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Biomimetic Control of Calcite Morphology with Homopolyanions

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Abstract

Biom mineralization is an intricate process that relies on precise physiological control of solution and interface properties. Despite much research of the process, mechanistic details of biom mineralization are only beginning to be understood, and studies of additives seldom investigate a wide space of chemical conditions in mineralizing solutions. We present a ternary diagram-based method that globally identifies the changing roles and effects of polymer additives in mineralization. Simple polyanions were demonstrated to induce a great variety of morphologies, each of which can be selectively and reproducibly fabricated. This chemical and physical analysis also aided in identifying conditions that selectively promote heterogeneous nucleation and controlled cooperative assembly, manifested here in the form of highly organized cones. Similar complex shapes of CaCO₃ have previously been synthesized using double hydrophilic block copolymers. We have found the biomimetic mineralization process to occur interfacially and by the assembly of precursor modules, which generate large mesocrystals with high dependence on pH and substrate surface.

Introduction

Mineralized biomolecular materials, as well as the processing strategies used to create them, are sources of fascination to materials scientists. Particularly impressive is the degree of exquisite morphological control with which organisms manipulate minerals composed of multivalent ions. Despite their high lattice energies, biology physically tunes them to suit a variety of specific needs (optical, mechanical, etc).¹ Bone, abalone nacre, sponge spicules, and the brittlestar skeleton are examples of complex multifunctional materials, whose properties are derived from precise control of morphology. The resulting hierarchically ordered composite structures incorporate very small fractions of organics and yet demonstrate greatly enhanced strengths and toughnesses.² It is of fundamental interest to understand the controlling physiological processes and a practical concern to harness such synthetic capability for the development of inexpensive, strong, lightweight composite materials (e.g. for biocompatible replacement materials, as for bone repair).³

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Supporting Information Available. Enlargements of SEM images from Figure 1, SEM image of paired bud morphology, SEM image of cones on their growth substrate, TEM images and diffraction pattern of rice morphology, and photographs and diffraction pattern of millimeter-scale cones. This information is available free of charge via the Internet at <http://pubs.acs.org>.

Control over mineralization has been gained from investigating effects of additives on mineralizing solutions. Such additives include inorganic ions (e.g. Mg^{2+}),⁴ organic molecules,⁵ Langmuir-Blodgett monolayers,⁶ self-assembled monolayers,⁷ various synthetic polymers, and extracted biopolymers.^{8,9} Anionic macromolecules are of particular interest biomimetically, because the proteins involved in CaCO_3 mineralization typically have low isoelectric points (PIs) and contain large fractions of phosphoserine, aspartate or glutamate residues. The precise role of acidic polymers remains controversial however, and several equally plausible hypotheses have been proposed. As antiscalants, they prevent mineralization by disturbing nucleation events;^{10,11} in contrast, as growth initiators they induce local supersaturation via ion sequestration.^{12,13} As habit modifiers, usually as part of a matrix, they selectively initiate crystal growth of specific faces, often by providing appropriate spatial periodicity.^{14,15} They may also stabilize amorphous calcium carbonate (ACC), sometimes prior to subsequent mineralization.¹⁶

Other more complicated polyanion functions have been suggested. In one mechanism, demonstrated by Gower et al., polymers induce liquid precursor precipitate (PILP) micron-sized droplets, which can subsequently direct crystal growth into rods via a dynamic and iterative process.^{17,18,19} The PILP droplets can also coalesce and be molded into a variety of non-equilibrium morphologies. The SLS mechanism reported by Gower is an elegant demonstration of the way a polymer *itself* can change the crystal growth mechanism and give rise to a new morphology without the use of a mold. In oriented attachment, crystallite assembly is mediated by a partially capping polymer.^{20,21} Such precise, oriented construction has also been suggested to yield mesocrystals of CaCO_3 ^{22,23,24,25} as well as structured crystals of BaSO_4 .^{26,27} Double hydrophilic block copolymers (DHBCs) have been suggested as important for dynamic stabilization of growing surfaces.²⁸ Lastly, polyanions can also self-associate in the presence of Ca^{2+} to yield complex coacervates, which can serve as microspherical templates.^{29,30}

