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Calcium carbonate films and helices grown in solutions of poly(aspartate)

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Abstract

Addition of charged polypeptides, in particular poly(aspartate), to supersaturated solutions of calcium carbonate (CaCO_3) leads to unusual morphologies in the inorganic phase. The polymer causes deposition of CaCO_3 films, which appear to act as membranous substrates that mediate the growth of CaCO_3 crystals. Spherulitic vaterite aggregates with helical extensions, and distorted calcite crystals that contain spiral pits, have been produced. A correlation has been noted between the *structures* of the crystalline films and those of the crystal aggregates associated with them: i.e. films of spherulitic texture yield electron diffraction patterns characteristic of vaterite and support the growth of spherulitic vaterite aggregates whereas calcitic single-crystal mosaic films carry rhombohedral calcite crystals. These films and aggregates exhibit features reminiscent of the morphologies observed in biogenic minerals. © 1998 Elsevier Science B.V. All rights reserved.

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1. Introduction

Composite materials have proven useful in a variety of technologies because of the enhanced properties that can be obtained from combining disparate components into a single materials system. An attractive approach to composites fabrication involves concurrent synthesis of components,

so that each may influence the other as it is formed, and thereby control the shape and properties of the final composite material. This approach finds precedent in the natural process of matrix-mediated biomineralization, where the organic component, e.g. a protein matrix or a vesicular membrane, exerts control over the final morphology of the mineral phase by providing nucleation sites or spatial restrictions [1–4]. Morphological control can also be accomplished by adsorption of soluble additives onto specific faces of growing crystals, altering the relative growth rates of the different

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crystallographic faces and leading to different crystal habits [5]. Not only can very elaborate morphologies be obtained, as is evident in the hard tissues of many organisms, but the size, texture, and orientation can also be mediated in biogenic minerals. Because the proteins that have been found to be associated with biominerals are usually highly acidic macromolecules [1–5], we have examined simple polyelectrolytes, such as the sodium salts of poly(aspartic acid) and poly(glutamic acid), as modulators of the growth of ionic crystals. In this study, we have used calcium carbonate (CaCO_3) as the inorganic phase, because the crystals are easily characterized, and because CaCO_3 morphology has been shown to be subject to control in biomineralization processes.

2. Experimental procedure

2.1. Solution crystallization

In a synthesis similar to that described by Addadi et al. [6], the polyelectrolyte of interest was added to a calcium chloride solution, into which carbonate was introduced via vapour diffusion. Glass coverslips (2.2 cm diameter), used as substrates, were cleaned by soaking overnight in an acid solution of Nochromix, rinsed with distilled water, and dried with acetone. Each of the coverslips was placed in a Falcon polystyrene petri dish (3.5 cm diameter) containing 2.7 ml of a filtered 12.5 or 20 mM calcium chloride solution (freshly prepared with boiled doubly distilled H_2O). Appropriate microliter volumes of the dissolved polymer (0.8 mg/ml of poly(α ,L-aspartate), $M_w = 14\,400$, Sigma; or $M_w = 6000$, ICN; or poly(α , β -D,L-aspartate), $M_w = 6850$, Sigma) were transferred to the dish by micropipet. One dish, containing no additives, was run as a control for each set of experiments. The dishes were then covered with parafilm, which was punched with 3 needle holes, and placed in a large desiccator. Three small vials (5 ml) of crushed ammonium carbonate were also covered with parafilm punched with one needle hole and placed in the desiccator. After 3 days (7 days at $T = 4^\circ\text{C}$), the coverslips were removed, rinsed with distilled water and ethanol, and allowed to dry at

room temperature. The coverslips were examined by optical microscopy, and then gold coated for scanning electron microscopy on a JEOL 35cf SEM instrument.

