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On Biomineralization: Enzymes Switch on Mesocrystal Assembly

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Supporting Information

ABSTRACT: Cellular machineries guide the bottom-up pathways toward crystal superstructures based on the transport of inorganic precursors and their precise integration with organic frameworks. The biosynthesis of mesocrystalline spines entails concerted interactions between biomolecules and inorganic precursors; however, the bioinorganic interactions and interfaces that regulate material form and growth as well as the selective emergence of structural complexity in the form of nanostructured crystals are not clear. By investigating mineral nucleation under the regulation of recombinant proteins, we show that SpSM50, a matrix protein of the sea urchin spine, stabilizes mineral precursors via vesicle-confinement, a function conferred by a low-complexity, disordered region. Site-specific proteolysis of this domain by a collagenase initiates phase transformation of the confined mineral phase. The residual C-type lectin domain molds the fluidic mineral precursor into hierarchical mesocrystals identical to structural crystal modules constituting the biogenic mineral. Thus, the regulatory functions of proteolytic enzymes can guide biomacromolecular domain constitutions and interfaces, in turn determining inorganic phase transformations toward hybrid materials as well as integrating organic and inorganic components across hierarchical length scales. Bearing striking resemblance to biogenic mineralization, these hybrid materials recruit bioinorganic interactions which elegantly intertwine nucleation and crystallization phenomena with biomolecular structural dynamics, hence elucidating a long-sought key of how nature can orchestrate complex biomineralization processes.

INTRODUCTION

Nature’s toolbox for material genesis inspires synthetic routes toward functional materials with intricate morphologies, patterns, and organizations.1–6 Biogenic pathways exhibit exquisite control over the size and structure of hybrid materials across several length scales. However, a lacuna of understanding persists for bioinorganic interactions of functional and regulatory significance that underlay the development and organization of biomaterials. In consideration of biologically controlled nucleation and crystallization, the formation and (de)stabilization of metastable amorphous mineral precursors, mechanisms of phase transformation as well as the sophisticated unification of organic and inorganic building units into functional mesostructured materials represent fascinating, however less understood, fundamental aspects of biomineralization.

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Structural studies on calcareous elements from echinoderms have shed some light on biomineralization pathways. Constructed by the transport and transformation of amorphous precursors, the sea urchin spine emerges as a biogenic mesocrystal composed of co-oriented calcite particles organized in a space-filling manner within a cement of amorphous mineral and biomolecules. This superstructure reconciles seemingly inconsistent material properties that enable a single crystal-like diffraction behavior and concomitantly a conchoidal fracture behavior typical of amorphous materials. Having explored structure–property relations of these biominerals, the extent of biochemical and biophysical regulation imposed upon nucleation and crystallization reactions toward achieving hybrid crystalline superstructures requires attention.

The intricate organic–inorganic interactions involved in sea urchin skeletogenesis is reflected by the diversity of biomineral-associated macromolecules. SpSM50 is an abundant nonglycosylated matrix protein in the organic matrix of the sea urchin spine. Localized in Golgi membranes and extracellular mineralization compartment as well as occluded within the biomineral, SpSM50 is potentially multifunctional, regulating early nucleation stages and subsequent phase transitions of the mineral phase. Recent investigations also indicate that spine-associated proteins modulate the early regime of mineral nucleation, inhibiting nucleation and transiently stabilizing amorphous minerals and also forming assemblies and hydrogels, which can control the texture and internal structures of emergent mineral crystals. Exemplified by proteins such as SpSM50, PM27, SM29, and the SM30 family, the prevalence of C-type lectin-like domains (CTLDs) in the biomineral proteome suggest distinct contributions toward mineralization. In this manner, the functional aspects of specific nanoscopic bioinorganic interactions and interfaces as well as the relative positions of biomolecular players in the regulatory cascade of mineral nucleation and crystallization require further elucidation.