Although various polyanions have demonstrated all of the above-mentioned roles to modulate CaCO_3 morphology, in-depth evaluation of the polymer's roles under varying physical and chemical conditions is often lacking. In many cases, it has been presumed that a specific polymer performs a single important function (and hence directs only one or few potential morphologies). Herein, we show that even simple homopolyanions can induce many CaCO_3 morphologies, simply by altering experimental conditions, and in turn effectively change the polymer's function. The implementation of ternary diagrams enables rapid screening of morphology over a wide range of chemical conditions, revealing many possible roles for one polymer. This facilitated the identification of regions of controlled assembly, which are more representative of biological processes. The crystallinity and polymer distribution of the various morphologies are also analyzed, with particular regard to a highly oriented, layered structure.

Experimental Section

Polymer solutions

All polymers were dissolved in DI H_2O and diluted to 10mM per monomer. Polyacrylic acids, sodium salts (PAA), were received as follows: PAA 90 kDa ($\text{PAA}_{90\text{k}}$) (Aldrich), PAA 15 kDa ($\text{PAA}_{15\text{k}}$) (Aldrich), PAA 6 kDa ($\text{PAA}_{6\text{k}}$) (Polysciences), and PAA 2 kDa ($\text{PAA}_{2\text{k}}$) (Aldrich). Poly-L-aspartate (PLD) (30 kDa) and poly-L-glutamate (PLE) (14 kDa) were received from Sigma and dissolved in deionized H_2O . Polystyrenesulfonic acid (PSS) (17 kDa, 18 wt.% in H_2O) was received from Aldrich and neutralized with NaOH (Fisher).

Synthesis of fluorescein-labeled PAA

6'-Aminofluorescein was received from Fluka, 1-Hydroxybenzotriazole hydrate (HOBt) was received from Aldrich, and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) was received from Pierce. To a 100mM (per monomer) polyacrylic acid solution, 6'-aminofluorescein was added to saturation (less than 5% per monomer), and the pH was adjusted to 4.5 using 0.01M HCl (EMD). A separate solution, containing 2 equivalents of HOBt and 5 equivalents of EDC (per 6'-aminofluorescein), was added to the first solution while maintaining a pH of 4.5. Following 12 h of reaction, the product was thrice dialyzed against deionized water for periods of 1d.

Mineral Syntheses

CaCl₂ dihydrate (EMD Chemicals Inc.), NH₄HCO₃, (NH₄)₂CO₃, and NaHCO₃ (Fisher) were used as received and dissolved in DI H₂O freshly prior to synthesis. Typically, 10.0 mM (per monomer) of PAA, 1.00 M of a carbonate salt, and 10.00 mM CaCl₂ were micropipetted and immediately mixed to homogeneity (and transparency, with the exception of floc morphologies), in the order listed, in 0.5 dram shell vials (Fisherbrand, Type I Glass) to a total of 240 μ L. The resulting mineralization solutions were not capped but were arranged in a larger container that was placed in a humidity chamber, which was temperature controlled at 30 $\pm$ 1 °C for up to 24h. Solution volumes decreased by less than 1 μ L per hour. For analyses requiring dry or pure product, washing steps were performed by removing the supernatant, twice rinsing with pH 9 NaOH solution, and finally rinsing with deionized H₂O.

pH measurements

Measurements of pH were obtained of various mineralizing solution over the course of 2h, using an ORION* Thermo Electron Micro pH Electrode.

Optical Microscopy

Samples were collected with pipet tips, which were sometimes used to mechanically dislodge precipitates from the container, and samples were pipetted onto a microscope slide and observed with a Nikon Eclipse ME600 operating in transmission mode using a 20x lens.

SEM

Washed samples were pipetted and dried on a silicon wafer, and then sputtered with Pd/Au at 10 mA for 2 minutes. Samples were loaded into an FEI Sirion Sphera operating at 5kV.

