Polysaccharide chemistry regulates kinetics of calcite nucleation through competition of interfacial energies

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Calcified skeletons are produced within complex assemblages of proteins and polysaccharides whose roles in mineralization are not well understood. Here we quantify the kinetics of calcite nucleation onto a suite of high-purity polysaccharide (PS) substrates under controlled conditions. The energy barriers to nucleation are PS-specific by a systematic relationship to PS charge density and substrate structure that is rooted in minimization of the competing substrate–crystal and substrate–liquid interfacial energies. Chitosan presents a low-energy barrier to nucleation because its near-neutral charge favors formation of a substrate–crystal interface, thus reducing substrate interactions with water. Progressively higher barriers are measured for negatively charged alginates and heparin that favor contact with the solution over the formation of new substrate–crystal interfaces. The findings support a directing role for PS in biomineral formation and demonstrate that substrate–crystal interactions are one end-member in a larger continuum of competing forces that regulate heterogeneous crystal nucleation.

biomineralization | calcium carbonate | free energy | algae | crustacean

Efforts to decipher patterns of biomineralization have identified proteins and polysaccharides (PSs) as major components of the organic matrices associated with sites of calcification. In mollusks and other organisms including the red algae, coccolithophores, and foraminifera (1, 2) (Table 1), calcifying macromolecules are dominated by functional groups with an acidic character—proteins that are rich in carboxylated amino acids (14) and PSs that are highly sulfated and carboxylated (15, 16). Although a physical picture of these interactions is not well developed, this recurring affiliation of carboxylate and sulfate groups with zones of mineralization in organisms suggests specific roles for macromolecules in nucleation and growth. Early studies have led the biomineralization community to generally assume that charged proteins actively regulate mineralization. In contrast, PSs, such as the chitin found in the insoluble fraction of the mollusk shell, are thought to provide an inert scaffolding to support these proteins (17, 18).

Recent studies challenge this assumption with qualitative evidence that PSs can also promote calcium carbonate (CaCO₃) mineralization (19, 20) (Table 1). For example, specific orientations of chitin fibers with neutral functional groups promote the templating of CaCO₃ in the lobster carapace (13, 21), crab cuticle (21), and nautilus shell (22). More generally, the monosaccharide sequences along PS chains can influence biological function and their interactions with proteins (23). This suggests PS chemistry and interactions with proteins could regulate patterns of mineralization.

Anecdotal observations from *in vitro* studies also support the thinking that PSs influence mineral formation, but their specific effects are unclear. For example, PSs with higher carboxyl and sulfate content promote either faster (24, 25) or slower (26, 27) rates of CaCO₃ nucleation. Although these results seem inconsistent at first glance, the differences are likely attributable to the diverse experimental designs that were used across disciplines and, in some cases, with little control over chemical conditions. Although qualitative, insights from *in vivo* and *in vitro* studies provide evidence that the chemical structure of PSs can indeed control calcite nucleation. They also raise the question

of whether there is an underlying relationship between homologies of mineralization and the functional group chemistry of monosaccharide sequences, and more generally, the chemistry and structure of macromolecules.

To test this idea, we designed an experimental study to quantify the underlying energetic controls on calcite nucleation. A suite of high-purity PSs with regular monomer sequences and simple functional group chemistry were chosen as model compounds for the complex PSs found in calcifying organisms (Fig. 1). Heparin is the most highly charged PS of the group with carboxyl and sulfate groups, whereas amine groups give chitosan a small net positive charge. Two alginates provided a test of how stereochemistry influences nucleation through different proportions of the stereoisomers mannuronic and guluronic acid.

Using a flow-through method that controlled for solution chemistry (28) we quantified the number of crystallites that formed on each type of PS substrate. These were prepared by electrodeposition onto gold using established methods. Rate measurements assumed that each nucleus that achieved a critical radius subsequently continued to grow into an observable crystallite. The number of crystals per square millimeter was counted in a sequence of time-lapsed images to determine nucleation rates (Fig. 2 *A–D*, *Insets*) across a series of solution supersaturations, σ , defined by

$$\sigma = \ln \left(\frac{a_{\text{Ca}^{2+}} a_{\text{CO}_3^{2-}}}{K_{sp}} \right), \quad [1]$$

where a_i is the activity of species i and K_{sp} is the equilibrium solubility constant ($10^{-8.48}$ for pure calcite at 25 °C). The σ varied from 4.83 to 5.63 at a constant solution pH of 10.8 ± 0.1 , $[\text{Ca}^{2+}]/[\text{CO}_3^{2-}]$ of 1.0, temperature of 25.0 ± 0.5 °C, and total ionic strength of 0.007–0.013 M. Despite the high supersaturations that exceeded the solubility of amorphous CaCO₃, only calcite crystallites formed under these conditions, as identified by their rhombohedral morphologies and consistent with previous work (28).