Recent developments in the field of nucleation and crystallization suggest that early mineralization events involving ion clusters, liquid condensed phases, and amorphous particles significantly contribute to the selective emergence of crystal form, structure, and organization. It is important to address the structural dynamics and post-translational modifications of the biomacromolecules in relation to these distinct crystal precursors and their microenvironments under well-defined experimental conditions. Our interdisciplinary approach involves the application of recombinant biomineral-associated proteins in a mineralization protocol that enables precise control over solution parameters, in situ quantitative insights into the nucleation process, and time-dependent reaction sampling. The structural evolution of organic–inorganic complexes is accessed by using analytical ultracentrifugation (AUC) and liquid cell (LC)/cryogenic transmission electron microscopy (cryo-TEM). Collectively, these techniques construct a holistic overview of bioinorganic interactions involving distinct mineral precursors of solute, fluidic, and particulate compositions.

Given the indispensable role of the spicule matrix protein, SpSM50, in spine development, recombinant forms of the full-length protein and its constitutive domains (Figure S1) are purified as fusion products and applied as mineralization additives. An N-terminal small ubiquitin-like modifier (SUMO) tag facilitates the purification process and provides a means to control the expression and activity of the protein. These recombinant proteins are then used in combination with other biomolecules to study the effects on mineral nucleation and growth.
tag is utilized because of the high aggregation propensity and toxicity of untagged biomineral-associated proteins. Note the unique domain architecture of SpSM50 (Figure S1), with a folded N-terminal CTLD and a highly basic disordered glycine and proline rich domain (GRR).

■ RESULTS AND DISCUSSION

Under nonreducing conditions, the recombinant C-type lectin-like domain is ascertained as well-folded, with conformational responses to Ca\(^{2+}\) ions and functionality toward sugar binding (Figure S2). The affinity of the CTLD with specific sugar residues has mechanistic implications toward heterointeractions guiding the construction of organic scaffolds during biomineralization.\(^{13,24,25}\) The protein is applied during calcium carbonate (CaCO\(_3\)) nucleation in a potentiometric titration setup. Distinct nucleation profiles are elicited by the CTLD, determined by the conditions of solution pH as well as protein concentration, conformation, and redox states (Supporting Information, section 3). Considering the four cysteine residues in the CTLD sequence, the dependence of mineral nucleation profiles on redox environments reflect the complementarity and specificity of bioinorganic interactions between the solvent exposed amino acid residues of the CTLD and inorganic species such as ions, ion-clusters, and liquid-like and particulate precursors.\(^{12,20}\) During the progression of mineralization, the protein molecules cluster and mature in terms of size and density, as observed by cryo-TEM of samples collected at different nucleation stages (Figure 1). In the prenucleation regime (i.e., before the formation of solid mineral particles), protein-ion complexes become denser and self-associate, presenting size developments from about 10 nm to 5 μm. From ion complexation studies as well as dynamic light scattering and analytical ultracentrifugation, the observed structural transitions of the CTLD appear to be collectively driven by single ions, ion-clusters, and emergent mineral precursors, forming larger, asymmetric biomolecular self-associates for the latter (Supporting Information section S4). Following mineral nucleation, the CTLD protein forms sheet-like structures, which stack and form organic laminae (Figure 1b, arrow). Free Ca\(^{2+}\) ions also induce CTLD self-association toward sheet-like microstructures, although over longer time periods, indicating that the dynamics of ionic and pH conditions during mineral nucleation can enhance the kinetics of macromolecular self-association and supramolecular assembly (Figure S4C). Interestingly, certain lectins produce similar sheet-like nanostructures induced by divalent cations, wherein multiple factors such as sequence composition and structural dynamics contribute to the emergence of supramolecular organizations.\(^{26}\) As shown in Figure 1, the contributions of vesicular structures in the mineralization of the laminae assemblies are supported by spatial proximity to mineralized bodies and the drying-induced crystallization of vesicles toward calcite.\(^{19}\) Considering that scaffold self-assembly precedes crystallization of the inorganic phase and also the high solubility of the initially nucleated mineral phase deduced from titration experiments (Figure S3B), the mechanistic roles of the
capillarity associated with liquid-like mineral precursors can contribute to mineralization of the laminar scaffold. In view of the conformational stability of the CTLD toward Ca\(^{2+}\) ions as well as the distinct ion-association behavior (Figures S2 and S4A), factors such as ion bridging and hydrophobic interactions appear to induce macromolecular self-assembly and the subsequent emergence of functional bioinorganic interfaces.