XRD

Larger syntheses (up to 2.4 mL) of the various products were screened for morphological sameness with optical microscopy. Washed and dried products were collected for analysis, and scanned between 20° and 70° using a Philips XPERT Powder Diffractometer.

TEM

Washed products were suspended in water or absolute ethanol, and dry cast onto a lacey carbon TEM grid. Samples were analyzed with a T20 FEI Technai G2 Sphera Microscope operating at 200kV, to record bright field images and diffraction patterns on photographic plates.

Confocal Laser Scanning Microscopy (CLSM)

A Leica microscope equipped with an ArKr laser was used for performing laser scanning confocal microscopy. A 0.5 mL volume of fluorescein-labeled sample was loaded into a specially prepared glass slide containing a well below a coverslip.

Dynamic Light Scattering (DLS)

Mineralizing solutions were mixed to 2mL, with component proportions corresponding to selected morphologies. Samples were loaded into disposable sizing cuvettes and placed in a Malvern Zetasizer Nano ZS. Measurement durations were set to be determined automatically, and data were accumulated over the course of 3h. The correlation function was processed using the absorption, indices of refraction, and viscosity parameters corresponding to PMMA and H₂O.

Results and Discussion

In order to analyze the morphological space of the major chemical components, morphological maps were obtained in the form of ternary diagrams. Solution sets of constant volume were prepared by varying constituent proportions incrementally from stock solutions of CaCl₂, polymer, and carbonate salt. Because too dilute solutions can yield too little product or too little morphological diversity, and because too concentrated solutions can create conditions of uncontrolled precipitation, proper stock solution concentrations were determined empirically. In this way, they also yielded a nearly “optimized” ternary diagram that is spanned by many morphologies and also limits the variety of morphologies within individual solutions. This optimization also maximizes the sampling information obtained.

For the most detailed investigation (Fig. 1), 10 mM PAA_{15k} (all concentrations per-monomer) and 1.0 M NH₄HCO₃ were used. Components were mixed to a total of 240 μL, using stock solution increments of 16μL, in 0.5 dram flat-bottom glass vials, and the solutions were incubated at 30°C for 24h. The narrowest regions of the diagram were explored using even smaller stock solution increments, in order to clarify morphological trends. Products formed as the pH increased due to CO₂(g) evolution, typically requiring a lag time of at least 20m, at which point the pHs were 8.2; however, most growth occurred at pHs above 8.5. Over a dozen distinct morphological regions were identified, and all products were either calcite or amorphous. Each inset image represents a prominent or distinct morphology for its region of chemical space, and each composition and morphology can be synthesized reproducibly and selectively, although there is overlap across the guideline borders. Hybrid “paired buds” also appeared, between the “peanut” and “bud” regions (Fig. S2). Ordinary calcite rhombohedra, which occur along the rightmost edge of the diagram, are not shown. The diversity of shapes demonstrates that the multiple functions displayed by this simple polyanion strongly depend on solution conditions.

The ternary diagrams in this study display some general trends. High proportions of Ca²⁺ associated rapidly with both polymer and carbonate components, resulting in large, poorly defined aggregates. At high [CO₃²⁻], crystallization was highly favored as evidenced by increased faceting. High levels of polymer inhibited crystallization and yielded little precipitate, particularly at low [Ca²⁺]. The absence of polymer yielded the expected calcite rhombohedra. Low [CO₃²⁻] products were entirely microspherical liquid charge-neutralized polymer-Ca²⁺ complexes also known as coacervates. Increasing [Ca²⁺] and [CO₃²⁻] from this coacervate region induced flocculation of submicron precursors into larger sponge-like masses. This likely resulted from the interconnection of polymer aggregates, induced by mild electrostatic screening of the added salt, coupled with rapid solidification of ACC.

