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THE INFLUENCE OF POLYASPARTATE ADDITIVE ON THE GROWTH AND MORPHOLOGY OF CALCIUM CARBONATE CRYSTALS

A Dissertation Presented

by

LAURIE A. GOWER

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
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Department of Polymer Science & Engineering

THE INFLUENCE OF POLYASPARTATE ADDITIVE ON THE GROWTH AND MORPHOLOGY OF CALCIUM CARBONATE CRYSTALS

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DEDICATION

This work is dedicated to my father, Dr. Warren M. Barrett, who passed away during my second year at the University of Massachusetts. He was a man with a deep passion and respect for learning, and would have been very proud to have attended my Ph.D. defense.

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ABSTRACT

THE INFLUENCE OF POLYASPARTATE ADDITIVE ON THE GROWTH AND MORPHOLOGY OF CALCIUM CARBONATE CRYSTALS

SEPTEMBER 1997

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The addition of low levels of polyaspartate to a supersaturated calcium carbonate ($CaCO_3$) solution leads to unusual morphologies in the inorganic phase. Spherulitic vaterite aggregates with helical protrusions, and distorted calcite crystals that contain spiral pits, have been produced. The helical particles are coated with an inorganic membrane that appears to be responsible for the helical twist. The polymer also causes deposition of thin $CaCO_3$ tablets and films on the glass substrate. Two distinct types of films are deposited; the first is a mosaic of calcite crystals, and the second is spherulitic vaterite.

In situ observations of the crystallization reaction have determined that the thin-film morphology is a result of the phase separation of a hydrated $CaCO_3$/polymer liquid-precursor, whereby accumulation of isotropic droplets creates a coating on the substrate, and subsequent dehydration and crystallization yields birefringent $CaCO_3$ films. During the amorphous to crystalline transition, incremental growth steps lead to "transition bars" and sectored calcite tablets.

This *in vitro* system was originally modeled after certain aspects of $CaCO_3$ biomineralization, in which the soluble proteins extracted from biominerals tend to have high levels of aspartic acid residues. Based on the similarities between features exhibited by the products of this system and those in biominerals, an argument has been presented

to suggest that this polymer-induced liquid-precursor (PILP) process is involved in the morphogenesis of $CaCO_3$ biominerals. These features include the following: thin $CaCO_3$ tablets that grow laterally; tablets that express unstable crystallographic faces; non-faceted single crystals with curved surfaces; spatially-delineated single crystals; sectored calcite tablets; hollow-shell spheres; calcium carbonate cements; and magnesium-bearing calcites.

This work has demonstrated that a means of morphological control can be accomplished through non-specific organic/inorganic interactions, whereby the polyelectrolyte transforms the solution crystallization to a solidification process. Not only are such findings of significance to the field of biomineralization, but a better understanding of the interactions between polymers and inorganic materials may be expected to lead to new strategies for crystal and particle engineering.

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CHAPTER 1

INTRODUCTION

This dissertation will examine the influence of polymeric additives on the growth and morphology of inorganic crystals, with the particular system of study being that of polyaspartate additive in precipitated calcium carbonates. After some preliminary studies using an iron oxide system, the calcium carbonate ($CaCO_3$) system was ultimately chosen for its ease of synthesis and characterization, and in particular, because the wide range of morphologies found in $CaCO_3$ biominerals seemed to suggest that there is ample opportunity for manipulation of this inorganic system. It became apparent early on in the investigation that, indeed, $CaCO_3$ crystals could easily be altered by using additives, for a variety of randomly chosen polymers produced a whole spectrum of crystal morphologies (see Appendix A). A description of the different polymorphic forms of $CaCO_3$ is given in Appendix B, and some pictures of the typical morphologies of the three anhydrous forms are included.

It was these preliminary experiments that changed the direction of this project, which was originally intended to be an examination of "specific" structurally-based interactions with periodic polypeptides, but instead became an examination of "non-specific" chemically-based interactions with polyaspartate additive. Comparisons will be made throughout this report between "specific" and "non-specific" interactions, therefore these terms will be defined in the next section. The polyaspartate additive was chosen for a more intensive investigation because it produced some unusual $CaCO_3$ morphologies (described in Chapter 1). Although the influence of polyaspartate on calcite growth has already been examined by others (because of the prevalence of aspartic acid residues in proteins extracted from biominerals),[1-3] it became apparent that these studies had overlooked some interesting phenomena that might have relevance to biomineralization, as well as to materials science in general.

As is already apparent, the theme of biomineralization will be prevalent throughout this dissertation because many of the theories developed by those in this field pertain to organic/inorganic interactions, where such interactions are believed to play a crucial role in the regulation of biomineral properties. Thus, this introductory chapter will include a brief discussion on biomineralization, with an emphasis on the role of organic additives in mediating $CaCO_3$ properties. The three subsequent chapters will be presented in the form of three inclusive papers, which describe the results of this project in essentially chronological order. The relevance of this work to biomineralization will be discussed in Chapter 4, which relies on pre-existing theories on biomineralization, as introduced in the first chapter, and the morphological characterization described in Chapter 2, and the process description of Chapter 3. The concluding Chapter 5 will tie these results in with the pre-existing theories to form a generalized theoretical scheme of the processes involved in biomineralization, and will propose how an understanding of such processes might be utilized by the materials engineer.

The Influence of Polymers on Inorganic Crystal Growth

Specific versus Non-Specific Interactions

The issue of specific versus non-specific interactions is discussed throughout this dissertation because of its relation to theories on the mediation of crystal morphology by organic additives. These terms will be defined as follows:[2] Specific interactions are considered to be structurally-based, whereby the atomic arrangement of the additive has a geometrical or stereochemical match to specific lattice planes of the crystal, such that it adsorbs preferentially to those planes. Non-specific interactions are considered to be only chemically-based, whereby the additive adsorbs to various crystal faces in a more-or-less random fashion.

The adsorption of an additive, and especially a large molecule such as a polymer, will usually inhibit or retard the growth of a crystal; the degree of inhibition will depend on the concentration, molecular weight, type of interaction, etc..[2] Because of this inhibitory action, polyelectrolytes have found use as anti-scale agents in industrial processes. Less potent inhibitory interactions may allow for some crystal growth, but will usually alter the morphology of the crystal products. In the case of non-specific interactions, the crystals tend to become poorly defined. For example, Addadi et al.[2] describe non-specific interactions as leading to crystals with rounded edges and rough, stepped faces. Similar morphologies were seen for several of the polymeric additives described in Appendix A. In the case of specific interactions, a change of crystal habit can be brought about by blocking or reducing the growth rate of those specific faces to which the impurity has adsorbed.[4] The slower growing faces will become larger, relative to the unaffected faces. A specific interaction can also lead to the expression of new faces, which would normally not be stable under the given set of conditions.

While the morphological alterations described above arise from the interactions of an additive with a crystal surface, non-specific interactions can also include chemical interactions between the polymer and the reacting species, prior to the formation of a solid interface (i.e. in anti-scale agents). While additives are more commonly considered to be inhibitors, it has been suggested that some additives can act as accelerators of crystal growth,[5] perhaps through stabilization of the forming nucleus through surface-active properties,[6] or through concentration of ionic species.[7] The latter case has been demonstrated for the sulfated fraction of the organic matrix in mollusk shells, in which it was shown that calcium binding by the polyelectrolyte induced local anion binding, which then induced secondary calcium binding This type of non-specific interaction has been termed ionotropic nucleation.[7, 8]

Lastly, polymers can cause aggregation or dispersion of crystals and particles, especially when electrostatic interactions are involved. Aggregation effects were seen for some of the polymers listed in Table A.1, and in particular for polyaspartate, polyglutamate, and poly(methacrylic acid). Control over aggregation is a fundamental issue for many industries in which the dispersion of particulates is critical to the handling and final properties of the products.

Soluble Additives versus Insoluble Matrix

While soluble polyelectrolytes usually inhibit crystal nucleation and/or growth, polymers bound to, or in the form of, an insoluble matrix can behave as substrates for crystal nucleation and growth.[2, 9] Heterogeneous nucleation on a charged substrate may be promoted by ionotropic effects, or more specifically, through templating interactions. Polymers, and in particular proteins, can adopt conformations that lead to well-defined secondary structures (e.g. a β-sheet or α-helix), and this can provide an organized array of functional groups that are positioned to interact with the crystal in a specific fashion. In this context, β-sheet structures that contain uniformly-spaced carboxylate groups on the side chains have been implicated as a possible matrix for specific interactions in biomineralization.[10, 11] In particular, a "stereochemical effect" has been described by Addadi and Weiner,[4] in which it was shown that calcium-loaded acidic proteins from a bivalve shell (*Mytilus californianus*) adopt a β-sheet conformation that binds in a stereospecific fashion to similar faces of different calcium salts. The stereochemistry relies on the carboxylate groups of the polymer, which are oriented approximately perpendicular to the plane of the crystal face, so that the protein-bound calcium ions can optimally complete their coordination polyhedrons. Similar orientational effects have been seen with other substrates; for example, [0001] oriented vaterite crystals were grown under compressed monolayers of stearate, and the interactions were attributed to a cooperative effect of both electrostatic and stereochemical matching.[10] In both of these cases, it is the uni-charged (001) planes that are at the interface. The most common orientation for biogenic $CaCO_3$ crystals is also with the *c*-axes perpendicular to the plane of the matrix,[9] thus the stereochemical effect may be involved.

An insoluble matrix can also be in the form of a compartment. Compartmentalization is deemed a fundamental aspect of biomineralization,[5, 9-11] or at least in the case of biologically controlled mineralization, in which a high degree of control is exerted over the properties of the mineral phase (as opposed to biologically induced mineralization). The compartment may or may not be polymeric, such as for example vesicles versus extracellular matrix. It is believed that such compartments provide spatial delineation to the growing mineral phase, and thereby determine the size and morphology of the crystal within. While this concept is easily rationalized for molding of an amorphous precipitate, it is not obvious how this could be accomplished for a crystal, being that the crystallochemical driving forces that lead to faceting are generally quite strong. The ability to mold a single crystal within a compartment has not been experimentally verified, and most evidence would suggest otherwise. For example, iron oxide particles grown within reconstituted protein cores of magnetoferritin still adopt a faceted habit.[12] It has been suggested that the compartment wall could contain inhibitor molecules that cause cessation of crystal growth when the boundary of the compartment is reached.[9] In particular, this idea was offered as a possible explanation for the development of the unusual morphology of the sea urchin spine, to be described in the next section.

Biomineralization

A variety of minerals are found in living organisms, ranging from calcium phosphates in teeth and bones, to amorphous silica in diatoms, to iron oxides in magnetotactic bacteria. For the sake of brevity, this introduction will strictly focus on $CaCO_3$ biominerals, and the reader is referred to several outstanding review articles and books on the general subject of biomineralization.[5, 9-11] However, one point to keep in mind is that many of the fundamental concepts pertaining to the manipulation of mineral phases may be similar, even though the final properties will be quite different due to the inherent differences in chemical and crystal structures (including amorphous precipitates).

Control of Biomineral Properties

For the sake of illustration, two examples of $CaCO_3$ biominerals will be described, the nacre of mollusk shells, and the spines of sea urchins. Unless otherwise stated, the information in this section can be found in the general review articles mentioned above. These examples were chosen because they illustrate quite nicely the various types of control that can be accomplished in biomineralization, and because they will frequently be referred to throughout this dissertation as comparisons are made to their unusual morphologies. These two biomineral systems have been widely studied in this field because of the high degree of control that is exerted over the individual crystals which compose the mineral phase, and because the composition and organization of these crystals leads to enhanced mechanical properties. Certainly, an understanding of the mechanisms responsible for such control would be of value to the materials scientist.

Mollusk Shell. The first example is the mollusk shell. The mollusk shell is composed of discrete sections that can be distinguished by their morphological characterisitics, and these sections are frequently composed of different crystal polymorphs. For example, in *Mytilus californianus*, a well-studied bivalve, the outer layer of the shell is composed of calcite crystals with a prismatic structure, while the inner layer, called the nacreous layer, consists of lamellar sheets of aragonite. Each lamellae (typically half a micron thick) is composed of an array of aragonite tablets that have preferentially grown in the lateral dimension and fused to form a continuous sheet, and the sheets are separated by a thin organic matrix (10 to 50 nanometers thick) called the conchiolin membrane.

Already, these initial observations pose some interesting questions pertaining to morphological control in biogenic minerals. For example, the tabular habit of the nacreous crystals is quite atypical of aragonite, which when grown *in vitro* forms acicular crystallites (see Appendix B). The restraint leading to lateral growth of the tablets is also puzzling, but is thought to arise from spatial delineation by the conchiolin membrane, or through cessation of growth by inhibitor molecules. Another point of interest to crystal engineers is the ability to control which crystal polymorph is deposited. For instance, in the above example, the nacreous layer is composed of aragonite while the prismatic layer is composed of calcite, both of which are formed within the same organism.