Subsequent to mineral nucleation, a structural transition occurs from proteinaceous sheets to multilamellar composites, which exhibit ED patterns typical of monocristalline calcite (Figures 2a and S5). The CTLD stabilizes the high-energy (001) calcite face, indicating that the protein actively interfaces with the crystalline phase, intrinsically reinforcing a mesocrystalline architecture (Figure S5). Detailed structural evaluation of titration-derived composites reveals a unique multilamellar mesocrystal exhibiting two distinct levels of hierarchy (Figure 2). First, mechanical disruption of the laminar organization produces crystallographic misaligned composite structures (Figure 2c,d). This reflects the co-orientation of crystalline domains constituting adjacent laminae. Second, the protease-mediated elimination of CTLD from single lamina produces a space-filling arrangement of irregular, calcite particles (Figures 2e and S5B), consistent with structural accounts of the biogenic mineral.  

AFM characterization of the demineralized organic template presents porous topographies, which structurally complements the dimensions of the mineral nanoparticles (Figure S5B). Thus, the mineralization-induced self-association of CTLD provides an organic framework conducive for the integration of inorganic constituents and organic assemblies toward crystalline, laminar composites.

Sedimentation velocity experiments provide solution-state insights into the bioinorganic interactions that underlay mesocrystal formation, wherein global pH and ionic conditions related to mineral nucleation augment CTLD self-assembly toward asymmetric shapes with high frictional ratios (Figure S4B). Under the applied chemical environments, the optimal stoichiometry for Ca\(^{2+}\) ion binding corresponds to less than one divalent ion for each carboxylate group within a protein monomer (about 0.1 Ca\(^{2+}\) ion for each aspartate/glutamate residue at pH 9.0). This suggests that non-Coulomb interactions such as hydrophobic forces also contribute to nanoscale interactions that underlay supramolecular architectures. In view of the mineralization process, the titration estimated enhancement in mineral hydration contents relative to ACC is 5.4 \(\pm\) 2.5 nm and 1.399 \(\pm\) 0.001 g/cm\(^3\) corresponding to the shell thickness and core density, respectively (Supporting Information section 6). The shell thickness suggests transitions from bilayer to multilayer protein shell architectures, induced by protein self-association and distinct interactions directed toward inorganic interfaces in the course of mineral nucleation. A bilayered shell also indicates the amphiphilic structure of the recombinant protein, originating from the globular N-terminal domain and the extended, more hydrophobic disordered region. On the other hand, the core is constituted by a highly hydrated amorphous mineral phase, with a 20–50% higher hydration content and a density considerably lower than that of hydrated amorphous CaCO\(_3\) (ACC; 1.49–1.62 g/cm\(^3\)). Given the possibility of protein molecules incorporated in the confined phase, the actual enhancement in mineral hydration contents relative to ACC is possibly greater. The estimated density of gel-like mineral precursors stabilized by small charged molecules is lower, about 1.2 g/cm\(^3\); hence, in the present study, the incorporation of protein species within the stabilized mineral core is plausible. In this regard, the confinement of highly hydrated mineral precursors appears to emerge from the localized self-assembly of biomolecules at the interfaces of kinetically stabilized nascent mineral nanodroplets, also reflecting fundamental contributions of confinement-mediated phase stabilization with morphological similarities to vesicles observed in vivo. 