With a further increase of [CO₃²⁻], there was a morphological transition from rapidly formed, interconnected masses into discrete microspheres in low yield, reflecting slower nucleation and weakened aggregation. Over extended periods, some of these exhibited PILP-like growth into rods.¹⁹ With modestly higher [CO₃²⁻], such microspheres nucleated the growth of larger conical morphologies, first with rod-like character and then with planar sides. At yet higher [CO₃²⁻], there was an increasing preference for growth of the initial microsphere over cone

formation. This distinguished the fibrous cones from the buds, which had larger, faceted spheres and shorter, less defined rod-like portions, and which indeed occupied a distinct region of chemical space. The microspheres showed an increasing tendency to pair with higher $[\text{CO}_3^{2-}]$. At the highest carbonate concentrations, cones and microspheres ceased to form, in favor of smaller, discrete shapes with increasing faceting. The rice, peanut, and hexagonal shapes decreased in size with greater polymer concentration, supporting PAA's role as nucleating agent.

Many of the morphologies reported here have been described previously, for either CaCO_3 or barium salts, but were formed by employing more complex polymers, instead of altering solution conditions. ACC in the form of flocs has been made previously, using polycations; 31 anionic PEO-b-PAA has been shown to stabilize ACC, although as nanoparticulates rather than flocs.³² Domes have been reported using polycations, although these were amorphous rather than calcitic.³³ Microspheres have been made, e.g., using PLD, although these were characterized as vaterite instead of calcite.¹³ Cölfen et al. reported the use of PEG-b-PMAA to form peanuts ("dumbbells"),³⁴ plotting part of a morphological map as a function of pH and the $[\text{polymer}]/[\text{CaCO}_3]$ ratio.³⁵ PEG-b-PEI-COOH has been used to make BaSO_4 peanuts.³⁶ Rice-like formations have been made using PAA³⁷ or PEO-b-PMAA.³⁸ PEG-b-PEI polymers with hydrophobic moieties of different lengths have been used to fabricate microsphere-, peanut-, quadruple-, hexagon-, and rice-like shapes.³⁹

Morphologies that are similar to the bud and cone structures have also been observed. "Petunias" were fabricated using carboxymethyl chitosan,⁴⁰ and "shuttlecocks" were made using PEG-b-PGL and PEG(84)-b-PHEE with varying degrees of phosphorylation⁴¹; these have been suggested to form around CO_2 microbubble templates.⁴² Cones were made with the combination of two different PAA MWs, and their oriented architectures were attributed to consecutive controlled growth via oriented attachment. Structures that appear similar to the fibrous cones reported here have also been made using PAA, but for BaCrO_4 or BaSO_4 rather than CaCO_3 .^{23,24,43} This study's microspheres and the precursors observed at the tips of the conical morphologies are likely related to the PILPs studied by Gower et al. Although their multi-domained films¹⁷ and rod-like structures¹⁹ are not described in this study's standard ternary diagram, they are readily accessible using different conditions, such as different substrates or different CO_2 diffusion rates.

Moreover, direct comparison of our results with those of many other studies is difficult, because different parameters such as these (substrate and CO_2 diffusion) can so radically alter morphology. For instance, previous studies have used different or unquantifiable methods of introducing CO_3^{2-} , different carbonate salts, and/or different polymers. Furthermore, the chemical space has been less completely determined for these other conditions and methods. From the results obtained here, it would be of considerable interest and benefit to conduct a more thorough analysis of morphological trends over chemical space for these systems as well.

Variations

In order to determine important factors for morphological control, several experimental parameters were varied. Additional morphological diagrams were constructed to test variations of: temperature, ionic strength, carbonate sources, polymer type, and PAA molecular weight. It was found that morphology was primarily controlled by $[\text{CO}_3^{2-}]$, as defined by pH, and by the extent of polymer/ Ca^{2+} association, particularly as functions of ionic strength and polymer type. Increments of 24 μL (10.0%) of the stock solutions were used to prepare solutions within the arrays to determine relative effects.