2.2. Diffraction techniques

Diffraction techniques were used for the determination of polymorphic composition of the CaCO_3 particles and films. The spherulitic polycrystalline particles (i.e., the rounded particles, some of which contain helical extensions) were scraped from the glass coverslip, crushed to a powder, and adhered to double-sided tape for mounting in a Debye–Scherrer X-ray apparatus (Ni-filtered $\text{Cu K}\alpha$ radiation) for wide-angle X-ray diffraction (WAXD) studies. Individual helices were separated from other particles by micro-manipulation on the optical microscope before being crushed and placed on carbon-coated copper TEM grids. Films, most of which were mosaics of single crystals, were scraped off the glass substrate and transferred to glass capillary tubes for WAXD analysis (the particles were removed and rinsed away prior to film removal). Films examined by electron diffraction (ED) were gently removed, while in solution, from the glass coverslip and micro-manipulated onto copper grids for electron microscopy. They were dried at room temperature, and a thin layer of gold was applied for calibration of camera length on the JEOL 100cx STEM used for ED analysis. For the single-crystal films, a series of tilt angles was used to obtain spacings of multiple zones, while the spherulitic films yielded oriented ring patterns.

3. Results and discussion

3.1. Aggregates containing helical protrusions

A control dish (without polymer), run with each set of reactions, produced rhombohedral calcite crystals approximately $40\ \mu\text{m}$ in size. It was expected that addition of a highly charged polyelectrolyte, such as poly(aspartate), would result in nonspecific inhibition of crystal growth [7]. Instead, it was found that low levels of polymer led to unusual morphologies in the inorganic phase. At

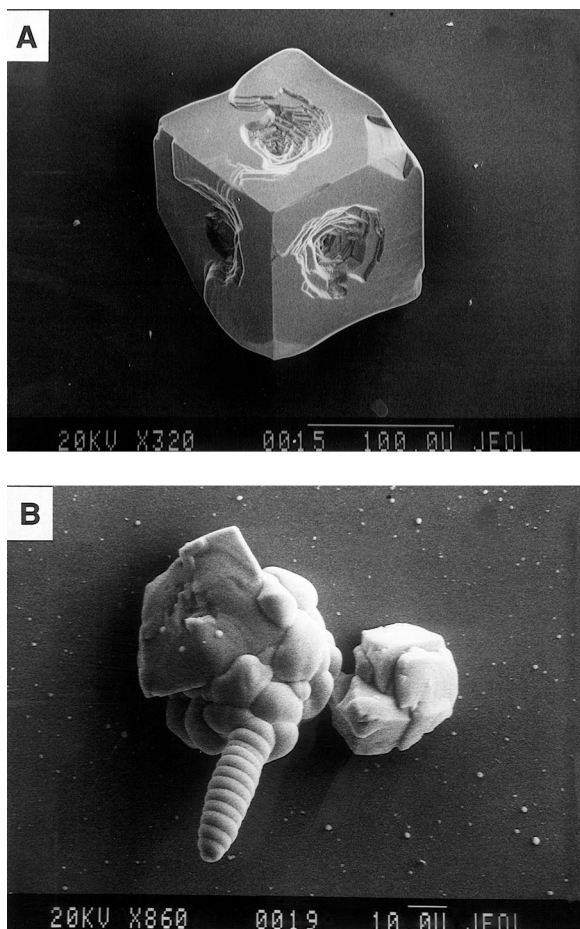


Fig. 1. Scanning electron micrographs of the unusual morphologies of CaCO_3 minerals grown in solutions of poly(aspartate). The control synthesis, not shown here, yields rhombohedral calcite crystals that are typically 20 to 60 μm in size. (A) A calcite crystal containing spiral pits resulting from the addition of 0.5 $\mu\text{g}/\text{ml}$ poly(α,L -aspartate) to the crystallizing dish. Bar = 100 μm . (B) A crystalline aggregate containing a helical protrusion resulting from the addition of 10 $\mu\text{g}/\text{ml}$ poly(α,L -aspartate). Lobed spherulites as well as distorted rhombohedral crystals are present in the aggregate. Bar = 10 μm .