In verification of the proposed mechanism, the simulation of mineralization-induced vesicles is performed by exposing rhodoamine-labeled SpSM50 to water:THF mixtures. Line plots of the fluorescence signal show the exclusion of proteins from the vesicle core, hence revealing the contributions of transient liquid–liquid interfaces as well as an antisolvent component in establishing vesicle shells (Figure S6C). Such conditions can be translated to mineralization scenarios, wherein divalent ions and their solute clusters aggregate protein solubility. Subsequently, the interfaces of nascent mineral droplets and particles provide adsorption sites for these precondensed macromolecules, in the present study producing hybrid vesicular structures. Comparison of the CTLD- and components with ED patterns suggestive of the [001] zone axis orientation of calcite (Figure 2). The nanoporosities decorating the ribbon surfaces also reflect a space-filling arrangement of constitutive nanoparticles. Overall, analogous structural and crystallographic features exhibited by the biomimetic and biogenic mesocrystals reveal the participation of the CTLD in bridging mineral nanocrystals within architectures exhibiting multiple levels of structural hierarchy. In this light, the remarkable superstructure of this biomimetic mineral might elucidate the prevalence of CTLDs among the members of the spicule proteome.
SpSM50- or GRR-controlled mineral nucleation phenomena shows that the hybrid supramolecular architectures are markedly distinct in form, structure, and crystallinity, with the inorganic phase inducing unique variations in the conformational and self-association states of the organic precursors. Therefore, during biomineralization, the organic—inorganic interactions encompassing biomolecular conformation and self-organization appear chemically programmed and elicit distinct nucleation and crystallization responses in relation to the physical states, interfaces, and structures of the maturing mineral phase viz. ions, ion-clusters, liquid condensed phases, and amorphous and crystalline particles. Following an analogous pathway, the structural transitions for the investigated proteins proceed from soluble species to discrete self-associates of higher order such as rods, sheets, and laminae (Figures 1 and S6A). In this sense, biomineralization phenomena appear to encompass “nonclassical pathways” for both the inorganic and organic phases, wherein the bioinorganic interface is highly dynamic, constituted with hybrid precursors with transient degrees of self-association, solvation, and structural dynamics that navigate across a highly complex energetic landscape to the final composite phase and organization.

To address the incongruous effects of the constitutive domains of SpSM50, i.e., the CTLD mediating mesocrystal formation and the LCD region effectively stabilizing mineral precursors within vesicles, we further characterize the mineralization behavior of the full-length protein. Previous studies show that sea urchin embryos exposed to inhibitors of matrix metalloproteases (MMPs) exhibit vesicle accumulation in the cytoplasm of mesenchymal cells and arrested biomineral development. Biochemical analyses of proteins from sea urchin skeletal elements also identify SpSM50 fragments at low molar masses relative to the full length version. These studies suggest that matrix metalloproteases participate in the maturation of biomineralization proteins, for instance enhancing the integration of partially proteolyzed macromolecules into growing crystals. This hints that extracellular matrix modeling in the context of homeostatic biomineralization necessitates selective proteolytic processing. Along this line, we examine the in vitro susceptibility of the recombinant SpSM50 toward proteolysis by a type IV collagenase (Figure 3a,c) and the sequence coverage of residual fragments (Supporting Information section 7), thus revealing a selective degradation of the LCD and a relative conservation of the CTLD. Wild-type blots of spine extracts developed using CTLD-specific primary antibodies also produce a 15 kDa fragment corresponding to the lectin domain, in addition to the full-length protein (Figure S8B).

With the disordered domain of SpSM50 identified as a putative target of proteolysis, the consequences of this post-translational processing on mineral formation are addressed. In reference experiments, the full-length SpSM50 significantly inhibits mineral nucleation and transiently stabilizes a highly soluble mineral phase (Figures 3 and S8A). Consistent with the reported stabilization of a hydrated amorphous mineral precursor, here we identify the functional contributions of vesicular confinement in stabilizing the mineral precursor against crystallization (Figure 3). Similar nucleation profiles elicited by the full-length SpSM50 and its constitutive LCD domain and a likewise vesicular entrapment of amorphous mineral precursor within proteinaceous bodies indicate that the independent effects of the disordered region on mineral nucleation supersede those of CTLD. However, combined applications of the matrix protein and collagenase in mineralization experiments diminish the time periods (i.e.,
supersaturation conditions) required for the nucleation of mineral particles in comparison to SpSM50 alone, an effect accentuated by increasing protease contents (Figures 3 and S8). Similar structural transitions from vesicles to mesocrystalline laminae are also identified in the presence of either the CTLD alone or SpSM50-collagenase mixtures (Figures 3 and S8B). The coapplication of SpSM50 and the protease leads to the formation of laminar mesocrystals that present single crystal-like diffraction and polarization patterns. Last, LC-STEM experiments demonstrate the existence of transient vesicles during mineralization performed in the presence of SpSM50 and type VI collagenase mixtures as well as for CTLD alone (Figure 3). In this manner, the crystalline products of the protease-guided mineralization pathway are structurally similar to the CTLD-mediated mesocrystals. Hence, the impacts of the nanoscale bioinorganic interactions between postsynthesis modified macromolecules and distinct mineral phases require attention. On these lines, post-translational modifications such as glycosylation and phosphorylation might have significant impacts on protein-guided pathways of mineral nucleation and crystallization.15,24,44