Temperature—Calcium carbonate's decreased solubility at higher temperatures was reflected in morphological changes in the ternary diagram. Refrigerated samples (4° C)

precipitated very little product, with the exception of buds. Products at 20° C were similar to the 30° C control products, except that cones formed at high $[Ca^{2+}]$, where flocs otherwise occur; this is likely because of decreased polymer association at this temperature. Low- $[Ca^{2+}]$ products (rice, peanut shapes) were larger, reflecting lower nucleation rates. Samples at 40° C also formed cones rather than flocs at high $[Ca^{2+}]$, and additionally formed cones with high [PAA]; low $[Ca^{2+}]$ products appeared smaller, however.

Molecular Weight—Because polymer molecular weight (MW) is known to affect both coacervation (polymer association increases with MW)²⁹ and the antiscaling properties of PAA (with an optimum around 5 kDa)¹⁰, morphological variation with MW was anticipated. Ternary diagrams constructed with different molecular weight PAAs, using constant 10mM per monomer stock solutions, and indeed, despite sharing some overall trends, the diagrams have notable differences (Fig. 2). PAA_{2k} was less potent, failing to inhibit precipitation at higher polymer concentrations, compared to all the higher MW polymers (Fig. 2b). High $[Ca^{2+}]$ did not readily yield flocs, instead promoting cones and rough domes, or no product at all for low $[CO_3^{2-}]$. PAA_{6k} solutions formed cones under more low-carbonate conditions than with other MWs, suggesting that this MW may be near optimal for controlled assembly (Fig. 2a). PAA_{90k} behaved contrarily, more readily forming flocs, and inducing cones only at [PAA] 3mM and below (Fig. 2c). Many shapes, such as peanuts and rice, became rough and indistinct.

Polymer Type—Polyanions PSS, PLD and PLE were also tested, and although they all clearly affected crystallization, only PLD yielded similarly controlled products to those made with PAA (Figs. 3a-c). Of this set, PLD and PAA were the only two to self-associate and form coacervates with Ca^{2+} at room temperature; PLE and PSS formed no precipitate or coacervate. This may imply the importance of a minimal level of dynamic Ca^{2+} binding.

Method of carbonate addition—The present study used aqueous carbonate salt stock solutions, which were mixed directly into mineralizing solutions. Compared with other methods of introducing carbonates, such as the sublimation/infiltration method, pre-dissolving carbonate offers several advantages: the concentration is more easily quantified, by a known initial amount; products are produced more quickly, with a typical lag time of only 3h instead of 8h+; and results are more reproducible because variables affecting the infiltration rate are eliminated. Furthermore, infiltration using crushed ammonium carbonate salts was found to produce some of the same morphologies, indicating that infiltration may also cause high carbonate content and that the methods operate similarly. Because it only offered advantages for these studies, direct addition was chosen.

Carbonate Source, and pH—All three carbonate sources were capable of inducing the morphologies reported here, but required different initial concentrations. The pH values of solutions with ammonium salts remained buffered near bicarbonate's pK_a , typically starting near pH 8.0 and ending around pH 9. In contrast, using sodium salt encumbered controlled precipitation into well defined shapes, because it induced more dramatic pH shifts; however, fine tuning pH and carbonate content made it possible to produce appropriate conditions, which verifies that ammonium is not essential to the process (Fig. 4).

Because an aqueous solution of 0.5 M $(NH_4)_2CO_3$ is equivalent to a 1.0 M NH_4HCO_3 solution with half of its carbonate content converted to $CO_2(g)$, the two salts are directly comparable. In fact, using such stock solutions yielded roughly equivalent diagrams, again suggesting that the initial concentration of carbonates is less important than adequate pH elevation.

Ionic Strength—The imbalance of carbonate compared to the other solution species (roughly 100-fold higher) is particularly striking because marine biomineralization occurs with reversed proportions, in which carbonates total to around 3mM. Since effects on pH change little after

about 100mM, another explanation for such high concentrations is needed. Therefore, the effect of increasing the ionic strength with the carbonate precursors was tested.