Results and Discussion

The number of calcite crystallites that nucleated and grew on the PS substrates was proportional to the driving force, σ , of the reactant solution (Fig. 2 *A–D*, *Insets*). Rates were also PS-specific, with the highest and lowest number densities measured for heparin and chitosan, respectively (Fig. 2 *A* and *D*).

To quantify the thermodynamic basis for these differences, we evaluated the linear portion of the nuclei versus time data using

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Table 1. Examples of polysaccharides found at sites of CaCO₃ mineralization from diverse phyla

Name	Structure	Polysaccharide	Ref(s).
Red algae (Rhodophyta)			
<i>Corallina pilulifera</i> (P. et R.)	Cell wall of thallus	Xylogalactan*, sulfated, methylated, agaran-like Floridean starch Alginic acid	3, 4
<i>Joculator maximus</i> (Manza)	Cell wall of thallus	Xylogalactan*, sulfated, methylated	5
Coccolithophores (Haptophyta)			
<i>Emiliania huxleyi</i>	Coccolith	Mannan*, sulfated, branched with uronic acids and methylated sugars	6, 7
<i>Pleurochrysis carterae</i>	Coccolith	Galacturonan*, sulfated, other uronic acids Glucuronan* with tartarate and glyoxylate residues	7, 8
Cyanobacteria (Cyanophyta)			
<i>Anabaena</i> spp.	Mucilaginous sheath	Pectin Glycosaminoglycan [†]	9
Foraminifera (Foraminifera)			
<i>Heterostegina depressa</i>	Test	Glycosaminoglycan [†] , sulfated	10
Mollusks (Mollusca)			
<i>Nautilus repertus</i>	Nacreous shell layer	β-Chitin	2
<i>Mercenaria mercenaria</i>	Extrapallial fluid	Glycosaminoglycan [†] , sulfated	11
Crustaceans (Arthropoda)			
<i>Carcinus maenas</i> (L.)	Cuticle	α-Chitin	12
<i>Homarus americanus</i>	Carapace	α-Chitin	13

*See references for more detail on structure and sequence.

[†]Glycosaminoglycan is a broad classification with ambiguous chemical structure.

classical nucleation theory (29, 30) (*SI Text, section S1*). The linear form of the general nucleation expression states that the flux of new nuclei onto a substrate is described by

$$\ln(J_o) = \ln(A) - \frac{B}{\sigma^2}, \quad [2]$$

where J_o is the steady-state rate of heterogeneous nucleation ($\text{m}^{-2}\cdot\text{s}^{-1}$); A is dependent upon kinetic factors that include rates of ion desolvation, attachment, and detachment; and slope, B , is given by

$$B = \frac{F\omega^2\gamma_{net}^3}{k_B^2T^2}, \quad [3]$$

where F is a nucleus shape factor, ω is the molecular volume of calcite, k_B is the Boltzmann constant, T is temperature, and γ_{net} is

the net surface free energy of the crystal–substrate–liquid system ($\text{mJ}\cdot\text{m}^{-2}$). Assuming that ω is relatively constant, the experimental variable within B is γ_{net} for a constant T . Because B is a proxy for the thermodynamic barrier to the nucleation (*SI Text, section S1*), its dependence upon γ_{net} represents the contribution of surface energies to the energy barrier. Eq. 2 gives a good fit to the rate data and shows that at the highest supersaturations substrates rich with carboxyl and sulfate functional groups (Fig. 2 *A–C*) promote the fastest nucleation rates, whereas the slowest rates occur on chitosan surfaces (Fig. 2*D*).

By evaluating the slopes of these trends, we find that values of B are PS-specific. The highest slopes, and thus the highest energy barriers to nucleation, are correlated with the charge-dense substrates [low-guluronic-acid (LG) alginate and heparin]. In contrast, nucleation onto chitosan substrates shows the lowest slope. Because B is directly proportional to surface energy cubed (Eq. 3), we can deduce that a low value of B is correlated with

Fig. 1. Polysaccharide chemical structures illustrate the functional groups attached to 2-carbon (red) and 6-carbon (blue) of each ring. (A) De-*N*-sulfated heparin contains amine, acetylamine, carboxyl, and sulfate groups. R is 80% H and 20% COCH₃. Ninety percent of carboxyl groups are in axial positions. (B and C) Alginates contain carboxyl groups with different proportions of guluronic acid monomer content. (D) Hyaluronate contains acetylamine and carboxyl groups. (E) Chitosan contains amine and acetylamine groups. (F) Cellulose, with only hydroxyl groups, was not used in study and is included for comparison.