Control is also exerted over the orientation of the aragonite tablets, which presumably arises from nucleation on the insoluble matrix. The aragonite tablets are oriented perpendicular to their *c*-axis, relative to the matrix, and thus express the (001) faces. The (001) faces are not expressed in the typical habits of aragonite (and calcite) because high surface energies are generated by the unicharged layer of Ca^{2+} ions in the (001) plane. Thus, it has been implicitly assumed that the (001) faces on the nacreous tablets are stabilized by the conchiolin membrane, or by preferential adsorption of an additive. In some species, it has been shown that the orientation is controlled in the *a,b*-directions as well, relative to the structured β-chitin/protein complex in the organic matrix.[9] Such orientational control would presumably require a highly specific interaction (i.e. epitaxy) during the nucleation event.

At this point, it is worth mentioning that another unrelated marine organism, called cyclostome bryozoan, has calcite tablets of a very similar morphology and *c*-axis orientation as the aragonite tablets in nacre.[13] While these tablets are less organized than nacre (and are called semi-nacre), it is possible that their similar morphological characteristics are generated by a similar mechanism.

Lastly, control is exerted over the location and organization of the nacreous tablets. For example, the nacreous tablets in some species are placed one on top of another in such a fashion as to form stacked hierarchical structures. However, there is a slight offset in the alignment of the stacks, such that the tablets overlap to form a brick-and-mortar type structure, and this imparts tough mechanical properties to the shell.

The issue of localization of crystals brings up an important point in regards to biomineralization, and that is the spatial and temporal relationship between the various processes occurring within the cell. This report makes no attempt to deal with the complexities that arise from cellular control, even though this is clearly a most important factor. Instead, the experiments presented here are based on a simple *in vitro* system, with the goal of developing a better understanding of the interactions that occur between organic and inorganic materials.

To summarize, it is seen that, in the mollusk shell, control is exerted over the size, shape, polymorph, orientation, and location of its crystal components.

Sea Urchin Spine. The second example is the spine of a sea urchin, and this example is included to illustrate another type of morphological control, the formation of spatially-delineated single-crystals. The spine is reported to be essentially a single-crystal of calcite (as determined by diffraction and epitaxial overgrowth experiments),[9, 14] but yet has a very convoluted and fenestrated structure that lacks the faceted morphology that one would expect to see for solution grown calcite. These large single crystals grow intracellularly within very large vesicles that are produced by the fusion of cell wall membranes into a large vacuole, inside of which the mineralizing vesicle forms. Spatial delineation by the vesicular compartment is believed to be the governing factor for the formation of these types of crystal morphologies, but as mentioned above, the mechanism by which this occurs is not well understood. This type of morphological control, the formation of single-crystals with smooth, curved surfaces, while quite different from that of the nacreous tablets, is found in a variety of other $CaCO_3$ biominerals.[10, 11] The formation of such non-equilibrium morphologies represents one of the classic mysteries of biomineral formation.

Control is also exerted over the texture and composition of these crystals. The spine was previously said to be composed of calcite, but more specifically, it is a magnesium-bearing calcite. Considering the high level of magnesium-ion (Mg-ion) present in seawater ($Mg^{2+} \approx 0.05$ M), this might not seem surprising; however, inorganically grown calcite does not usually incorporate high levels of Mg-ion (Mg-poor calcites are considered to be less than 1% $MgCO_3$), but instead, increasingly higher proportions of aragonite are formed as the concentration of Mg-ion is increased.[15, 16] However, magnesium-rich calcites are found in a variety of biogenic minerals, with the highest reported levels being around 30 wt.% $MgCO_3$ in calcareous algae.[15] Kitano et al.[17, 18] have shown several *in vitro* methods for increasing the magnesium incorporation into calcite, such as by using a carbon dioxide escape synthesis (with yields of up to 5.5% $MgCO_3$) and especially by adding organic calcium-complexing species, such citrate, pyruvate, and malate of sodium (with yields of up to 12% $MgCO_3$ from a solution of 0.4 g/l Ca^{2+} and 1.2g/l Mg^{2+} and 3g/l sodium citrate). Therefore, they suggested that the crystal form and the minor element distribution found in calcareous biominerals are a result of organic materials present in the parent medium of the marine organism.

An additional point of interest related to composition is the occlusion of proteins within the inorganic crystal. The conchoidal, glassy type fracture that occurs for this "single-crystal" of a calcite (calcite normally fractures along well-defined cleavage planes), is thought to be caused by disruption of crack propagation from the occlusion of proteins along specific crystallographic planes.[9]

To summarize for this second example, the following two additional types of control are added to the list: the control over crystal composition (which could include both inorganic and organic impurities/additives); and the formation of spatially-delineated single-crystals (which could be considered a type of morphological control).

Biomineral Constituents

Not all organisms show such a high degree of control as the above two examples, but one can summarize the overall types of control that are possible as follows: control over particle size, shape (morphology), polymorph, composition (including texture), orientation, and location. Being that most organisms are restricted to a relatively limited set of reaction conditions (such as low temperature and pressure in an aqueous environment, with a relatively limited number of species to work with), it is quite amazing that this level of control can be accomplished. Basically, the only means for controlling the properties is through cellular regulation of the reacting species and additives. Knowledge of how such control is accomplished in biomineralization should prove beneficial to the materials scientist.

As described above, organic constituents can be divided into the following three groups according to the role they play in the regulation of biomineral properties: a compartment, an insoluble matrix, or a solubilized additive. In the latter case, this dissertation will show a new and unexpected type of interaction for polyaspartate, and will build an argument to suggest that this type of organic/inorganic interaction is involved in biomineral formation. This previously undescribed role for an additive, which is based on non-specific interactions, will be defined as a process-directing agent, rather than a structure-directing agent. It will be shown that, through a transformation of the solution crystallization process to a solidification process, a fundamental type of morphological control can be attained, and that this polymer-induced process offers a viable explanation for many of the properties described in the above two examples.

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CHAPTER 2

CALCIUM CARBONATE FILMS AND HELICES GROWN IN SOLUTIONS OF POLYASPARTATE

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, such as 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 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] I 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; calcium carbonate ($CaCO_3$) was chosen 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.

Experimental Procedures

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 vapor diffusion. Glass coverslips (2.2 cm in 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 in diameter) containing 2.7 ml of a filtered 12.5 mM or 20 mM calcium chloride solution (freshly prepared with boiled doubly-distilled water). Appropriate microliter volumes of the dissolved polymer (0.8 mg/ml of poly(α,L-aspartate), Mw=14,400, Sigma; or Mw=6000, ICN; or poly(α,β-D,L-aspartate), Mw=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, 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°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 (SEM) on a JEOL 35cf SEM instrument.

Diffraction Techniques

Diffraction techniques were used for the determination of polymorphic composition of the $CaCO_3$ particles and films. Spherulitic crystals 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 Cu Kα radiation) for wide-angle x-ray diffraction (WAXD) studies. Helices were separated from other particles by micro-manipulation on the optical microscope before being crushed and placed on carbon-coated copper transmission electron microscopy (TEM) grids. *In situ* films, most of which were mosaics of single-crystal films, were scraped off and precipitated within a glass capillary tube 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 TEM grids. 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 d-spacings of multiple zones, while the spherulitic films yielded oriented ring patterns.

Results and Discussion

Aggregates Containing Helical Protrusions

A control dish (without polymer), run with each set of reactions, produced rhombohedral calcite crystals approximately 40 μm in size (see Appendix B). It was expected that addition of a highly charged polyelectrolyte, such as polyaspartate, would result in non-specific inhibition of crystal growth.[7] Instead, it was found that low levels of polymer led to unusual morphologies in the inorganic phase. At a concentration of 0.5 μg/ml, the rhombohedral habit of calcite became distorted, and in some cases, large spiral pits formed in the centers of the crystal faces, as can be seen in Fig. 2.1A (note-photographic figures are placed at the end of each chapter). At this level, the inhibitory action was strong, and only a few, larger crystals were formed. In the concentration range of 1-5 μg/ml, the calcite crystals tended to form aggregates, and at higher levels (5-30 μg/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. 2.1B). 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(α,β-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.

X-ray diffraction shows that these rounded particles are composed of vaterite, a metastable polymorph of $CaCO_3$, and electron diffraction on crystals obtained from a crushed helix confirms that the helical extensions are also vaterite (Table 2.1, 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. 2.2A): This membrane is also clearly visible when the helices are fractured (Fig. 2.2B). Occasionally, the helices are hollow (Fig. 2.3A), and the spherulitic crystals appear to have grown inward from the membrane template (Fig. 2.3B) 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 µg/ml) of polyaspartate, a highly birefringent inorganic film forms in streaks and patches on the glass substrate (Fig. 2.4). Because the particles discussed above are frequently associated with such films, it seems likely that it is the film that ultimately becomes the "membrane" surrounding the particles. It was initially believed that the spherulitic particles had wrapped around pieces of dust to form the helical structures, however, the fact that some of the helices are hollow suggests that the membrane plays a key role in producing the helical twist (Fig. 2.3). This would be

Table 2.1

Lattice d-spacings determined by diffraction techniques (Å)

WAXD lobed particles (dried)[*]	ED crushed helix (dried)[*]	ED spherulitic film (dried)[*]	WAXD mosaic films- cumulative (wet)[†]	ED mosaic film- type 1 (dried)[†]	ED mosaic film- type 2 (dried)[‡]
	4.23	4.22	3.91	3.88	4.18
3.56	3.58	3.57	3.07	3.06	3.97
3.28	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.

[*] matches d-spacings given for vaterite in the *International Center for Diffraction Data (1995)* x-ray powder file #24-0030.

[†] matches d-spacings given for calcite #24-0027.

[‡] reasonable match to d-spacings given for ikaite ($CaCO_3 \cdot 6H_20$) #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[10] or by mechanical stress (see section note 2.1).

consistent with empirical observations of the corresponding relationship between systematic variations in reaction conditions and the relative number and type of helices formed. For example, the following trends have been observed: Under a given set of reaction conditions, an increase in temperature, polymer concentration, or polymer molecular weight, typically produces more helices and less film (with the exception that higher polymer concentrations produce more film as well), and the helices tend to be long and thin (e.g. 20 μm wide x several hundred microns long for helices produced at 21°C with 20 μg/ml poly-L-aspartate of 6000 m.w., versus 200 μm wide x 250 μm long for helices produced at 4°C with 10 μg/ml poly-L-aspartate of 14,400 m.w.). Interestingly, an accidental warming spell in the cold room led to helices that had substantial curvature along the helical axis.

Calcium Carbonate Films

The inorganic polycrystalline films produced by the polyaspartate additive are typically of submicron thickness, and form in a variety of textures. They also appear to influence the type of particles deposited on them. For example, it has been noted that mosaic films, composed of interconnected single crystals, tend to carry aggregates of rhombohedral calcite crystals (Fig. 2.4A), whereas films with a spherulitic texture have spherulitic vaterite particles (lobed and helical) associated with them (Fig. 2.4B). Although the 50-200 μm sized 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 2.1, columns 3 through 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 2.1, column 6). The hexahydrate is an unstable form of $CaCO_3$ which can be produced at low temperatures[11] or through addition of aspartic acid[12] or polyphosphates.[10-13] Observations of this system 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 on the purity of the crystals,[10] 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 (see section note 2.1), 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, 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.

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? Sims et al.[14] have also described "helicoid 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. It is noted, however, that the ruptured membranes that they describe are rarely observed on the helices in this system, and only minute amounts of the polyelectrolyte are required to produce these effects; even x-ray microanalysis of the helical structures and films does not resolve the organic constituent.

Garcia-Ruiz and Amorós[15] 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 examined in this project (see Appendix A), only polyaspartate has yielded helical outgrowths and well-defined inorganic films on the glass substrate (although poly(glutamate) produces membrane covered calcitic aggregates).

Semblance to $CaCO_3$ Biominerals

To my knowledge, the *in vitro* precipitation of a calcitic mineral in the form of a film has not been observed before. It is noted, however, that some features of these products resemble the morphologies found in biogenic minerals. In particular, the nacre of mollusk shells consists of lamellar sheets of aragonite tablets (or calcite in bryozoan semi-nacre),[16] separated by a thin organic matrix called the conchiolin membrane.[17-20] The thickness of these $CaCO_3$ mineral layers (about 0.5 μm) is similar to that of the $CaCO_3$ films reported herein. In fact, the thinner calcitic films, such as those pictured in Fig. 2.5, 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 products of this polyaspartate system (Fig. 2.5A). Micrographs of mollusk nacreous layers typically show the complex hierarchical organization of the $CaCO_3$ tablets,[19] but when pictured in plan view, the growing surface of mollusk nacre resembles the layered films and tablets reported here.[5, 17, 20]

Conclusion

This chapter has described 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. The deposition of calcite and vaterite films appears to influence the types of crystal aggregates subsequently formed, as well as the twisting of the vaterite spherulites into helical structures. The following chapters will address the mechanism of film formation that is induced by polyaspartate, and the relevance of this mechanism to biomineralization.

Section Note

Note 2.1 Personal communication with S.A. Morse, co-author of *Crystal Identification with the Polarizing Microscope*, by R. E. Stoiber and S. A. Morse (Chapman & Hall, N. Y., 1994).[21] Conoscopy of the film patches yields single-crystal interference figures, which in some cases appear biaxial (the isogyres have borders of red and blue due to dispersion, and the hyperbolic legs separate, but remain in the field of view as the stage is rotated), but it is possible that these are pseudo-biaxial figures produced by strain in the thin film structures. This is suggested because the optic angle was not very well defined, and seemed to vary among samples.

