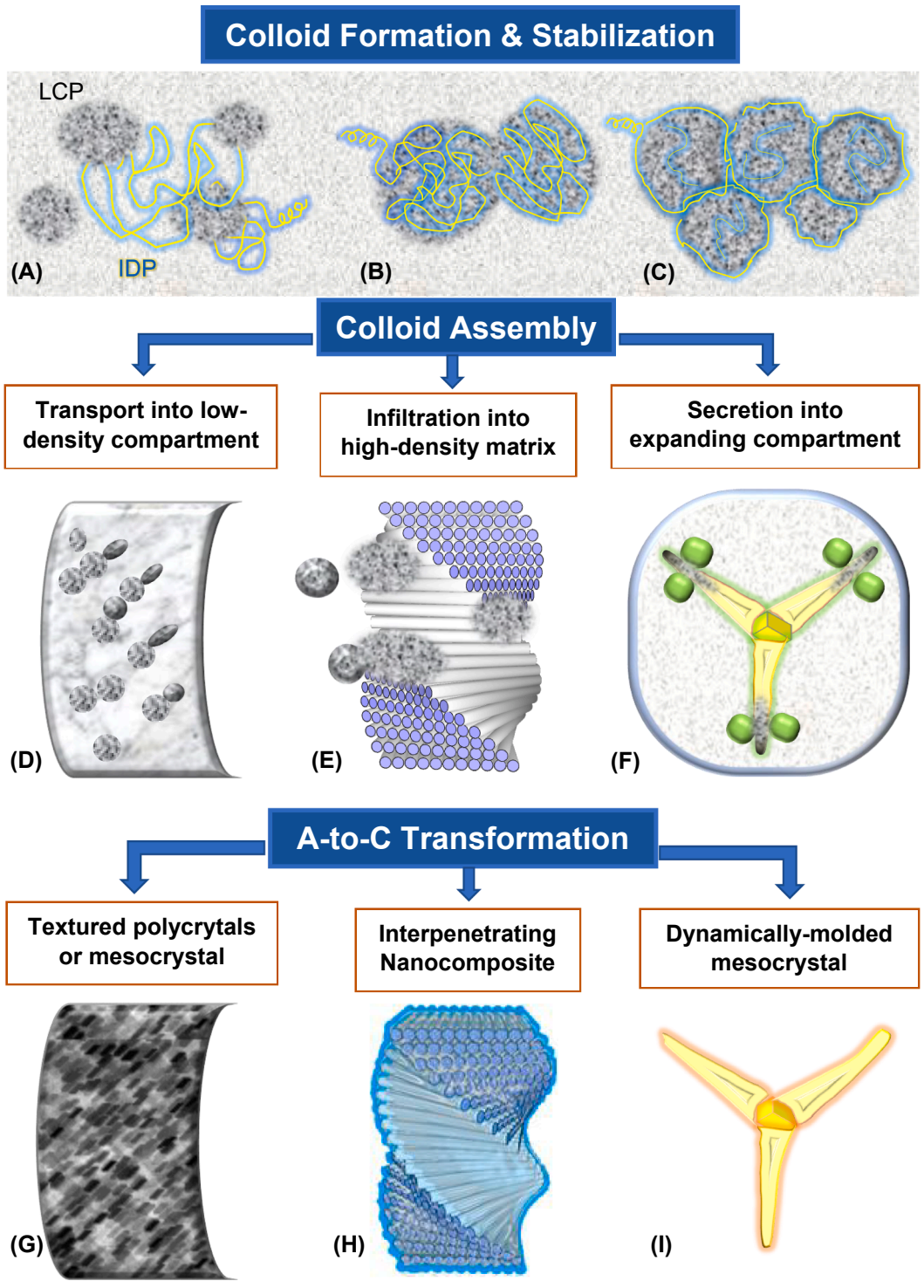
Fig. 4. Non-classical crystallization pathways and significance of CAT to biomineralization. **(Left)** A variety of non-classical crystallization pathways have been resolved in recent years, which firstly demonstrates the power of *in vitro* model systems. The pathway most relevant to biomineralization is arguably the liquid droplet pathway. However, even though a liquid condensed phase exists without polymer, without polymer additive, the result would simply be the euhedral "Bulk crystal" represented as the final product. **(Right)** An adaptation of this popular schematic shows the "Droplets" pathway takes a dramatically different turn when polymer is added, as discovered by the PILP system. The liquid condensed phase is sequestered in larger quantity with polymer, and most importantly, is stabilized long enough to enable the pseudomorphic transformation. This provides a means for delivering the hallmark of biominerals, the molding of species-specific nonequilibrium morphologies. The second hallmark of biominerals is their mesocrystalline texture, which has also been demonstrated with the PILP model system. Occluded organics may be organized along specific crystallographic planes due to the symmetric exclusion of impurities into the transition bars, or it could become entrapped randomly, and especially concentrated at grain boundaries and phase boundaries, such as between temporally deposited secretions of precursor. The third hallmark of biominerals is the interpenetrating composites that are created through infiltration of the precursor phase into organized matrices. Although the mechanism of infiltration remains controversial, it does not occur without polymer additive, and some type of precursor phase is visualized both *in vitro* and *in vivo* (in bone). Given that infiltration would not be expected to occur with solid particles (which are sometimes visualized in cryo-EM as being much too large to fit within the narrow confines of collagen fibrils), there apparently is some 'fluidic' quality to this precursor phase that allows its infiltration into matrices, which Gower's group proposed occurs through capillarity. Adapted reprint from (De Yoreo et al., 2015), with permission from AAAS.