Figure 4. (a) Scheme illustrating different stages of the formative process of crystalline minerals involving (1) a population of stable, structurally dynamic ion-clusters, (2) at a critical ion activity, the liquid–liquid demixing of mineral nanodroplets and the subsequent nucleation of (3) amorphous calcium carbonate and (4) crystalline polymorphs. (b) Schematic representation of the multifunctionality of SM50 (1) initially forming vesicles that confine and stabilize a liquid-like amorphous mineral form. (2) At the site for mineral growth, MMP activity specifically cleaves the disordered domain of SM50 (pink), which is crucial for vesicle stability. This initiates the phase transformation of the amorphous phase to calcite under regulation of the residual CTLD (blue). Conditions of pH and supersaturation drive the assembly of CTLD to scaffolding structures. (3) The destabilized fluidic (and possibly transient particulate45,46) precursors infiltrate and mineralize the organic laminae, generating calcite mesocrystals. (c) Structural similarities between vesicles trafficking mineral precursors and laminar modules from the sea urchin spine (left) to SM50-based vesicular structures and CTLD-mediated calcite mesocrystals (right). Scale bars represent 1 μm, 200 nm, 500 nm, and 1 μm, left to right. (*The left-hand image in panel c is reproduced with permission from ref 32. Copyright 2014 National Academy of Sciences.)
networks and supramolecular assemblies emerge as vital in directing the phase transformations of minerals toward crystalline superstructures. During mineralization, the proteolytic processing of structural extracellular proteins with low sequence complexities emerge as crucial for providing optimal inter- and intramolecular interactions that guide the mesoscale self-assembly of inorganic nanocrystals. Isolated from the LCD region, the lectin domain organizes the transient fluidic and possibly particulate amorphous mineral precursor into calcitic mesocrystals consisting of polydisperse and irregular, however crystallographically coaligned, mineral particles organized in a space-filling manner. Intertwined with the transformation of highly hydrated amorphous minerals, the mechanism of mesocrystal formation is based on defined bioinorganic interactions affecting the amalgamation and mesoscale assembly of mineral phases and supramolecular assemblies. Given the metastable nature of the mesocrystal structure, the mechanistic roles of transient nanocrystalline domains as intermediates during biogenic and synthetic crystallization also require attention.

With proteomic studies revealing diverse sets of biomineral-associated macromolecules, addressing their synergistic functions and relative positions in the regulatory cascade will assist the identification of molecular checkpoints and interactions underlying the integration of supramolecular assembly and mineral growth and structure. In addition, with emerging possibilities of performing mineralization reactions at near physiological pH conditions using macromolecules with near native compositional and structural states, the prospects of elucidating critical questions in the field of biominalization such as the polymorph selectivity of minerals as well as the contributions of bicarbonate species and liquid condensed phases are emerging. Lastly, the dynamics and ultrastructure of precrystalline entities and stabilizer populations will help further elucidate phase transformation pathways concerning biominalization. Further investigations on the synergetic relations between distinct mineral precursors and biomacromolecules under physiological conditions will help resolve nature’s blueprint for hierarchical materials, opening avenues for smart additives that control crystallization processes at different length and time scales.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscentsci.8b00853.

Experimental details and supplementary sections (1–8) related to protein expression and purification, mineralization—demineralization methodologies, biochemical assays, and solution and structural analytical investigations (PDF)

Supplementary Video 1: Proteolysis switch for mesocrystal assembly (AVI)

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Notes
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