Supplementary Information

This section contains experiments that are not included in the above chapter, but that extend the interpretation of some of the data that has been presented. The epitaxial overgrowth technique[22] (described more fully in Chapter 4) can provide information as to the orientation and type of crystal polymorph that is present in the aggregates and films. Fig. 2.6 shows lens-shaped overgrowths that are found on helical particles, consistent

consistent with the premise that the membrane surrounding the helical particles is a densely packed spherulitic vaterite, as had been surmised by the fact that spherulitic films generally surround the lobed-particle aggregates, and the corresponding spherulitic extinction pattern in polarized optical microscopy of hollow-type helices. In contrast, well defined rhombohedral overgrowths have been observed on all faceted aggregates and films that are composed of calcite (Fig. 2.7).

Fig. 2.1 Morphologies of $CaCO_3$ crystals grown in solutions of polyaspartate. The control synthesis, not shown here, yields rhombohedral calcite crystals that are typically 20 to 60 microns in size (see Appendix B). (A:top) A calcite crystal containing spiral pits resulting from the addition of 0.5 μg/ml poly(α,L-aspartate) to the crystallizing dish. Bar = 100 μm. (B:bottom) A crystalline aggregate containing a helical protrusion resulting from the addition of 10 μg/ml poly(α,L-aspartate). Lobed spherulites as well as distorted rhombohedral crystals are present in the aggregate. Bar = 10 μm.


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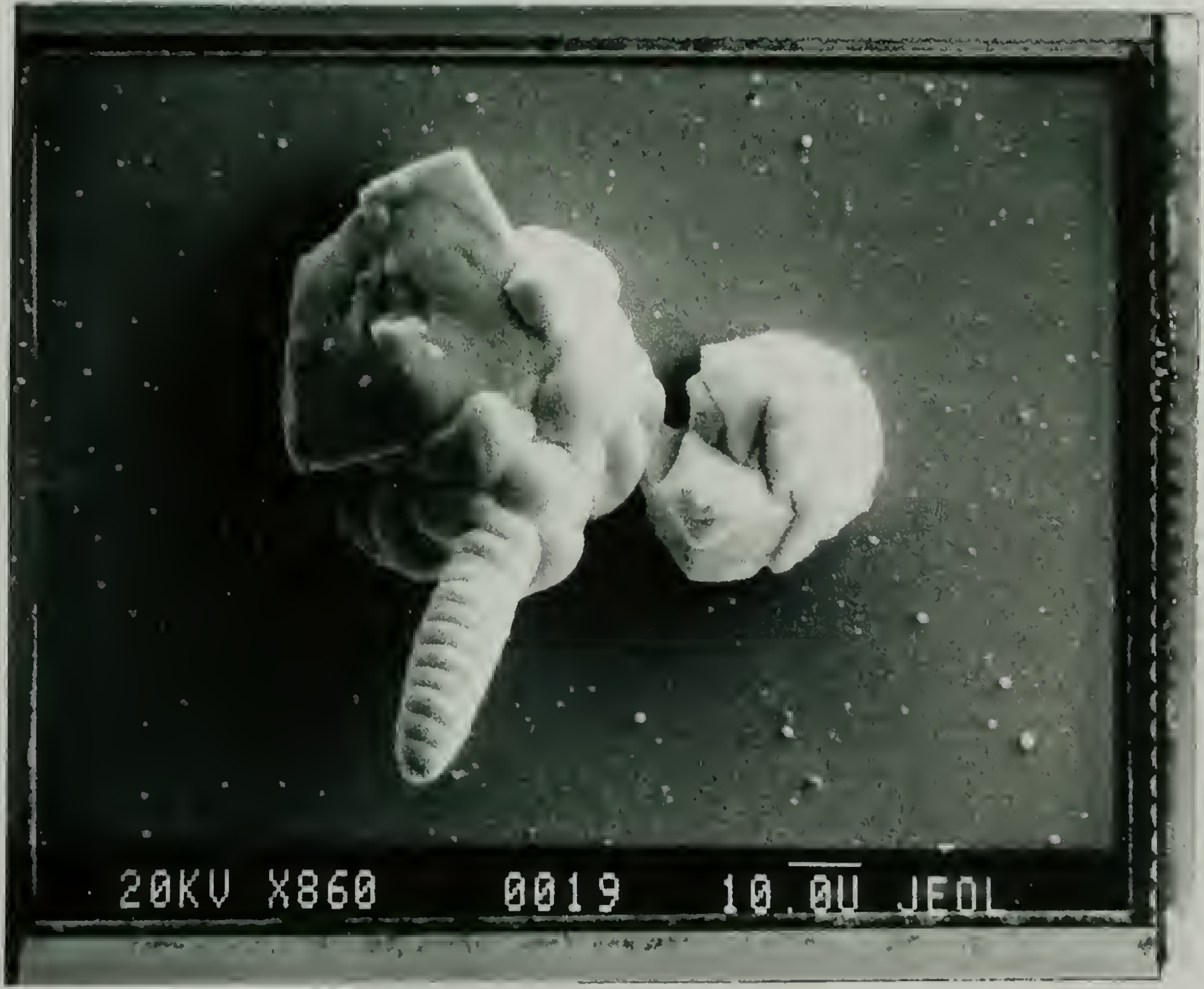
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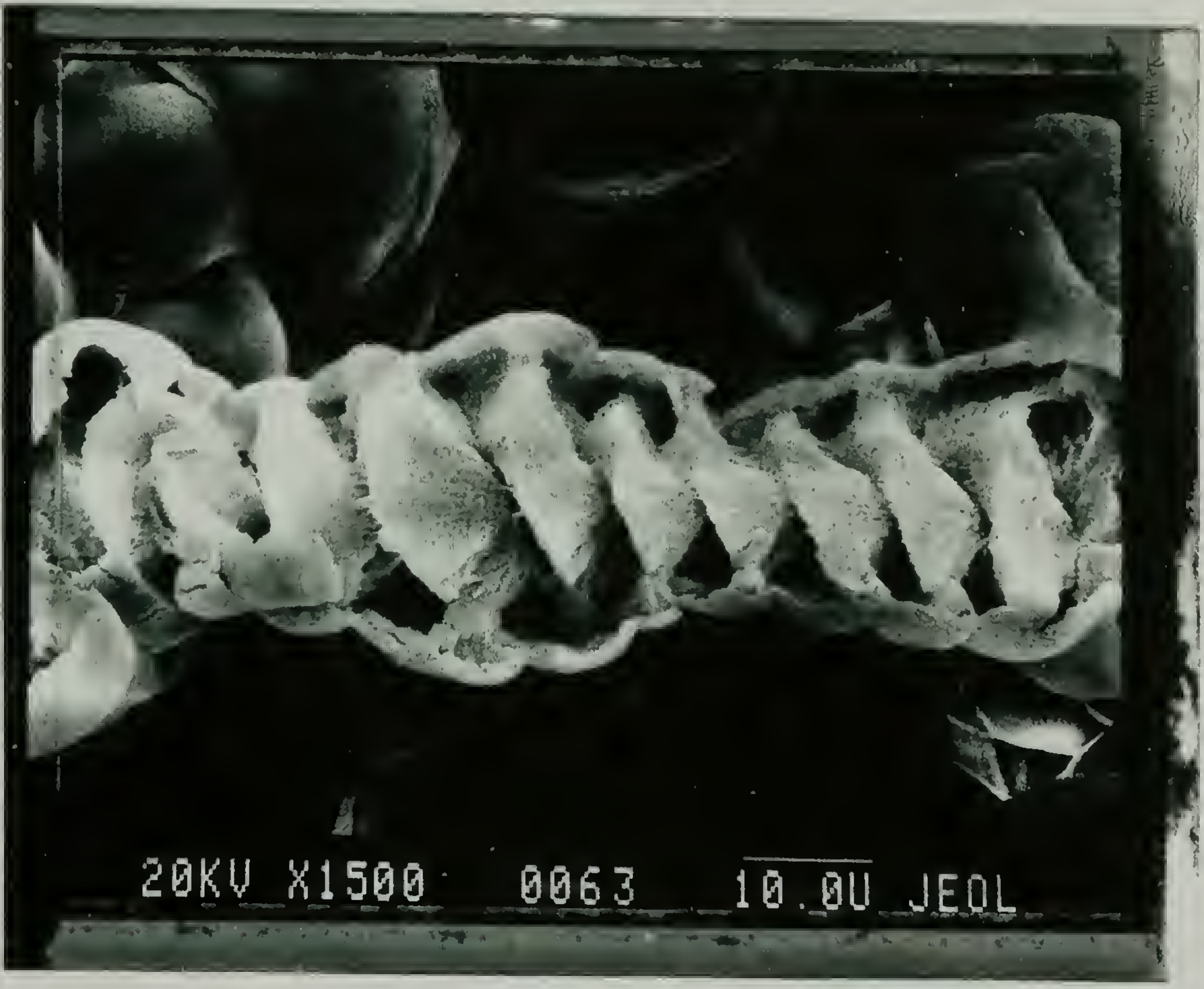
Fig. 2.2 Textural analysis of helical particles. The following two techniques show that the helical particles are spherulitic and surrounded by an outer membrane. (A:top) This helical particle was etched with 0.2% acetic acid for 10 minutes, neutralized with 0.1N NaOH, and then rinsed with distilled water.[22] The surrounding membrane appears to have been more resistant to the acid, and the polycrystalline texture is revealed. Bar = 10 µm. (B:bottom) This helix was fractured, revealing an outer membrane and polycrystalline core. Several helices were fractured using a micro-manipulator on an optical microscope, and they all showed similar membranes. Bar = 10 µm.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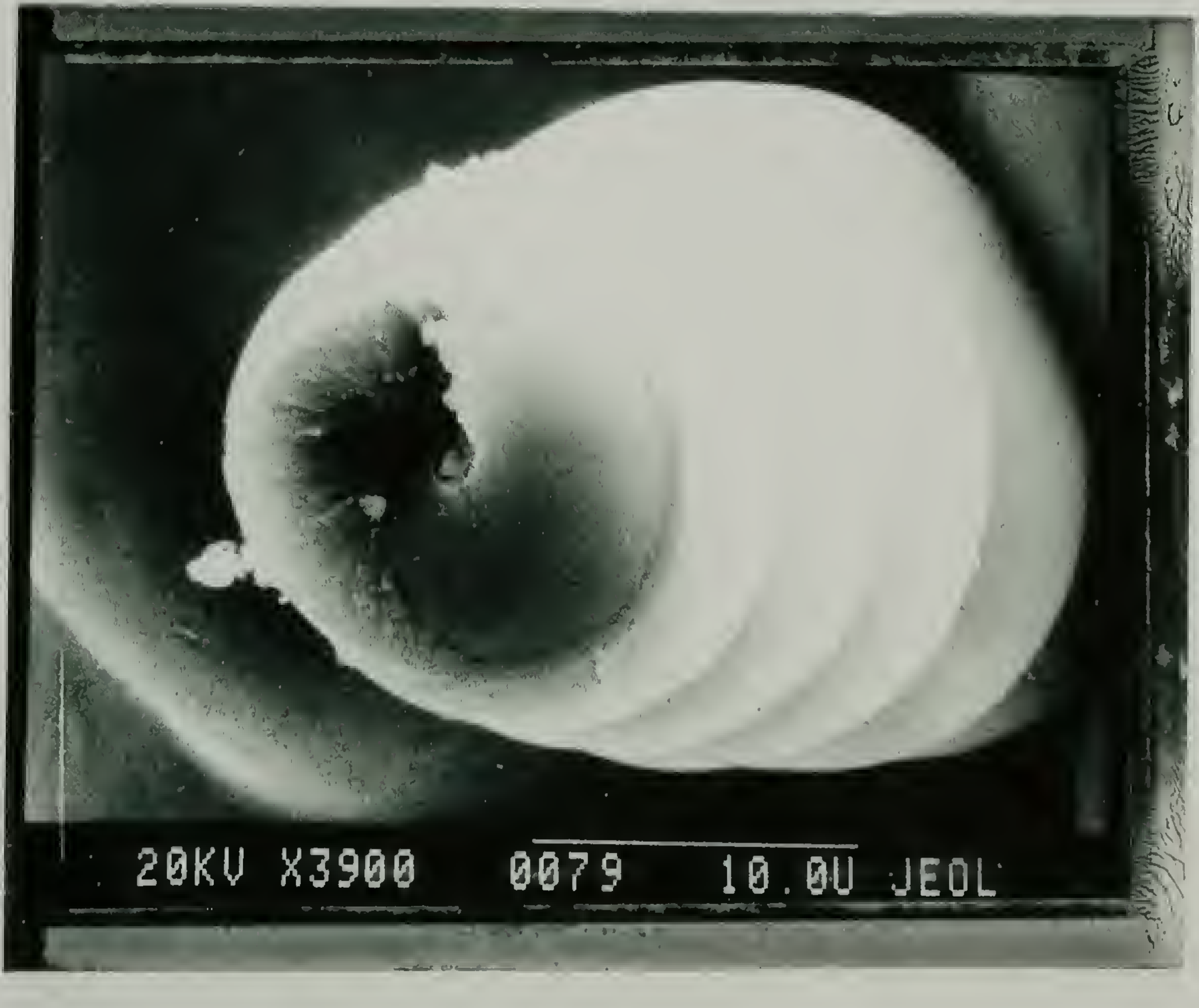
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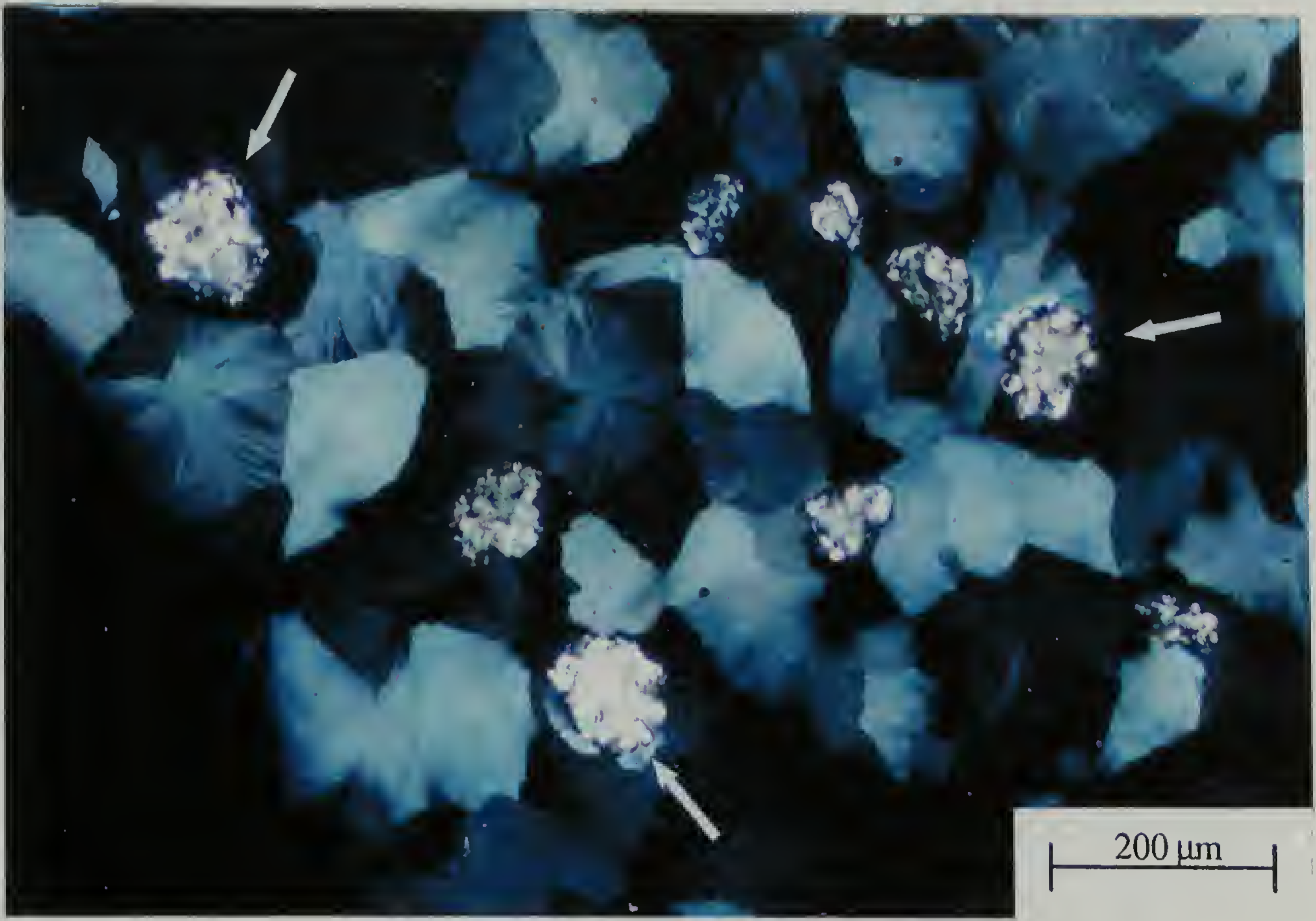
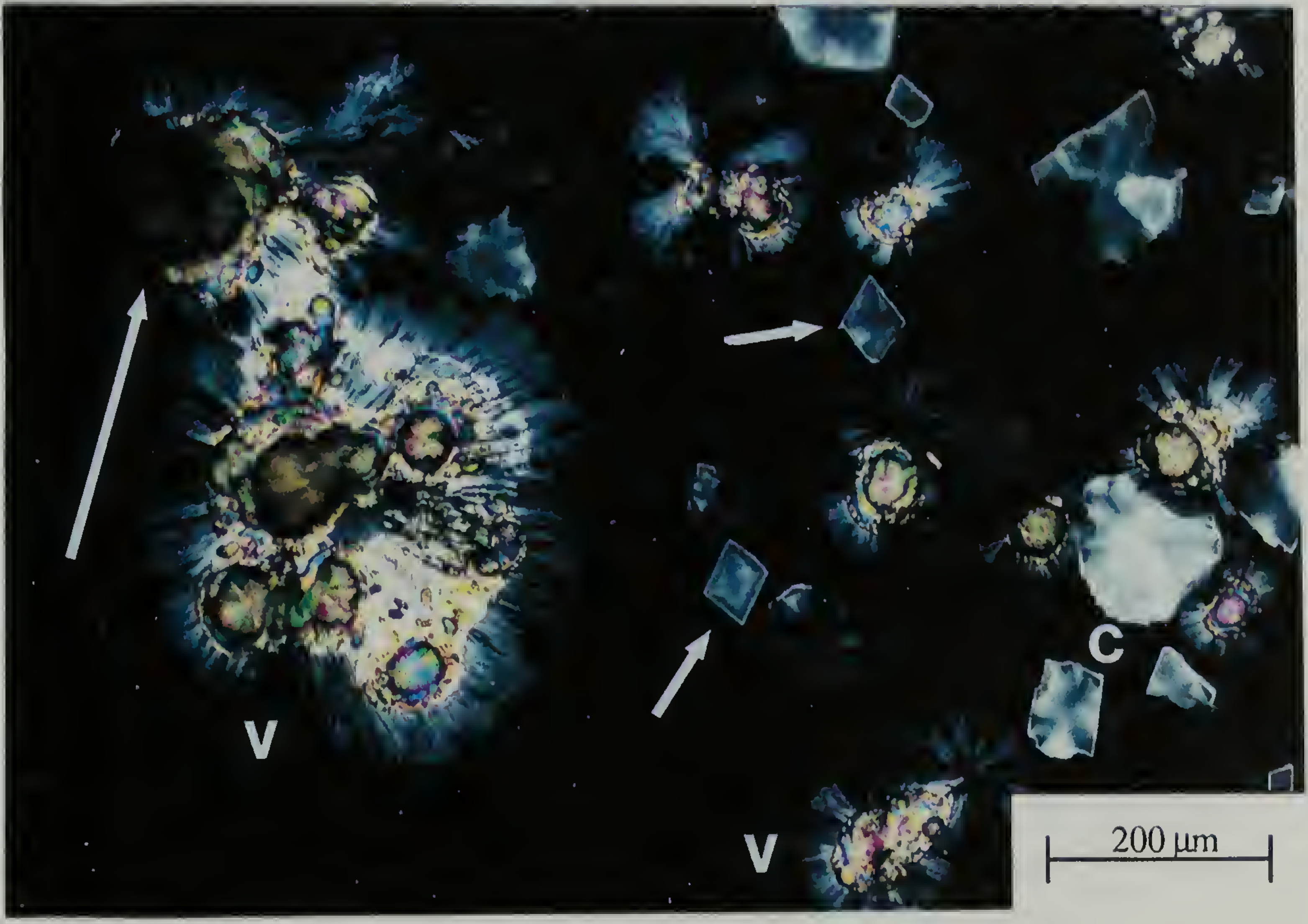
Fig. 2.3 Hollow helical particles. (A:top) The hollow helices appear transparent on the optical microscope, and are highly birefringent when viewed under crossed polars. The solid helices have a different appearance on the optical microscope; the spiral lines are not visible across the dense golden-brown helix. (B:bottom) The same hollow helix fractured by micro-manipulation. The fracture is different from that of the solid structures, as shown in Figure 2.2B. An internal core, such as dust particles, does not appear to be present in the hollow helices, which implies that the membrane is somehow responsible for the helical twist. Bar = 10 μm.