protect cells, to finding evolutionary advantage of using a sequestered phase that naturally deposits in the form of a protective mineral coating/shell or within an intercalating matrix (like chitin, collagen). In the field of paleontology, it is now much easier to sort out the relationships between organisms when one understands that the differences between these strikingly different mineral morphologies is perhaps more based on the organic matrices and substrates which modulate the attachment and organization of the precursor colloids (as opposed to evolution of a complex set of crystal binding proteins).

7. Continuing value of the PILP *in vitro* model system for studying CAT

With the conceptual premise of CAT, the key questions then become, how do the biopolymers collect ions and stabilize the amorphous colloids long enough for them to transform via pseudomorphic transformation, thereby yielding species-specific single crystals with molded non-equilibrium morphologies? This usually occurs in some type of mineral deposition vesicle or syncytium (Beniash et al., 1997). Alternatively, how do the precursor colloids interact with fibrous matrices, collagen in vertebrate bone (Olszta et al., 2007), chitin in invertebrate exoskeletons, to infiltrate into the interstices and yield interpenetrating composites? We know a lot about these self-organizing matrices and compartments, but how they interact with and modulate the assembly of the precursor colloids is really the next stage (in our minds) of solving the biomineralization puzzle.

On the inorganic side, while the PILP process seems like a simple physicochemical process of polyelectrolyte interaction with ion clusters, there are still challenges in capturing and measuring the properties of the PILP phase, particularly as it is a dilute and dynamically changing system, both in composition and consistency (Kim et al., 2007; Dai et al., 2008; Dai et al., 2008). One can imagine that *in vivo*, by the time such a phase is transported to where it is forming the biomineral, it will have likely densified into a viscoelastic liquid or gel (as observed in model systems via in situ AFM (Wolf et al., 2017), and/or these changes in consistency may very well be influenced by its interactions with the substrate or matrix (Kim et al., 2007), as well as the IDP process-directing agent. That PILP macrodrop under the bubble (Fig. 3B) never did crystallize (Gower and Odom, 2000), suggesting the thickness of precursor can be an issue. Confinement is known to stabilize ACC (Stephens et al., 2010), perhaps because impurities (polymer, water) can't be effectively removed; or the proper ratio of counterions can't be achieved; or the bicarbonate/carbonate ratio may not be adequate. Unlike many studies on calcium carbonate, most of the PILP experiments were done at a neutral pH, where bicarbonates are the dominant species. This may contribute to the inhibitory action in preventing classical nucleation, and as mentioned earlier, they might also provide a hydrogen bonding network that creates viscoelastic character of the phase. In other words, there are many more features that need to be studied, and clearly the biological aspects which haven't been addressed here are important with respect to the spatiotemporal secretion of matrices and mineral processing additives, but we believe that a further mechanistic understanding of the physicochemical processes involved in biomineralization can best be accomplished with a clean *in vitro* model system, such as the PILP system, which allows for in situ advanced characterization of CAT processes.