a concentration of 0.5 $\mu\text{g}/\text{ml}$, the rhombohedral habit of calcite became distorted, and in some cases, large spiral pits formed in the centers of the crystal faces (Fig. 1A). At this level, the inhibitory action was strong, and only a few, larger crystals were formed. In the concentration range 1–5 $\mu\text{g}/\text{ml}$, the calcite crystals tended to form aggregates, and at

higher levels (5–30 $\mu\text{g}/\text{ml}$), the calcite aggregates were replaced by aggregates of rounded particles. What is particularly interesting is that these lobed aggregates frequently grew helical protrusions (Fig. 1B). Many of the helices extend upward from the substrate, so it could easily be determined that both right- and left-handed helices were formed. It is not essential that the polypeptide be chiral to produce these helical structures; even achiral poly($\alpha,\beta\text{-D,L}$ -aspartate) produces helical protrusions. Through variation in the concentration of calcium or polymer, polymer molecular weight, or reaction temperature, longer helices were produced (up to 700 μm long), with up to 70% of the aggregates containing one or more helical protrusions.

Wide angle X-ray diffraction (WAXD) shows that these rounded particles are composed of vaterite, a metastable polymorph of CaCO_3 , and electron diffraction (ED) on crystals obtained from a crushed helix confirms that the helical extensions are also vaterite (Table 1, columns 1 and 2). When the mineral phase is etched with acid, the spherulitic texture of the rounded particles and helices can be seen, and a “membrane” which appears to be more resistant to the acid is also revealed (Fig. 2A). This membrane is also clearly visible when the helices are fractured (Fig. 2B). Despite its resistance to acid etching, the membrane is not simply a precipitated form of calcium poly(aspartate); indeed, the polymer concentration is sufficiently small that none of the organic component can be detected by energy dispersive X-ray spectroscopy. Occasionally, the helices are hollow (Fig. 3A), and the spherulitic crystals appear to have grown inward from the membrane template (Fig. 3B) rather than from a central core. A similar phenomenon occurs in some biogenic minerals; for example, in the otoliths of fish, spherulitic growth is induced by nucleation sites distributed along a surface, and the aragonite or vaterite crystals grow with their *c*-axes approximately perpendicular to the surface [1,8]. Pach et al. [9] discuss several factors, including viscosity, ionic interactions, and steric effects, which can give rise to oriented crystal growths at an organic–inorganic interface.

At higher concentrations (10–30 $\mu\text{g}/\text{ml}$) of poly(aspartate), a highly birefringent inorganic film forms in streaks and patches on the glass substrate

Table 1
Lattice spacings determined by diffraction techniques

WAXD: lobed particles (Å) ^a	ED: crushed helix (Å) ^a	ED: dry spherulitic film (Å) ^a	WAXD: wet mosaic films (Å) ^b	ED: dry mosaic film-type 1 (Å) ^b	ED: dry mosaic film-type 2 (Å) ^c
	4.23	4.22	3.91	3.88	4.18
3.56	3.58	3.57	<u>3.07</u>	3.06	3.97
<u>3.28</u>	3.32	3.30	2.51	2.50	3.74
2.73	2.72	2.74	2.31	2.30	3.02
2.06	2.11	2.13	2.11	2.11	2.69
1.82	1.81	2.06	1.93	1.94	2.50
		1.85	1.89	1.84	2.26
					2.09
					1.96
					1.86

–Underlined spacings indicate very strong intensity in WAXD powder patterns.

^aMatches spacings given for vaterite in the *International Center for Diffraction Data* (1995) X-ray powder file #24-0030.

^bMatches spacings given for calcite #24-0027.