21KV X1100 0091 10.0U JEOL

Fig. 2.4 Types of $CaCO_3$ films deposited on glass coverslips. (A:top) A mosaic film composed of a patchwork of interconnected calcite crystals (optical micrograph using crossed-polars). Calcitic aggregates frequently grow on the mosaic films (arrows). (B:bottom) Patches of spherulitic film composed of polycrystalline vaterite (marked with the letter v), interspersed with calcite single-crystal patches (marked with the letter 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 more like faceted tablets (short arrows). In this example, the edges of the tablets are thicker and appear as white rims.


200 µm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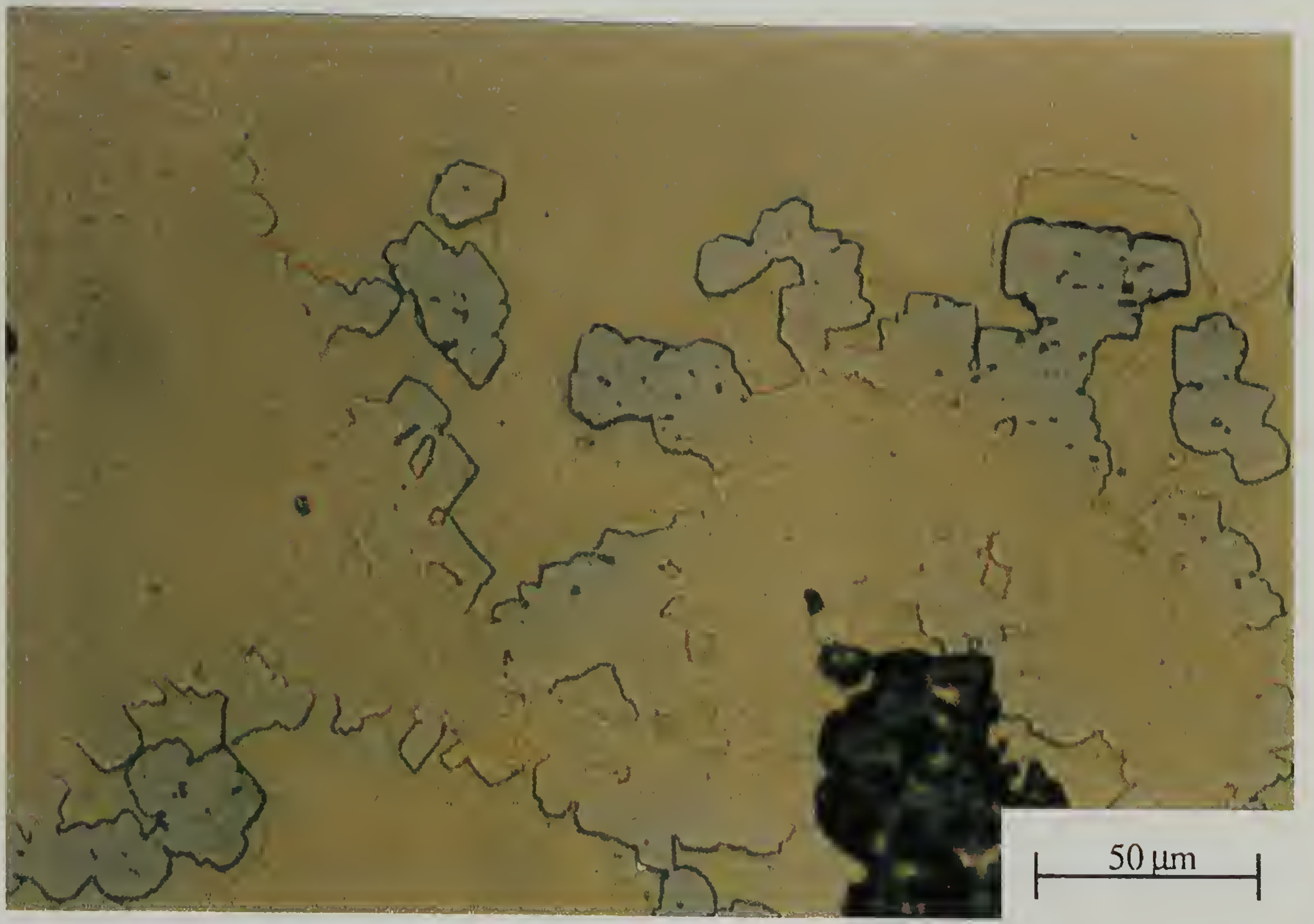
V
200 µm

Fig. 2.5 Features of $CaCO_3$ films. (A:top) Without crossed-polars, the layering of this film resembles micrographs of the growing surface of nacre (see, for example, Figures 2, 16, and 3c from references 5, 17, and 20 respectively). (B:bottom) The same film, using crossed-polars, shows a granular texture that is frequently seen on many of the 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.