It was found that flocs are immediately produced when low levels of carbonate (<100mM) are added to turbid solutions of complex coacervates containing PAA and Ca^{2+} . However, higher carbonate salt content eliminates the turbidity by increasing the ionic strength to inhibit coacervation, and in turn liberates solubilized polymer. Hence, in such solutions, there exists a situation of dually cooperative inhibition, in which an antiscaling polymer inhibits crystallization and the salt content inhibits polymer association. Breaking this cycle requires either heterogeneous nucleation by a high energy surface or a pH increase, which is prominent in these syntheses.

The effect of ionic strength was further tested by adding NaCl to standard starting solutions, to a concentration of 0.5 M. Only products with >4mM $[\text{Ca}^{2+}]$ were altered (mostly inhibited), and most strikingly, solutions that otherwise would yield flocs instead produced a small number of fibrous cones (Fig. 5). This reaffirms the importance of the ionic strength increasing function of carbonate salt for producing complex morphologies with anionic polymers.

Characterization

All products were analyzed by LOM, SEM, CLSM, and various diffraction methods. Conical morphologies (umbrellas, buds, and fibrous cones) were of particular interest because of their complexity and order, which are indicative of a controlled assembly process. The umbrella morphology is new and also exhibits particularly interesting qualities, which are highlighted below.

The mechanism by which the mineral structures grow was examined with real-time optical microscopy of solutions in Petri dishes or in specially made reaction slides containing shallow wells. A typical lag of >20 min preceded precipitation, except with coacervates and flocs, which form sooner—often upon initial mixing. Peanut shapes and other high- CO_3^{2-} morphologies formed while suspended in solution, before eventual sedimentation and further growth on the container surface.

In contrast to the other shapes, cones depend on directional growth at the container surface, following nucleation. The first step is the settling of small precipitates, which initiate growth at the substrate surface. Conical shapes proceed to grow at their bases, where they interface with the substrate, such that the cones point upwards (Animation S1). This provides direct evidence of the growth mechanisms, and is in contrast to previously proposed mechanisms for other similar conical or “flower-like” shapes.^{42,44} The growth direction was confirmed by SEMs of cones grown directly on Si wafer substrates (Fig. S3). The preferential growth of conical morphologies at the crystal/substrate surface suggests that the growth mode is sensitive to surface energetics. In fact, cones grown on different substrates were quite different in appearance, even when different glass surfaces were used (Figs. 6a-c); see also Characterization section below. Similar dependence has been previously observed⁴⁴ and might be utilized to initiate yet further morphological diversity.

SEM images of umbrellas highlight the organization involved during their growth process (Fig. 7a). The structures are evidently modular, indicating growth by stepwise addition of solution precursors, which appear nearly monodisperse despite the polydispersity in polymer starting materials. Their periodicity is reminiscent of patterns found in self-regulating systems such as Liesegang patterning,⁴⁵ and could reflect local fluctuations in pH or other chemical concentrations during formation. Fibrous structures do not appear faceted and may be formed by similar precursors which orient and smoothen, in a process that is akin to the SLS mechanism put forth by Gower et al.¹⁹ Dynamic light scattering measurements confirm that particles on

the order of 200 nm are present in mineralizing solutions for at least 5h. These precursor “seeds” have been observed previously, and are thought to be amorphous.^{16, 32, 46} They have been identified as the cause of granular superstructures in non-classical crystallization processes.³⁷

The crystallinity of products was determined from either powder XRD, single crystal XRD, or TEM, depending on product size, yield, and morphological purity. All crystalline products were indexed to calcite, the thermodynamically most stable form of calcium carbonate. Of note are the flocs, which indicate one means of achieving conditions that stabilize ACC.

Microspheres diffracted as calcite and are widespread across the diagram with a gradual transition from flocs to microspheres. Umbrella fragments analyzed with TEM surprisingly revealed that the modules that make up the structure (Fig. 7a) diffracted as single crystals, confirming the precise, oriented arrangement directed by the growth process (Fig. 7b). Rice shapes were characterized by TEM analysis and appear as single crystals, but are composed of smaller, ~20nm grains (Fig. S4).