^cReasonable match to spacings given for ikaite ($\text{CaCO}_3 \cdot 6\text{H}_2\text{O}$) #37-0416, except for the 3.74 spacing. The larger spacings given for the hexahydrate were not observed within the tilt range available. The 4.18 and 3.97 spacings eliminate the possibilities of calcite, aragonite, vaterite, and monohydrate, unless they were distorted by the additive [12] or by mechanical stress [14].

(Fig. 4). Because the particles discussed above are frequently associated with such films, we suggest that it is the film that ultimately becomes the “membrane” surrounding the particles; the fact that some of the helices are hollow suggests furthermore that the membrane plays a key role in producing the helical twist. The relationships between reaction conditions and the number and type of helices formed are complex. Lower temperatures (e.g. 4°C) favour film formation and produce fewer helices relative to room temperature reactions, and helices grown at reduced temperatures tend to be short and stout (e.g. 200 µm wide × 250 µm long). Trends are also noted with respect to polymer concentration, in that higher concentrations produce more films and helices (to a limit of ca. 20 µg/ml). Lower molecular weight poly(aspartate) (e.g. polymer of viscosity-average molecular weight 6850) was found to be substantially more effective at producing films and helices than longer chains (molecular weight 36 300).

3.2. Calcium carbonate films

The inorganic polycrystalline films (typically of submicron thickness) form in a variety of textures

and appear to control the type of particles produced. Mosaic films composed of interconnected single crystals, tend to carry aggregates of rhombohedral calcite crystals (Fig. 4A), whereas films of spherulitic texture have spherulitic vaterite particles (lobed and helical) associated with them (Fig. 4B). Although the 50–200 µm patches within the mosaic films frequently contain many defects, they exhibit single-crystal extinction in polarized light microscopy, whereas the spherulitic films show moving cross lines as the microscope stage is rotated. These two film textures can also be distinguished by electron diffraction; the single-crystalline mosaic films yield single-crystal spot patterns, while ED patterns of the polycrystalline spherulitic films display oriented arcs. Electron diffraction also reveals that these different film textures correspond to different CaCO_3 phases; spacings that match those of vaterite are found for the spherulitic films, whereas calcite spacings are obtained for some of the single-crystalline films (Table 1, columns 3–5).

Some films exhibit spacings which do not match any of the three anhydrous CaCO_3 polymorphs (calcite, vaterite, and aragonite), but instead show a reasonable match to the hexahydrate form of CaCO_3 (Table 1, column 6). The hexahydrate is an

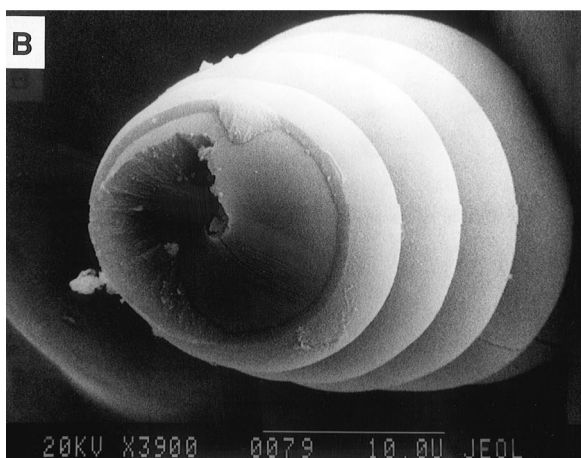
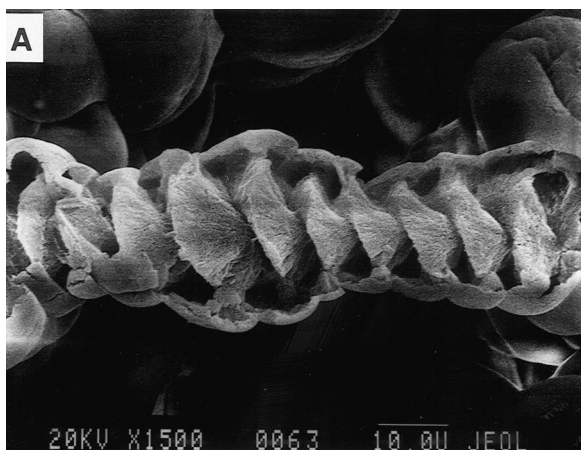