50 µm

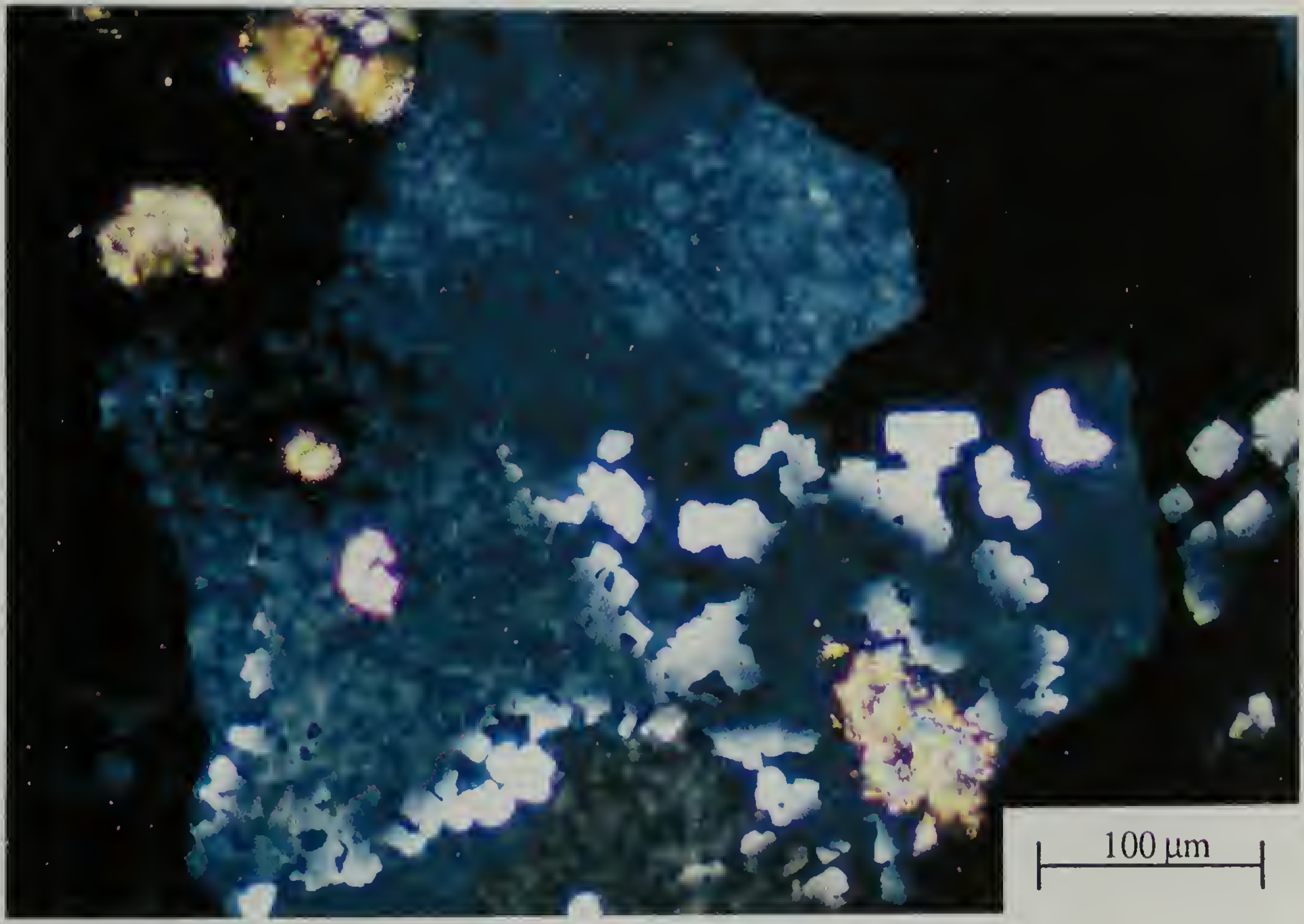

100 µm

Fig. 2.6 Epitaxial overgrowth technique applied to vaterite aggregates.

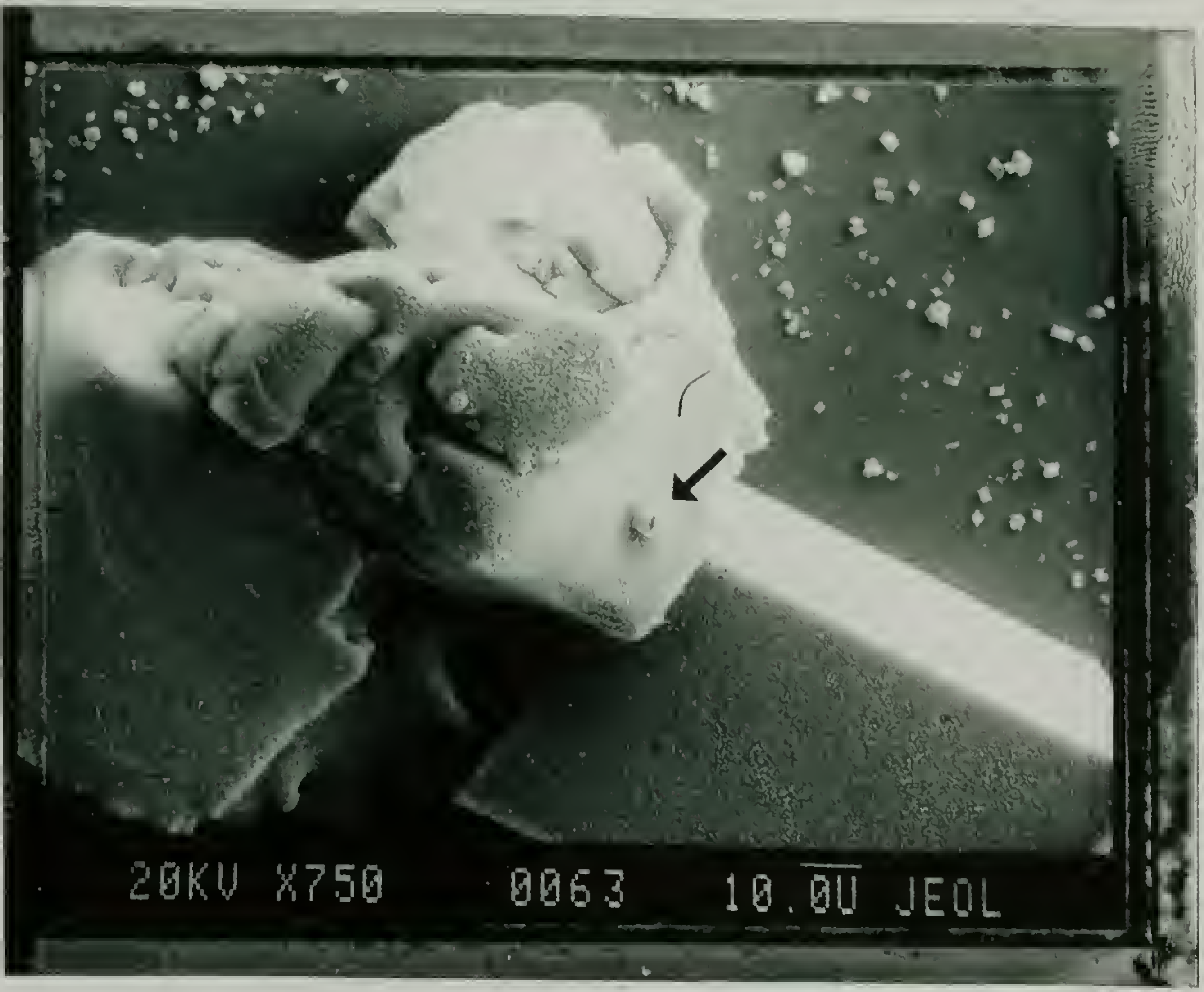
(A:top) Scanning electron micrograph of a $CaCO_3$ aggregate in which tiny lens-shaped overgrowths are found on top of the helix. In general, the helix membranes and spherulitic films do not readily support epitaxial overgrowths, and only a few lens-shaped crystals are seen to be scattered about. Thus it is assumed that the helix membranes are composed of densely packed vaterite (as are the spherulitic films), such that the polycrystals may be too small to promote nucleation. Bar = 10 μm.

(B:bottom) In this example, it appears that the outer membrane of the helix has been dislodged, or may have been removed during the bleaching procedure, and allowed for the well-aligned radially overgrowths. Note, similar lens-shaped crystals have been reported for vaterite, which grows in an aligned configuration under compressed monolayers of stearate.[23] Bar = 1 μm.


20KV X750 0061 10.0U JEOL


20KV X6500 0068 1.0U JEOL

Fig. 2.7 Epitaxial overgrowth technique applied to calcite aggregates. (A:top) The overall distortion of the calcite crystals composing this aggregates is apparent. Bar = 10 µm. (B:bottom) A higher magnification view of the region indicated by the arrow in the top picture shows that the epitaxial overgrowths adopt the typical rhombohedral habit that would be expected for calcite crystals. Bar = 1 µm.


20KV X750 0063 10.0U JEOL


20KV X7500 0062 1.0U JEOL

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CHAPTER 3

POLYMER INDUCED DEPOSITION OF CALCIUM CARBONATE FILMS VIA A LIQUID PRECURSOR

Introduction

In the previous chapter, it was shown that the addition of polyaspartate to a calcium carbonate ($CaCO_3$) crystallizing solution resulted in some unusual crystal morphologies, including calcitic films, aggregates, and helical vaterites. This chapter will describe direct observations of the crystallization reaction, obtained by *in situ* optical microscopy, which show that a new and unusual process is responsible for the deposition of the $CaCO_3$ films. The synthesis was modified, relative to the previous chapter, so that a glass reaction chamber could be placed under the objective of an optical microscope (illustration provided in Fig. 3.1). In brief (see experimental section for details), $CaCO_3$ crystals were grown by slowly saturating a 20 mM calcium chloride solution, containing 20 μg/ml polyaspartate, with ammonium carbonate vapor for several days. A glass coverslip was placed over the crystallizing dish and in contact with the calcium chloride solution, and infusion of ammonium carbonate vapor occurred through a gap created by resting the coverslip on a trapped air bubble (caused by slightly under-filling the dish), or through gaps in a grease sealant connecting the dish and coverslip.

Experimental

$CaCO_3$ crystals were grown by equilibrating approximately 1.5 ml of a 20 mM $CaCl_2$ solution (made with freshly boiled and cooled dd-H_2O and $CaCl_2$ from Fisher Scientific), containing 20 μg/ml polyaspartate (appropriate volumes micropipetted from an 0.8 mg/ml aqueous solution of poly-α,L-aspartate, Mw= 6000: ICN; or poly-α,β,D,L-aspartate, Mw=6850: Sigma; sodium salts), with ammonium carbonate vapor for up to

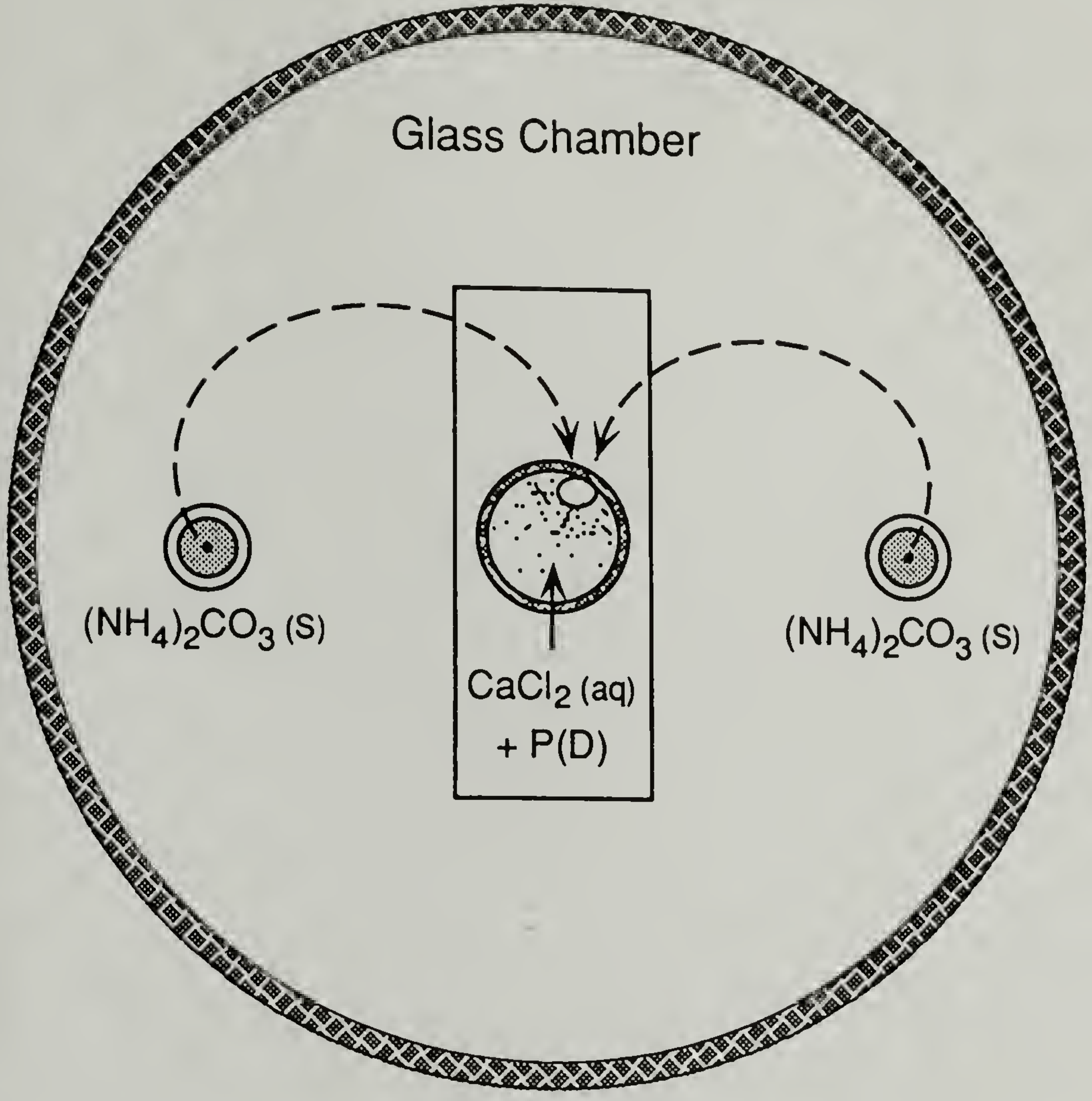


Fig. 3.1 Experimental setup for *in situ* observations of the crystallization process.