Fig. 3. Kinetic measurements provide insights to controls on thermodynamic barriers to nucleation. (A) Barriers are inversely correlated with charge density such that near-neutral PSs present the lowest barrier to calcite formation. (B) Measurements of surface free energy in air, a proxy for γ_{SL} , provide evidence for a competition between solvent and crystal interactions with the PSs that correlate with hydrophilicity. (C) Relationship between energy barrier and charge density can also explain the very high energy barrier measured on carboxylated SAMs. Offsets between SAMs and alginates of different chain lengths and stereochemistry suggest functional group structure and conformation also tune barrier height. SEs of B are derived from the linear fits of Eq. 2 to data in Fig. 2.

The trends in Fig. 3 *A* and *B* thus suggest the possibility of a broader relationship that could explain calcite nucleation behavior onto substrates ranging from neutral to highly charged. Measurements of energetic barriers to calcite nucleation onto carboxylated self-assembled monolayers (SAMs) provide insights into the influence of highly charged functional groups that are found in proteins and PSs in mineralizing systems (28). Due to the greater hydrophilicity of these substrates, this method of measuring γ_{S-air} cannot be used because the contact angles lie outside of the accessible range. Instead, we estimate the surface charge density from pK_a values of functional groups and packing density of the SAMs or PSs (*SI Text, section S4*). Fig. 3C shows that, indeed, charge density is correlated with the barrier height to calcite nucleation along a single trend that includes the SAM substrates.

The implication of Fig. 3C is that charge density through functional group chemistry has a primary control on the barrier to nucleation through differences in γ_{SL} . Small offsets in B between the two alginates and the carboxylated SAMs (Fig. 3C) suggest that differences in the structure of substrates with similar charge densities further regulate the barrier height. This conclusion is supported by recent simulations showing that variations in head group geometry that arise from SAM–monomer interactions cause subtle differences in substrate–crystal interfacial energies (35). Thus, nucleation barriers are largely controlled by substrate charge density through γ_{SL} and further regulated by small differences in γ_{SC} through subtle variations in structure.

Our results demonstrate the energy barrier to calcite nucleation is determined by a competition between the energetics of crystal nucleus attachment to a substrate and creating new interface (γ_{SC}) versus the energetics of eliminating a solvated substrate (γ_{SL}). By minimizing the change in free energy through these thermodynamic drivers, the systematic relationship between nucleation and the chemistry and structure of substrate environments can be understood. Hydrophilic substrates prefer to maintain the substrate–water interface, thus presenting a high barrier to making a new substrate–crystal interface. In contrast, neutral to less-hydrophilic surfaces present a lower thermodynamic barrier to displacing water to form a new substrate–crystal interface and thus to mineral nucleation. This interpretation of the experimental data expands upon the conclusions of earlier computational studies that explored the importance of surface ionization, as well as the competition between the energies of the substrate–crystal and liquid–crystal interfaces (36).

At first glance, the higher energy barriers we estimate for nucleation onto charged surfaces are counterintuitive given the

long-standing perception that negatively charged functional groups promote nucleation by strong interactions with Ca^{2+} ions (14, 15, 37, 38). Moreover, a number of studies observe that calcite crystallites are preferentially formed on carboxyl-patterned regions, in sharp contrast to the surrounding hydrophobic methyl-functionalized areas (39–41).

To understand why these seemingly disparate observations are consistent, recall that energy barriers are constant for a given substrate–liquid–crystal system and nucleation rates are dependent upon σ^{-2} . Thus, for any two substrates, there must be a value of σ above which, paradoxically, the one with the higher value of B gives faster nucleation rates (Fig. 2E). Because the studies described above were conducted at very high levels of supersaturation, the charge-dense macromolecules produced the most crystallites over short time intervals. Fig. 2E shows that as supersaturation is lowered these trends eventually flip and neutral substrates with lower energy barriers promote the fastest rates of nucleation (Eq. 2). This physical picture reiterates the point that whereas energy barriers to nucleation at a given supersaturation are constant and substrate-specific, the local supersaturation environment has the overarching control on relative rates of nucleation onto charged versus neutral substrates.

The findings suggest a simple mechanism-based framework for understanding nucleation as a continuum of substrate–liquid–crystal interactions that are governed by acidic and neutral chemical domains, irrespective of macromolecular class. The possibility of regulating the onset of nucleation by this construct suggests the need to revisit the long-standing assumptions regarding the roles of neutral and acidic macromolecules at sites of calcification. If near-neutral macromolecules present a lower energy barrier to nucleation by promoting the formation of new substrate–crystal interfaces, an important part of the mineralization picture may be currently missing.