Fig. 2. Two techniques show that the helical particles are spherulitic and surrounded by an outer membrane. (A) A helical particle that has been etched with 0.2% acetic acid for 10 min, neutralized with 0.1 N NaOH, and then rinsed with distilled water [23]. The surrounding membrane appears to be more resistant to the acid, and the polycrystalline texture is revealed. Bar = 10 μm . (B) A helix that has been fractured, revealing an outer membrane and polycrystalline core. The helices were fractured using a micro-manipulator on an optical microscope. Bar = 10 μm .

unstable form of CaCO_3 which can be produced at low temperatures [10] or through addition of aspartic acid [11] or polyphosphate [10–13]. Our observations are consistent with these reports, in that much more film is produced at 4°C than at room temperature. The limited stability of the hexahydrate phase is reported to be highly dependent

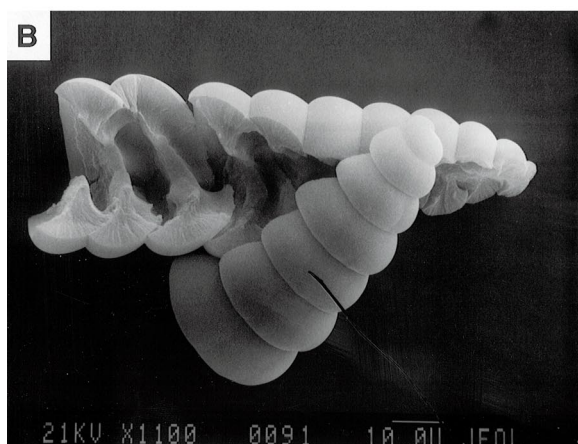
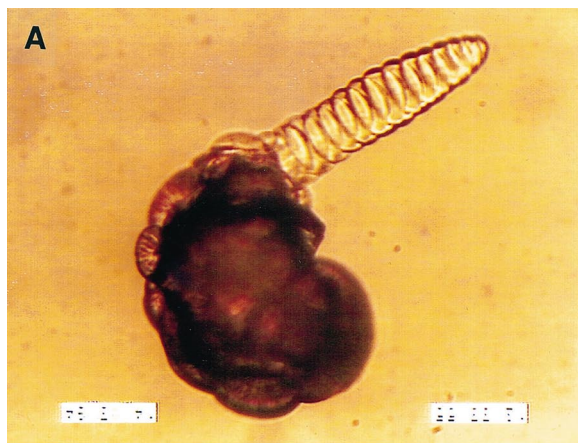


Fig. 3. Occasionally, the helices are partially hollow. (A) The hollow helices appear transparent on the optical microscope, and are highly birefringent when viewed under crossed polars. The solid helices have a different appearance on the optical microscope; the spiral lines are not visible across the dense helix. (B) The same hollow helix fractured by micro-manipulation. The fracture is different from that of the solid structures, as shown in Fig. 2B. Bar = 10 μm .

on the purity of the crystals [12], and can make it difficult to obtain reliable diffraction data. Conoscopic examination of the films in polarized optical microscopy shows biaxial interference figures for some of the films [14], consistent with the monoclinic structure of the hydrated forms of CaCO_3 (calcite and vaterite are uniaxial structures). This was particularly the case for the bottom layers of some films which grew in layers,