three days; the $(NH_4)_2CO_3$ from EM Science is a mixture of variable proportions of ammonium bicarbonate and ammonium carbamate, which decomposes to NH_3 and CO_2.

The crystallizing dish (a glass ring, 5 mm high x 22 mm in diameter, epoxied onto a glass slide) was covered with a 22 mm glass coverslip that was in contact with the calcium chloride solution (coverslips were cleaned by soaking in a Nochromix/H_2SO_4 solution overnight). The covered dish was placed into a round glass chamber (8 mm high x 15 cm in diameter). Two vial caps, each containing approximately 0.2 g of freshly crushed ammonium carbonate powder, were also placed in the chamber, covered with parafilm, and punched with a needle hole. The chamber was then covered and sealed with a thin glass plate (0.1 mm in thickness x 15 cm in diameter), and placed under the ultra-long-working-distance objective of an Olympus BX-50 optical microscope for *in situ* observations.

Results

This experimental setup yields more film deposits, but fewer vaterite aggregates and helices, relative to the synthetic conditions in Chapter 2. (Note, the formation of helices can be favored by using plastic wrap instead of the glass coverslip). In general, most of the crystal products are formed on the top glass coverslip, and mainly in the vicinity of the gaps for carbonate infusion, where large swirls and layers of film are deposited. The reaction times in this experimental setup are quite variable (ranging from 2 hours to infinity), and seem to be sensitive to the environmental conditions, such as the temperature (typically 21°C) and humidity (moisture affects the decomposition of the ammonium carbonate powder). Once again, characteristic calcite crystals of rhombohedral habit are produced in the control reactions (without polymer).

These *in situ* experiments have revealed that the $CaCO_3$ films result from a polymer-induced liquid-precursor (PILP) process. The overall PILP process is depicted in Fig. 3.2 (figures are at the end of the chapter), where for the purpose of representation, it has been divided into four stages that illustrate various features and components of process. The remaining figures in this chapter provide evidence, or examples, of each of these components.

PILP Process

Early in the reaction, a cloud develops where the ammonium carbonate vapor diffuses into the crystallizing solution. On the optical microscope, the cloud is seen to consist of small (≈ 3 μm) isotropic droplets. It is difficult to photograph these small moving droplets, but accumulations of the liquid-precursor phase are frequently found on the bottom surface of the trapped air bubbles provided by the *in situ* setup (Fig. 3.3A). The precursor phase flows like a slightly viscous liquid when disturbed. Isotropic gel-like globules are sometimes seen on the glass coverslip, but these thicker structures tend to remain amorphous, become encrusted with crystallites, or dissolve away.

The precursor phase accumulates on the glass substrate to form a thin isotropic coating, which then solidifies and crystallizes into a birefringent $CaCO_3$ film. Fig. 3.3B provides evidence that the $CaCO_3$ films are created by coalescence of the precursor droplets, where it can be seen that they do not always fully coalesce. Droplets that settle late in the reaction may adsorb to the crystalline films and aggregates without fully coalescing; they still tend to have the same optical extinction as the underlying crystal, suggesting that they crystallize by iso-epitaxy. The partially coalesced droplets give the films a granular appearance, as had been noted in the previous chapter in Fig. 2.5A. In some cases, droplets with a different crystallographic orientation are adsorbed as well (Fig. 3.4A); such droplets were most likely partially crystalline before they precipitated. Droplets that crystallize while still in solution resemble the free-form droplets that appear at the clearing-point in liquid crystal systems (Fig. 3.4B).[1] In the *in situ* setup, where the droplets accumulate on the upper substrate, the resulting films tend to be less granular because they are not prone to gravitational settling effects.

In the next stage of the PILP process, the isotropic films solidify. Sometimes they are fairly soft (e.g., when scratched with a needle, a groove is formed, as in a fine granular solid), or in some cases they are brittle (when scratched with a needle, cracks are formed). When the precursor films transform, patches of birefringence nucleate and spread across the film, along with what appear to be "wrinkles" (Figs. 3.5 to 3.7). Depending on the synthetic conditions, the degree of wrinkling is variable, ranging from tablets with no observable wrinkles, to ridges, to micro-facets (Fig 3.5A, see excursus in section note 3.1). The spacing and orientation of these wrinkles are usually quite uniform, resulting in modulated crystals with ridges spaced about a micron apart. The orientation of the ridges and the crystallographic orientation appear to be correlated, as is evident in Fig. 3.5. Because of this correlation, and the fact that the wrinkles are created during a phase transition, the terminology "transition bars", which is used in liquid crystal systems,[1] seems justifiable here (see section note 3.2).

The transition bars, which progressively form along zones that emanate from the original point of nucleation, have orientations that differentiate the sectors in the calcite tablets. Triangular-, rhombohedral-, and hexagonal-shaped tablets have bars that divide them into 3, 4, or 6 sectors respectively (Fig. 3.7, and depicted in Fig. 1C). Incremental growth steps are also seen in the spherulitic films (Fig. 3.6B), but in this case, concentric circles are formed by the radial advance of the vaterite polycrystals. While most of the calcite tablets have sectors with linear transition bars, some contain sectors with wavy wrinkles as well (Fig. 3.6B). Based on the premise that the wrinkles (transition bars) are orientationally correlated with the crystallographic lattice, it follows that the wavy wrinkles may be indicative of micro-defects. One could imagine that the dehydration aspects of this process would lead to shrinkage stresses that could generate micro-defects. In support of this, it has been noted that some (but not all) wavy-wrinkled sectors exhibit a slight fluctuation in the birefringence.

Finally, lateral growth of the tablets/patches ensues across the precursor film, while wrinkles become filled in, or anneal, resulting in a smooth coherent film (demonstrated in Fig. 3.5). This explains why wrinkles and sectors are not seen in the final products. However, artifacts of these features are occasionally seen, such as in the differential birefringence pattern shown in Fig. 3.7B. In some cases, the films thicken and/or form a new layer of tablets as well, as was seen in Fig. 2.5A of Chapter 2. It has also been observed that amorphous films will dissolve if a large crystal aggregate grows in the vicinity, presumably due to a local depletion of ions.

PILP Products

The end result of this PILP process is that unusual $CaCO_3$ crystal morphologies are generated, i.e. thin tablets (faceted to anhedral); rounded droplets; and mosaic, swirl- or sponge-like films (not pictured), etc.. It should be pointed out, however, that an assortment of crystal products are formed under these conditions, including aggregates of rhombohedral crystals (as seen in many of the pictures), some of which tend to have a "molten" appearance (Fig. 3.8A), and lobed aggregates of vaterite that have helical protrusions (Fig. 3.8B). The mixture of crystal products in this system presumably arises from a competition between the solution and solidification processes.

Depending on the reaction conditions, the calcite crystals deposited by the PILP process have variable defect concentrations, as judged by the textures exhibited in polarized light microscopy. For example, the poly-α,L-aspartate commonly produces streaks of mosaic film, where the individual crystal patches are seen to contain a high degree of defect texture, especially towards the periphery (Fig. 3.7B). On the other hand, the poly-α,β,D,L-aspartate tends to produce thicker, isolated tablets, that have uniform extinctions (Fig. 2.4B). If the crystallization is carried out at a lower temperature (T= 4°C), which has been found to promote the PILP process, thick films are produced that contain a variety of textures (Fig. 3.9A). In some films, an undulatory extinction is observed, implying that there is a gradual shift in orientation of the crystal lattice. In many cases, the defect patterns are correlative, i.e. with biaxial strains (Fig. 3.9B), and most likely result from shrinkage stresses where the film is constrained by the substrate.[2]

Discussion

Crystals grown from a solution typically have faceted habits, where the faces that are expressed afford a lower surface energy to the crystal.[3, 4] It is well known that impurities in the solution can modify the crystal habit,[4] and studies using stereospecific additives have shown that selective adsorption can be used to alter the growth kinetics of those specific faces, thereby modifying the crystal habit.[5] In this system, however, it is clear that the classical solution crystallization theory does not provide an adequate explanation for the morphogenic action of the polyaspartate additive. Instead, the non-equilibrium morphologies generated by this additive are seen to arise from a solidification process, in which the shapes and textures of the products are reminiscent of crystals grown from a liquid-crystalline state (see section note 3.2),[1] or a chemical precursor,[2] or a melt, rather than a solution.[4, 6] Presumably, the crystals transform from a hydrated precursor, so perhaps the best analogy is that of "growth from molecular complexes", described by Grisdale[3] (for ferrite crystals grown from the vapor phase) as having an "unmistakable impression of the growth of crystalline material by the accretion, merging, and solidification of fluid droplets or lamellae."

How does the polymer induce this process? Based on an argument provided by Dickens and Brown,[7] it was suggested in the first chapter that the hydrophilic polymer might stabilize a $CaCO_3 \cdot 6H_2O$ precursor. Although the hexahydrate may well be an intermediate in the PILP process (and the Ostwald-Lussac rule of stages would predict such a case, see section note 3.3),[8, 9] this report shows that the action of the polymer goes beyond this, and actually stabilizes a liquid precursor. As to how this would occur, the following suggestion is offered: The polyelectrolyte could play a dual role, serving to concentrate the ionic species, while delaying the nucleation long enough to generate a metastable solution. Certainly, both sequestering and inhibitory effects have been described for polyelectrolytes, separately, and especially in the context of biomineral systems. For example, it is known that polyelectrolytes, including solubilized acidic macromolecules, almost inevitably delay the crystallization of a salt.[5, 10] On the other hand, Greenfield et al.[11] describe a coordinated ion binding by a charged polymer bound to a matrix (i.e. the hydrophilic, sulfated fraction of the organic matrix found in mollusk shells). They report that the binding stoichiometry of one calcium for every two ester sulfates is not altered, but the calcium binding induces local anion binding, which then induces secondary calcium binding. They suggest that the resultant local high concentration may then bring about "ionotropic nucleation".[11, 12] They even point out that the initial deposit would probably be amorphous, followed by transformation to a crystalline phase.

To some extent, my results support the ionotropic model, in that phase separation is only seen after the addition of carbonate species (see section note 3.4), and that an amorphous phase is deposited. In contrast, though, the polymer in my system is not attached to a substrate, and although it may promote the formation of a highly supersaturated phase, it likely inhibits the nucleation, until it is either removed or overwhelmed (by dehydration). Secondly, and more importantly, it is clear that the dual role the polymer plays in the PILP process extends beyond nucleation aspects, where it is seen that the physical attributes of a distinct liquid-precursor phase can dramatically alter the morphology of the crystal products. Thus, I consider the highlight of these results to be, *the demonstrated conversion of a solution crystallization to a solidification process by a polymeric additive.*

There are reports in the older literature that also mention liquid-type precursors in the formation of calcium carbonates:[13-15] For example, Brooks et al.[13] describe "gelatinous precipitates" that occur under quiescent conditions using magnesium-ion and Calgon additive; while Hunt[14] describes gels that are generated without additive at high reactant concentrations (≈ 0.5 M); and "droplets" of $CaCO_3$ and water have been reported to occur during the formation of spherulites from metastable solutions.[15, 16] Although Brooks et al.[13] discuss the stabilization of metastable crystal phases (i.e. the hexahydrate), I feel the significance of such observations in terms of morphological influence has hitherto been overlooked. This is understandable considering that metastable supersaturated solutions generally give rise to spherulitic growths due to excessive nucleation.[16] Apparently, the inhibitory action of the polymer allows for the formation of both spherulitic and single crystals from the induced metastable solution.