Implications

There are a number of implications for interpreting the origins of biominerals that form in organisms and for better controls on materials synthesis. By establishing a physical basis for how macromolecule chemistry favors or inhibits biomineral nucleation, it may be possible to interpret patterns of skeletal biomineralization through geologic time using an inverse approach. That is, differential expression of PSs and proteins with varying charge density, in concert with local supersaturation, regulate the placement, timing, and extent of mineral nucleation. For example, modern mollusks induce mineralization at epithelial membranes within neutral chitin matrices using acidic proteins, hydrophobic silk proteins, and glycosaminoglycans in the growth

front of the shell (19, 41–43). Recall that the chitin and glycosaminoglycans found in organisms are, in fact, glycoproteins (17, 18, 44).

These results also cast light on the processes of crystal templating and epitaxy. Heterogeneous nucleation theory has long recognized the contributions of interfacial energies as the primary drivers to minimizing the overall energy of the system (45). However, conventional wisdom focused on epitaxy mechanisms and assumed that substrate–crystal interactions were predominant in nucleating environments (30, 46). Our findings show this assumption must now be reconsidered within a broader mechanistic framework whereby the drivers to nucleation are a continuum of substrate–liquid–crystal interactions. This explains why the basic tenets of classical nucleation theory have always allowed for the possibility that poor substrate–crystal interfaces can promote nucleation. We show this can occur by eliminating a less favorable substrate–liquid interface. By considering nucleation as a continuum of interactions, a new paradigm for understanding diverse nucleation phenomena may emerge.

Materials and Methods

Preparation of PS Films. PS substrates were deposited onto gold-coated Si wafers (Platypus Technologies) using established electrodeposition protocols (47–50). The electrodeposition apparatus consisted of two ~1.5-cm² wafers that were held 15 mm apart by a Teflon spacer and connected to an electrophoresis power supply using stainless steel alligator clips. For each type of film, the electrodes were submerged in 0.05% solutions of LG sodium alginate (<50% guluronic acid; Novamatrix), HG sodium alginate (>75% guluronic acid; Novamatrix), sodium hyaluronate (Novamatrix), chitosan chloride (75–90% deacetylated; Novamatrix), and de-*N*-sulfated heparin (20% *N*-acetylated; Sigma Aldrich), prepared at room temperature by gently mixing into 18.2 MΩ-cm ultrapure water for 1 h. The film was deposited by applying 20 V for 15–25 min. The integrity of PS film was confirmed before and after crystallization by SEM imaging or Alcian Blue (Sigma Aldrich) staining of carboxylated PSs.

Measurement of Nucleation Rates. Rates of heterogeneous calcite nucleation were measured using a flow-through method that maintained controlled

supersaturation conditions for the duration of each experiment. Experiments began by placing a freshly prepared substrate inside an acrylic glass flow chamber (25-mm diameter and 1.5-mm depth) and sealed with a glass coverslip. Solutions of 3–7 mM CaCl₂·2H₂O (≥99%; Sigma Aldrich) and Na₂CO₃ (99.997%; Alfa Aesar) were prepared and placed in two polypropylene syringes that were connected to the flow chamber with Tygon tubing and a T junction. The syringes were loaded onto a high-precision syringe pump (PHD 2000 Infusion; Harvard Apparatus) and dispensed at a constant flow rate of 30 mL/h. Before each crystallization experiment, the substrate, flow chamber system, and tubing were flushed with distilled deionized water. The experiments were conducted at a suite of solution supersaturations, σ , ranging from 4.83 to 5.63, but were held at constant pH of 10.8 ± 0.1, constant [Ca²⁺]/[CO₃²⁻] = 1, and constant temperature of 25.0 ± 0.5 °C. The short residence time of solution in the flow chamber allowed us to assume that solution pH and σ remained constant for the entire experiment.

Each experiment proceeded by collecting time-lapse images of crystallites that formed onto the substrates as a series of time-lapse images under an optical microscope at 10× magnification. Rates of nucleation were determined using only data within steady-state nucleation conditions. We assumed all nuclei that achieve a critical size grow into an observable crystallite. The maximum duration of an individual experiment was 1 h, at which time the substrate was removed and rinsed with ethanol.

Measurements of Surface Free Energy. The γ_{s-air} measurements were conducted using a well-established method (32–34). Static contact angle, θ , was measured with a goniometer on electrodeposited PS substrates using three test liquids: 18.2 MΩ-cm ultrapure water, glycerol (99.5%; Fisher), and ethylene glycol (99.7%; Aldrich) with known polar and dispersive components to surface free energy (γ_L^P and γ_L^D). Six angle measurements (three left and three right) were made on three replicate droplets of each test liquid (SI Text, section S3 gives details).

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