Confocal Laser Scanning Microscopy (CLSM)—In order to determine the polymer distributions within various morphologies, a fluorescently labeled polymer was used in the syntheses (a morphological ternary diagram constructed with the labeled polymer did not deviate from the control), and confocal microscopy was used to gather cross sectional images; these images and their three-dimensional reconstructions are available in the supplementary information. The polymer was integrated throughout the structures despite their crystalline properties, although some cross-sections (Animations S2-5) revealed internal regions from which the polymer was partially excluded.

The polymer distributions in the conical products demonstrated some discontinuity, reflecting the organization seen in SEM images. The nucleating tips found on many cones usually had the highest concentration of polymer, followed by the longitudinal “legs.” In agreement with structures observed with electron microscopy, umbrella fragments had a periodic, striped distribution of polymer that defines the border between horizontal layers of what must be crystalline (Fig 7c). A similar effect was evident in images of PILP-type growths, which showed dark (crystalline) rods surrounded by a sheath of excluded polymer (Fig. 7d).⁴⁷ This patterning is likely due to micro-phase separation, in which the crystallizing mineral partially excludes polymer. Similar mechanisms have been hypothesized for hierarchical biomineral structures,^{47, 48} and importantly represents a new methodology of achieving periodic, layered composite materials via cooperative organization on the photonic length scale.

The combined results of the ternary diagrams and the various characterization procedures suggest mechanisms similar to those depicted in Scheme 1. As previously suggested, mineralization begins with metastable solutions, in which dynamic nanoscale complexes form and dissolve in equilibrium, as polymer inhibits $\text{Ca}^{2+}/\text{CO}_3^{2-}$ nucleation and ionic strength inhibits coacervation. This mutual inhibition ceases with an appropriate pH increase, and the precursor aggregate nuclei stabilize. The position in the ternary diagram dictates their properties and their subsequent development to: solidify, crystallize, aggregate, coalesce, precipitate, or assemble interfacially. Their precise compositions and formation mechanisms are unknown, but the various precursor entities might exist as: polymeric coacervates, with or without carbonates; $\text{Ca}^{2+}/\text{CO}_3^{2-}$ solution nuclei or solidified microspherical “emulsion-like forms” capable of undergoing further transformations⁴⁹; transient “micro-ion” coacervates³⁹ of $\text{Ca}^{2+}/\text{CO}_3^{2-}/\text{H}_2\text{O}$; or PILP droplets. Peanuts might also form according to the separate rod-to-dumbbell mechanism,⁵⁰ but the minor presence of similarly sized single spheres and the existence of the quadruple morphology both indicate that colloidal pairing may occur. Centrifugations of conical-product solutions, throughout the mineralization process, yielded only solid precipitates. Therefore the precursors in this region may be distinct from liquid PAA/

Ca^{2+} coacervates, and the fibrous-cone PILPs may be only transiently liquid—or else unstable when aggregated.

Umbrellas are one example of a morphology that forms interfacially, such that the precursor modules successively position themselves into crystallographic alignment at the base. While oriented attachment cannot be ruled out from these experiments, it is reasonable that in these aqueous media of high ionic strength, the precursors are first amorphous and then crystallize once they have fused with the growing structure. Supporting this hypothesis was the observation that a solution of amorphous flocs slowly transformed into several large cones (Fig. S5). Following fusion to the growing structure, crystallization induces micro-phase separation, partially excluding the polymer into layers.