Regarding morphology, others have shown that crystals grown in polymeric gels are subject to viscous effects that limit the diffusion of ions, which thereby influences the morphology.[17, 18] A distinction is made here, however, that the polyaspartate, in and of itself, is not a gel (the $CaCl_2$/polyaspartate solution alone does not phase separate); nor is the PILP phase (it flows like a slightly viscous liquid). It is this induced phase, in its entirety, that precipitates, unlike crystals grown in a gel. This is an important distinction in terms of morphological control, because a separate liquid phase can conceivably be manipulated.

Conclusion

This chapter has demonstrated a novel process, in which a polymer transforms a solution crystallization to a solidification process. The solidification aspect arises from the fact that the polymer induces the phase separation of a liquid precursor. Precursor chemistries find important uses in materials engineering,[2] and this novel precursor process, generated by low levels of a polymeric additive in a low temperature and aqueous environment, may prove beneficial in the engineering of thin films and particulates in an environmentally friendly and cost effective manner. At this point, it is difficult to predict if a similar process could be generated in any systems other than calcium carbonates because I have only examined the process at the physical, or macroscopic level. Further studies, directed towards developing a understanding of the PILP process at the molecular level, will address this issue of generality, as well as contribute to our knowledge of the interactions that occur between polyelectrolytes, ions, and crystals.

Section Notes

Note 3.1 It is assumed that a volume reduction must be associated with the dehydration of the liquid precursor, but it is not obvious why this would be manifested in the form of modulated structures. One possible explanation might be that the ridges help reduce the surface energy, analogous to crystal faces that are roughened due to the expression of more stable micro-facets. This is suggested because the tablets in Fig. 3B appear to contain micro-facets; however, micro-facets are not readily apparent on most of the modulated tablets I have examined (by light microscopy).

The fact that wrinkles are not present in the final crystal products would seem to contradict this notion that micro-facets are formed to lower the surface energy. Indeed, the resultant film in Fig. 3B exposes the relatively unstable (001) face of calcite, even though it is the same film that I suggested contained micro-facets. Therefore, an alternative explanation is offered for the formation of these modulated structures that is based on the analogy between these "wrinkles", and the "transition bars" that occur in liquid crystal (LC) systems. The transition bars in LCs are reported to occur in impure samples only, and presumably arise in heterogeneous regions in which both phases coexist.[1] It is plausible that the liquid precursor in the $CaCO_3$ system, which has polymeric impurities, could undergo a related phenomenon. The fact that "wrinkles" are sometimes filled in with crystalline material of another orientation (Fig. 3D) seems to suggest that latent isotropic precursor is still present in the grooves of the modulated tablets.

Note 3.2 A variety of features seen in the PILP products appear similar to those in liquid crystal systems,[1] i.e. clearing-point droplets, transition bars, wedges, stepped drops, disclination defects, pseudo-π disclinations (not pictured), and mosaic to spherulitic film textures. However, this is not intended to imply that there is orientational order within the PILP phase; rather, the optical textures and morphologies may be similar because, in both systems, the crystals are formed by a phase transformation process, where physical constraints on the isotropic phase influence the morphology and texture of the crystalline phase. It should be pointed out, however, that in LCs the phase separation is induced by orientational order, and this is not the case in the PILP process.

Note 3.3 The Ostwald-Lussac rule[8] is described by Falini et al.[9] as being an empirical observation that predicts that the initial mineral formed from a solution supersaturated with respect to more than one mineral is the one with the highest solubility.

Note 3.4 Phase separation occurs during the introduction of carbonate species in a solution already containing polymeric additive, thus it may be more accurate to describe this process as a carbonate-induced, polymer-stabilized, liquid-precursor (CIPSLP) process. Indeed, previous reports[13-15] indicate that excess reactants, especially carbonate, can lead to metastable solutions. But under the synthetic conditions employed here, phase separation is not observed without polyaspartate additive. Therefore, the simpler phrase PILP has been used, which emphasizes the role of the polymer in overall process, from sequestering ions to generate a metastable solution, to inhibiting the nucleation event.

Fig. 3.2 Illustration depicting the stages and features of the PILP process. (A) Isotropic droplets from a phase-separated liquid precursor accumulate on the substrate. (B) The droplets coalesce to form a continuous isotropic film. Some post-precipitate droplets may be partially solidified, or crystalline, and do not fully merge with the film. (C) The isotropic film becomes birefringent as crystal tablets nucleate and grow. Transition bars progress along growth fronts, dividing the tablets into sectors. Tablets that nucleate in different crystallographic orientations show a variety of sectorization patterns. (D) The tablets expand laterally to form a continuous mosaic film.

Polymer-Induced Liquid-Precursor Process

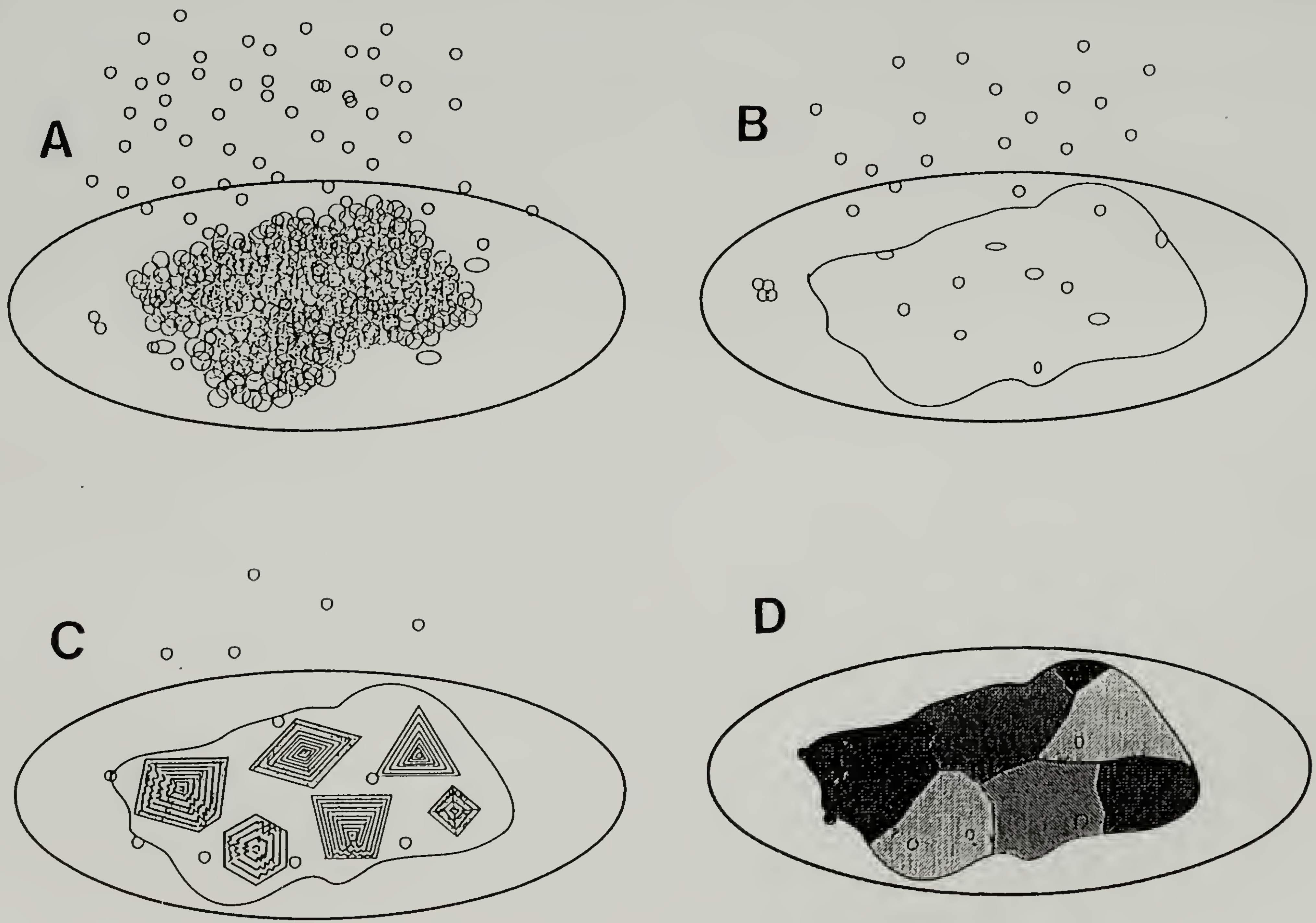
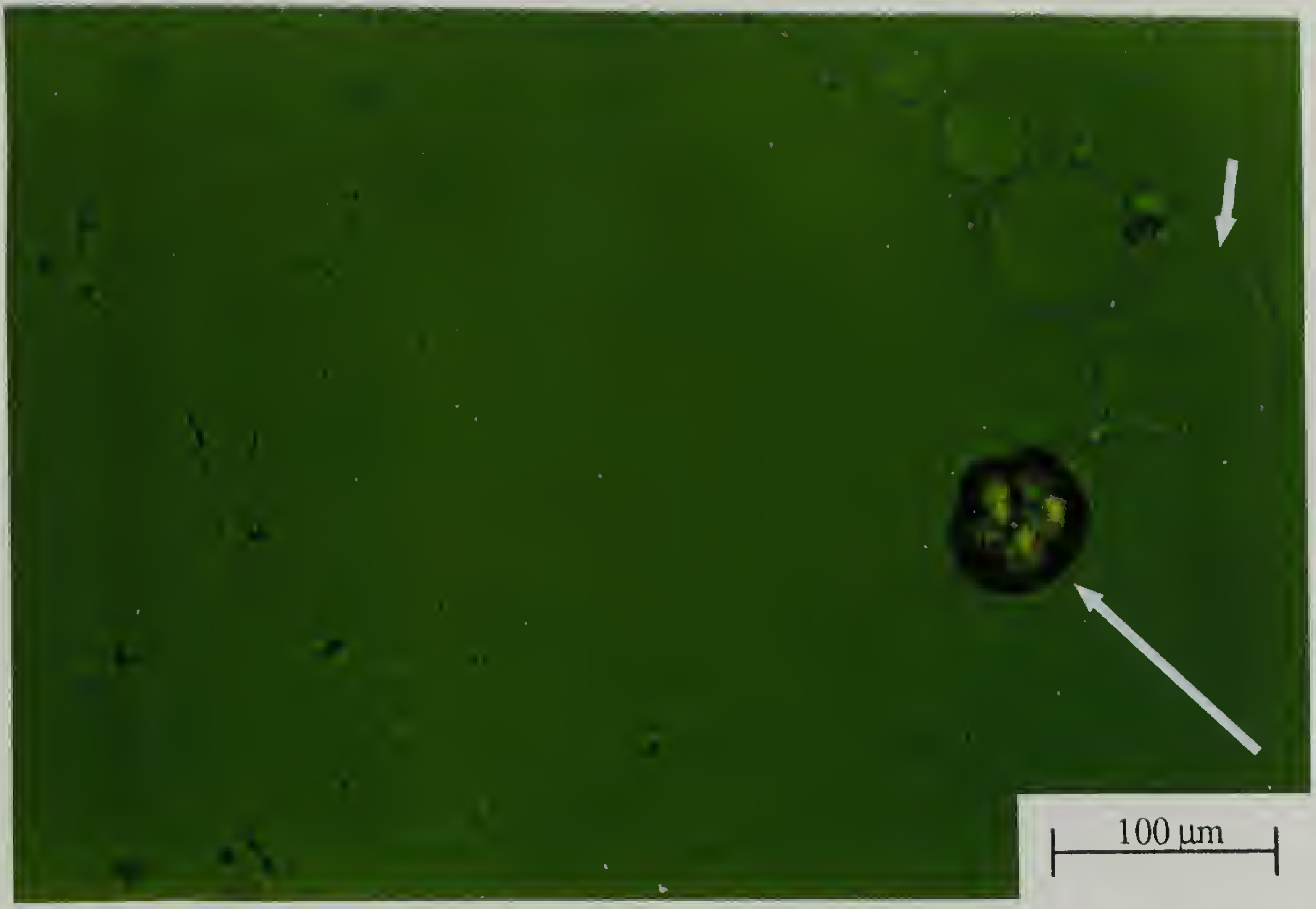