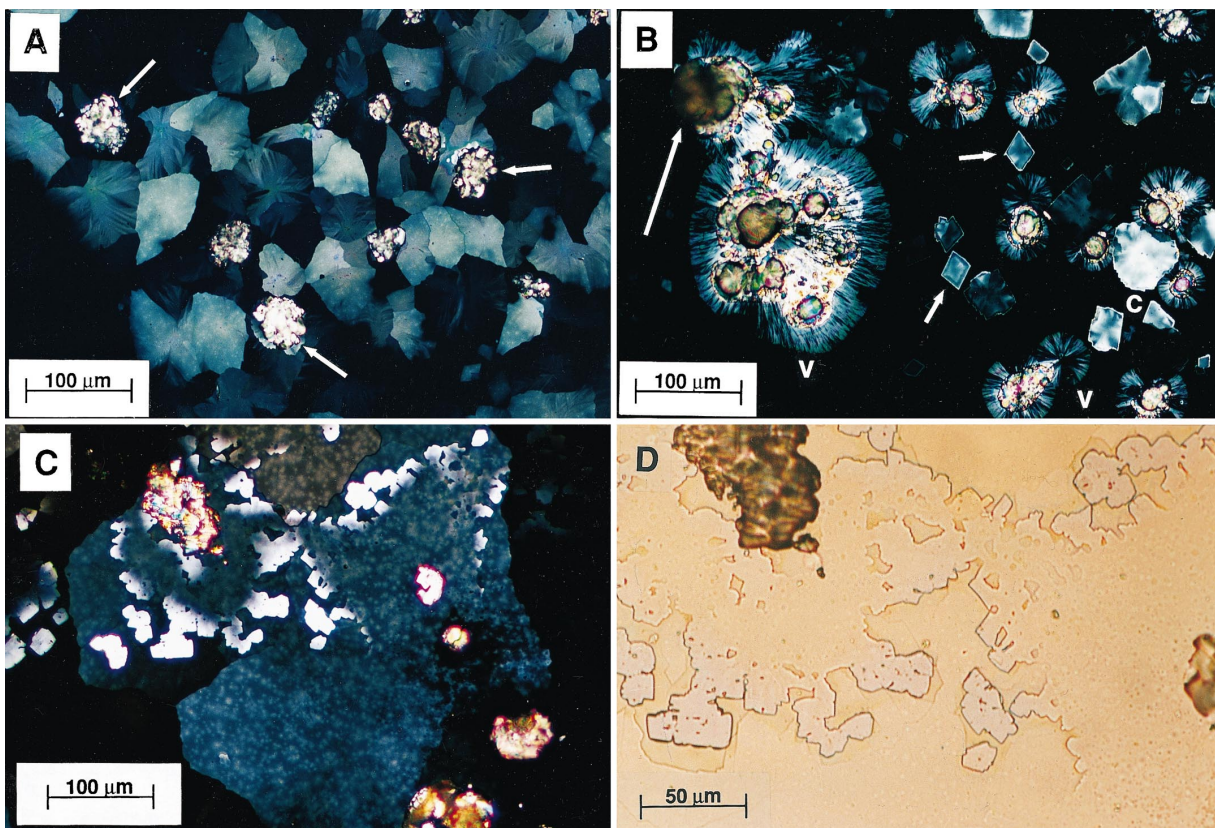


Fig. 4. At higher concentrations of polyaspartate (20 $\mu\text{g/ml}$), an inorganic film forms on the glass coverslip in addition to the membrane on the particles. Under crossed polars on the optical microscope, the film is highly birefringent. (A) A mosaic film composed of a patchwork of interconnected calcite crystals. Calcitic aggregates frequently grow on the mosaic films (arrows). (B) Patches of spherulitic film composed of polycrystalline vaterite (marked with v), interspersed with calcite single-crystal patches (marked with c). Note, the spherulitic film is not always circular in form, but is termed spherulitic because of the radial type growth of the polycrystals. Lobed vaterite aggregates have grown on the spherulitic film, including a helix in the center of the top left aggregate (which is blurred due to the limited depth of focus of the optical microscope, long arrow). These films were produced using poly(α,β -D,L-aspartate), which tends to yield thicker and less connected films, where the morphology of the calcite crystals is now in the form of faceted tablets (short arrows). In this example, the edges of the tablets are thicker and appear as white rims. (C) A granular texture is seen on many of these films. Layering is also a common feature, which can be seen here as white patches that stand out due to the higher-order interference color of the thicker crystal. (D) Without crossed-polars, the layering of this film resembles micrographs of the growing surface of nacre (see, for example, Figs. 2, 16, and 3c from Refs. [5,20,22], respectively).