Conclusions

Polyanionic additives are capable of inducing a multitude of morphologies in crystallizing systems, thereby exhibiting various functions in the process. Polymers behave as antiscalants, ACC stabilizers, nucleation sites, and inducers of precursor assemblies—often demonstrating multiple functions at once.⁵¹ This latter “precursor assembly” phenomenon is a useful paradigm for explaining how polymers may simultaneously exhibit these various roles in the form of emergent, modular components and hence create diverse crystal morphologies, dependent on conditions. We have shown that exploring the space of chemical components, using ternary diagrams, is useful for elucidating such morphological diversity. With such diagrams, one can rationally study conditions that promote specific shapes, that selectively promote ACC, or that induce controlled assembly processes. We have thereby demonstrated fundamental connections among previous studies, having shown that all such properties can be achieved by the same polymer. This is of particular benefit to a field that has lacked standard synthetic methods for analyzing the effects of solution additives. The methodology may provide a deeper understanding of biomineralization and indicate new methods for synthesizing high performance composite materials. Given the potential range of possible growth modes, proper characterization in future studies of both synthetic and biological polymers will require testing across chemical space.

The various morphologies were shown to be dependent on several critical parameters, and each can be controlled to reproducibly select mechanisms of crystal growth: low temperature inhibited most assemblies, polymer molecular weight affected the inhibition potency and the degree of association, and polyanions that formed complex coacervates with Ca^{2+} had a more dramatic role in directing crystal assembly. The growth mode of conical morphologies was found to be highly sensitive to substrate properties. Carbonate salt content affects the pH by both buffering and inducing pH increase; pH, in turn, acted as a synthetic “switch” to assembly—a common method in biological systems. Importantly, carbonate salts also control ionic strength, which is especially critical for suppressing polymer association. The direct carbonate addition method, in contrast to other methods such as vapor infusion, was useful for inducing “dual inhibition,” for creating a slow pH increase with time to modulate crystallization, and for providing a concrete measure of carbonate content. We have shown that all of these variables can be modulated to select for certain modes of growth, of both crystalline and amorphous materials.

This systematic investigation has revealed a controlled, interfacial, modular assembly process involving organic/inorganic phase separation. This is manifested in the form of novel, highly organized umbrella morphologies with periodic polymer/calcite layers in crystallographic alignment. Since the PAA and PLD used in these studies are simple and polydisperse, the process is quite general, and such a mechanism should also be accessible with more complex or organized polymers, similar to those used in biomineralization. The amorphous-crystalline

transition method that is common to many biological processes most probably occurs in the growth of complex crystalline phases, with features of alternating organic/inorganic layers, high crystallographic alignment with registry between layers, and, as recently characterized, compositions made up of smaller interconnected crystallites into mesocrystals.^{52,53,54} This controlled-assembly approach represents an easily reproducible method for arranging synthetic materials into periodic structures, with the promise of kinetically and thermodynamically engineering the process to attain desirable mechanical or optical properties.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Standard Morphological Ternary Diagram. The maximum concentrations are displayed at the corners. Inset optical images share the same $100\mu\text{m}$ scale bar. Colored regions and microspheres were all identified as calcite. Enlarged version of the SEM images can be found in the supplementary information (Fig. S1).

Figure 2.
Molecular weight variations (a) PAA_{6k}, (b) PAA_{2k}, (c) PAA_{90k}

Figure 3.
Representative optical micrographs of morphologies using (a) PLD, (b) PLE, and (c) PSS.

Figure 4. Fibrous cones fabricated using sodium bicarbonate. The initial pH was lowered to 7.3 with HCl, in order to compensate for the lack of ammonium buffering. Final concentrations were 5 mM Ca^{2+} , 1 mM PAA, and 45 mM NaHCO_3 .

Figure 5.
Cones made with 125mM NH_4HCO_3 and 500mM NaCl.

Figure 6.
Cones grown on different surfaces: (a) silicon wafer, (b) glass Petri dish, (c) polystyrene.

Figure 7. Umbrella fragments. (a) SEM, showing modular alignment. (b) TEM image, its diffraction pattern, and an image at an alternate angle revealing 2 connected planar sections. (c) Averaged projection confocal micrograph, showing striped pattern of polymer (scale bar 10 μ m). (d) Single plane confocal micrograph, showing a different, PILP-like growth and excluded polymer (scale bar 10 μ m).

Scheme 1.
Proposed mechanisms of formation of the various morphologies