(caption on next page)

CRediT authorship contribution statement

Laurie Gower: Conceptualization, Supervision, Project administration, Funding acquisition, Resources, Methodology, Visualization, Writing – original draft. **Jeremy Elias:** Investigation, Visualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Fig. 5. Schematic representation of the key stages of the 'Colloid Assembly & Transformation' (CAT) pathway. ***Colloid Formation & Stabilization:*** **(A)** The charged, intrinsically disordered proteins (IDPs) sequester liquid-condensed phases (LCP), which are considered to be comprised of prenucleation clusters (speckles). These IDPs are generally inhibitors to classical nucleation, which thereby transform the reaction to a multi-step non-classical crystallization process. There may be ordered domains on the IDPs (represented here as a helical extension), which could provide more specific biofunctionality, such as binding to matrix proteins, cell integrins, and forming crystal faces. **(B)** As the polymer stabilizes the LCP, more phase is generated. The colloids tend to be attracted to others and can coalesce as they accumulate. **(C)** Analogous to the PILP system, the colloids exhibit a viscoelastic nature and fuse together, thereby providing a moldable precursor phase. As the phase crystallizes, polymer and other impurities are excluded, while some become entrapped and occluded during solidification. ***Colloid Assembly:*** The colloidal precursors are assembled into species-specific morphologies within delimited reaction spaces. **(D & E)** This may be within preformed compartments, where the precursor colloids are somehow transported into the compartment where they are often observed to attach to fibrous structures that may be loosely packed within a hydrogel matrix **(D)**, or the precursors may infiltrate into a densely-packed self-organizing matrix **(E)**, such as chitin or collagen, both of which tend to adopt a nematic or cholesteric type of twisting structure created by 'molecular crowding' of concentrated fibers that generates a liquid crystalline phase. **(F)** A very different pathway occurs for some biominerals which are dynamically molded within intracellular 'mineral deposition vesicles' or within a multicellular fused syncytium. Cells and syncytium are represented here as green because they are often fluorescently labeled during *in vivo* analysis. In both intracellular and syncytium compartments, the biomineral is gradually molded within an expanding (not preformed) compartment. Given the very small amount of reaction space between cell membrane and forming mineral element, it seems as though a concentrated precursor phase must be transported across the membrane and slathered onto the forming mineral. In the classic example of the embryonic urchin spicule, a calcite seed crystal (rhomb shape) directs the crystallographic orientation of the newly deposited phase as it undergoes isoepitaxial crystallization, yielding a uniformly birefringent, single crystal under crossed polars (represented here as yellow). The darker regions at the tips represent amorphous regions where the precursors are still being secreted as the compartment is being expanding. ***A-to-C Transformation:*** All three pathways lead to occluded organics to varying degrees, but if the nucleation event is controlled such that the element is single-crystalline, it might be referred to as a mesocrystal (such as mollusk prisms, urchin spicules and spines). **(G)** However, many of the biominerals formed in a preformed matrix are textured polycrystalline, such as in the iron oxides of chiton and limpet teeth, where they may lead to textured crystallographic orientation that is directed by the fibrous matrix. **(H)** This is even more pronounced in the densely-packed matrices of collagen and chitin, whose fibrils template the orientation of hydroxyapatite nanocrystals in bone and dentin, or the chitin-guided orientation of calcium carbonate/phosphate in crustacean cuticles. In these dense matrices, the organic matrix is roughly 50 vol%, which yields interpenetrating nanocomposites with remarkable mechanical properties. **(I)** A pseudomorphic transformation of the dynamically molded element enables the entire crystal to have the same crystallographic orientation across bends and curves as it is molded into its species-defined non-equilibrium morphology. Although not shown here, the A-to-C transformation might occur through a random sporadic pathway across connected bits of precursor phase (as seen in mollusk nacre), or via spherulitic growth or plume-like splay across channels (as in corals), or it might follow along step-edges of crystallographic symmetry, similar to classical crystallization where growth spirals around dislocation ledges. In the latter case, one might expect the impurity proteins to be excluded and thus occluded along well-defined crystallographic planes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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