suggesting that the hydrated phase, which typically forms a platy habit, may serve as a precursor to the other more stable CaCO_3 phases. Dickens and Brown [13] have suggested that hydrophilic polymers might stabilize the nuclei of hydrated salts relative to the anhydrous forms, and a precursor phase might then influence the habit and identity of the final crystal products.

3.3. On the morphogenesis of helices

How does the organic polymer cause the inorganic film to precipitate, and how does this film mediate the morphology of the crystal products? Citing our preliminary unpublished report [15], Sims et al. [16] have also described “helicoïd outgrowths of stacked vaterite disks”, and proposed

a morphogenic mechanism based on an amorphous CaCO_3 -peptide gel that serves as a membrane that inhibits crystal growth, leading to episodic outgrowths. We note, however, that the ruptured membranes that they describe are rarely observed on the helices in our system, and only minute amounts of the polyelectrolyte are required to produce these effects. We reiterate that the film is not simply – or even predominantly – poly(aspartate); no film is deposited from poly(aspartate) solutions containing CaCl_2 until carbonate is introduced, and X-ray microanalysis is insufficiently sensitive to detect the organic material on either the film (or the helical membrane) surfaces.

Garcia-Ruiz and Amorós [17] have also reported alkaline-earth carbonates which form helical morphologies, such as braids that grow out of planar spherulitic aggregates. The presence of an ordered inorganic membrane appears to be a key feature in each of these systems, though the membranes are of different chemical compositions. Of all the polymeric additives we have examined, only poly(aspartate) has yielded helical outgrowths and well-defined inorganic films on the glass substrate (although poly(glutamate) produces membrane covered calcitic aggregates).

3.4. Analogies to CaCO_3 biominerals

To our knowledge, the in vitro precipitation of a calcitic mineral in the form of a film has not been observed before. We note, however, that some features of these products resemble the morphologies found in biogenic minerals. In particular, the nacre of mollusc shells consists of lamellar sheets of aragonite tablets (or calcite in bryozoan semi-nacre [18]), separated by a thin organic matrix called the conchiolin membrane [19–22]. The thickness of these CaCO_3 mineral layers (about $0.5\ \mu\text{m}$) is similar to that of the CaCO_3 films reported herein. In fact, the thinner calcitic films, such as those pictured in Fig. 4A, are iridescent, reminiscent of the beautiful mother-of-pearl texture that arises from diffraction of light across the thin tablets of nacre. Furthermore, the biogenic tablets composing the nacreous and semi-nacreous layers tend to vary from anhedral to polygonal shapes, similar to the

components of poly(aspartate) produced films and tablets (Fig. 4). Micrographs of mollusc nacreous layers typically illustrate the complex hierarchical organization of the CaCO_3 tablets [21], but when pictured in plan view, the growing surface of mollusc nacre resembles the layered films and tablets reported here [5,20,22].

4. Conclusions

This paper describes unusual morphologies generated by a polyelectrolyte-mediated crystallization of calcium carbonates, in which the polymer appears to stabilize the metastable hexahydrate and vaterite forms of calcium carbonate. Deposition of calcite and vaterite films influences the types of crystal aggregates subsequently formed, as well as the twisting of the vaterite spherulites into helical structures. Current studies address the mechanisms of film formation and helical growth induced by poly(aspartate), and the relevance of these mechanisms to biomineralization and to controlled deposition of inorganic coatings and films.

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