Fig. 3.3 Evidence of a liquid-precursor phase. (A:top) This picture was taken during *in situ* observations of the PILP process using the optical microscope, and shows the bottom surface of an air bubble that was used to create the gap for infusion of carbonate vapor (see Fig. 3.1). The large drops seen here (relative to the 3 µm size droplets formed in solution) are an accumulation of the PILP phase at the bubble-solution interface. These drops flow like a slightly viscous liquid when the bubble is disturbed. These thick accumulations do not crystallize readily; but in this example, a spherulite is present (long arrow), and one drop looks puckered, as though it has started to dehydrate (short arrow). (B:bottom) This scanning electron micrograph demonstrates that the calcitic films are composed of an accumulation of droplets, which in this case did not fully coalesce into a smooth film. Bar = 10 µm.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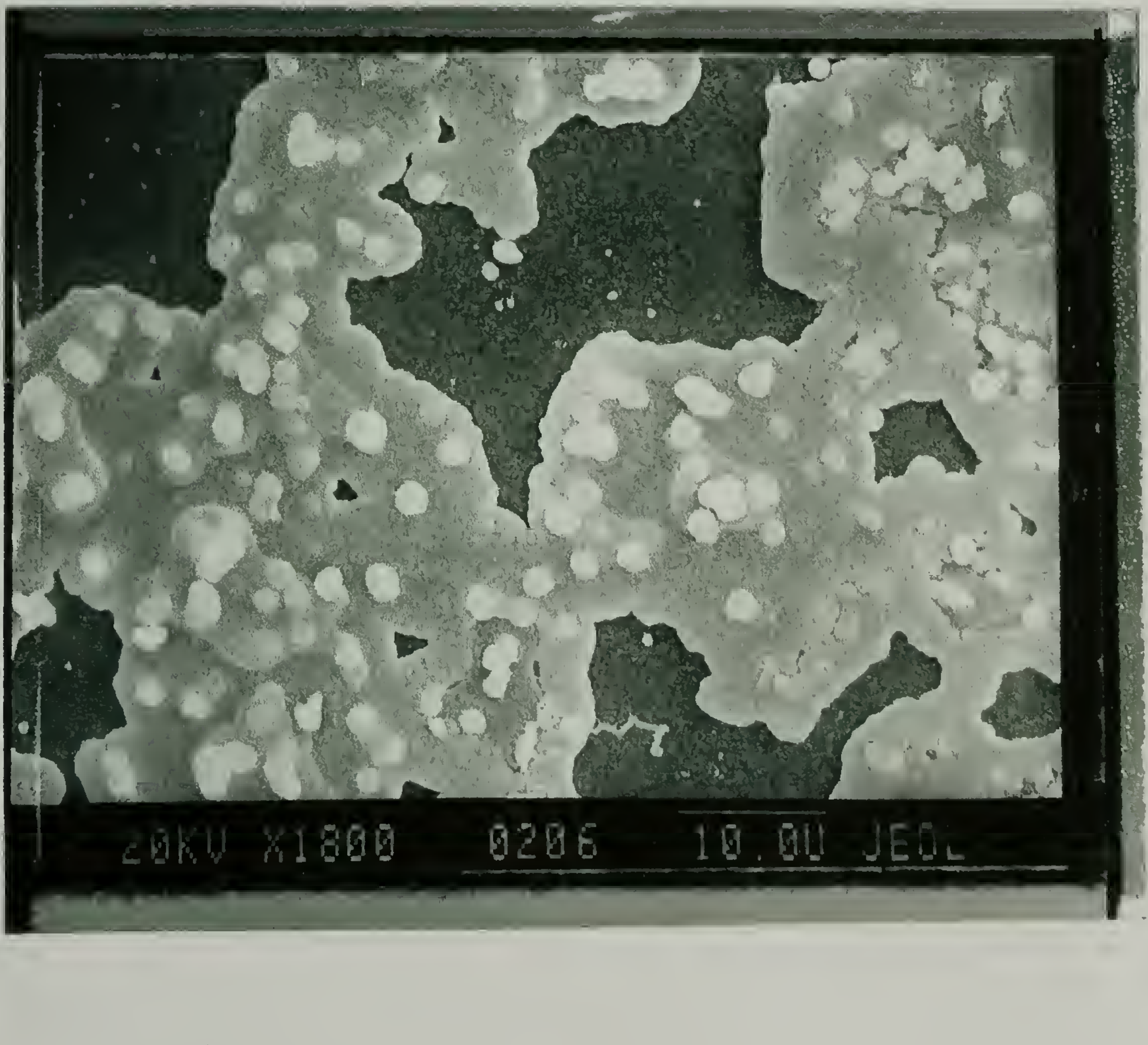
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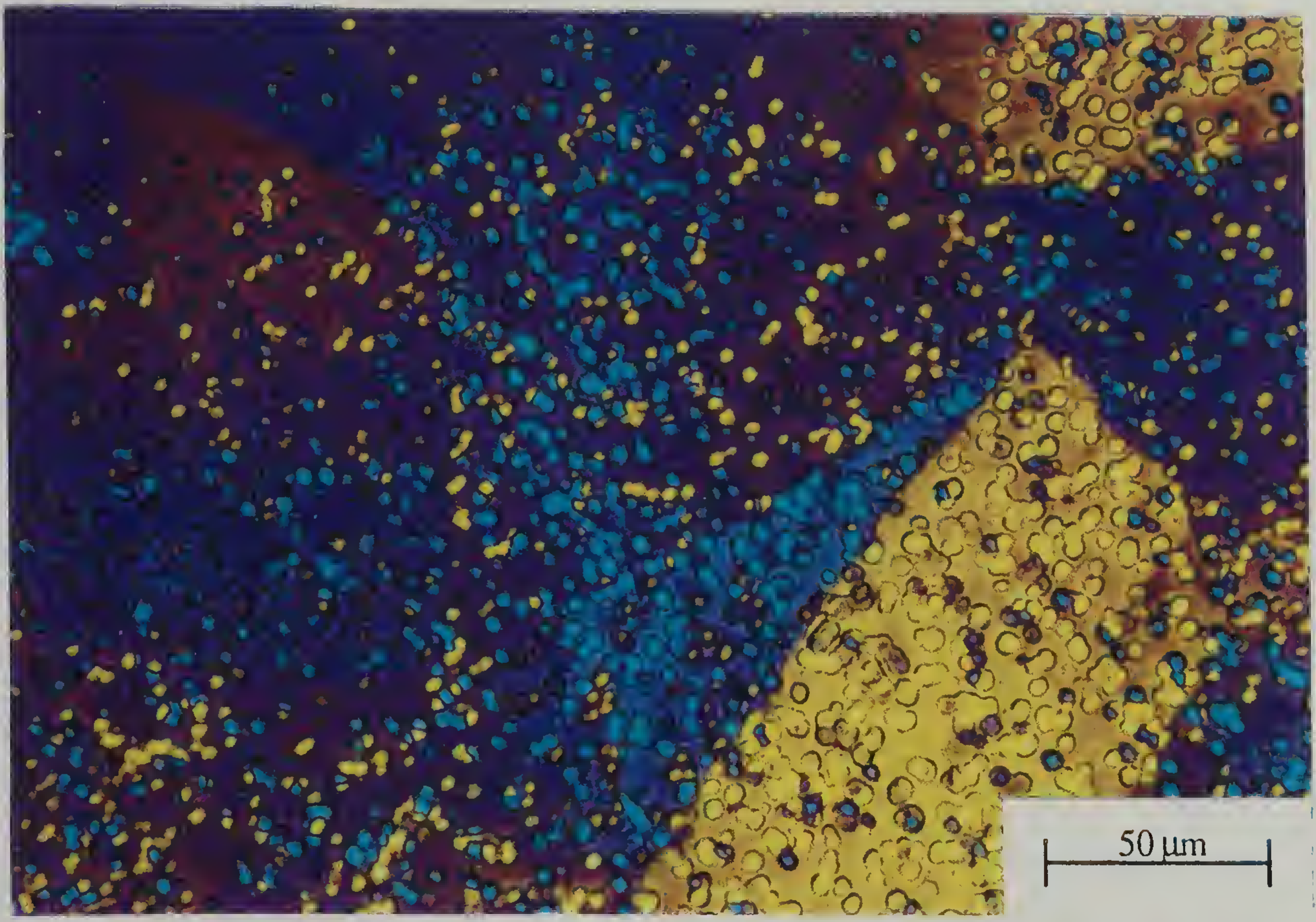
Fig. 3.4 Pre-solidification of precursor droplets. (A:top) This optical micrograph shows a calcitic film that was deposited on the bottom substrate, where post-precipitate droplets adsorbed to its surface. Some of the droplets did not crystallize epitaxially to the film, and were probably partially crystalline before they precipitated. (Note, using the 1st-order red plate with crossed-polars, droplet orientations different from that of the underlying film are seen as different colors). (B:bottom) These coalesced droplets, seen under cross-polars, bear a resemblance to clearing-point LC droplets because of the disclinatory type defects at the junctions. For example, as the sample is rotated, black threads gradually move across the crystalline droplets, different from that of a spherulitic maltese-cross pattern or a grain boundary defect. This is not intended to imply that there is orientational order in the PILP phase, but rather, the precursor phase was probably crystallizing within the droplets as they merged, such that a concomitant shrinkage led to a gradual shift in the orientation of the crystallographic lattice (see section note 3.2).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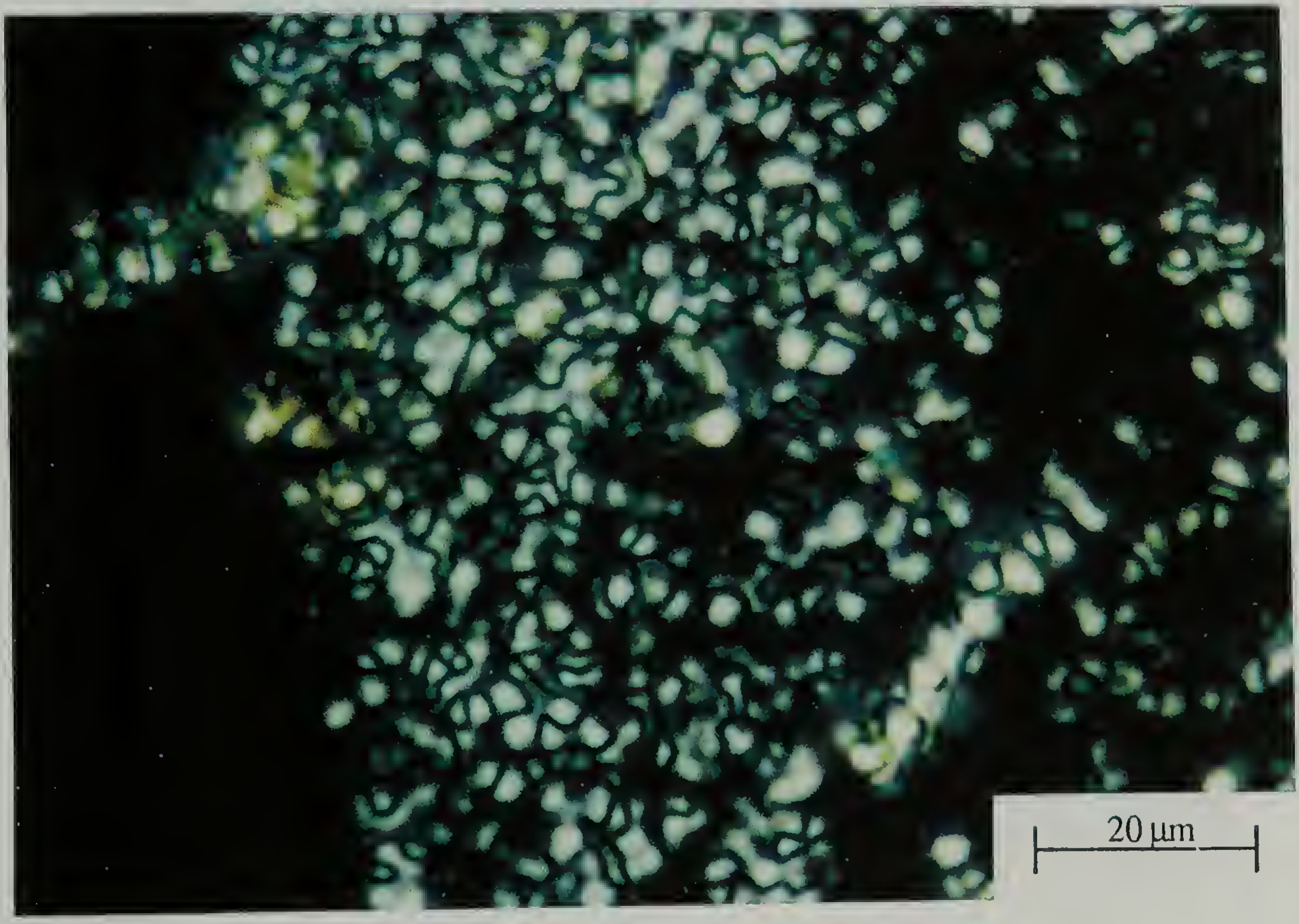
20 µm

Fig. 3.5 The amorphous to crystalline transformation. (A:top) At the beginning of the transformation, these heavily "wrinkled" tablets appear to contain "micro-facets". These optical micrographs were taken using the 1st-order red plate, so that both birefringent and isotropic material can be viewed under cross-polars. For example, the ≈ triangular tablet is magenta, and thus is oriented at, or near, the isotropic *c*-axis of calcite. (B:bottom) The same view at a later stage shows that the wrinkles have partly filled in, and the tablets have expanded laterally to form a continuous film. Even the micro-facets on the isotropic tablet have filled in, which now exposes the relatively unstable (001) face. Sometimes the wrinkles are filled in with crystalline material of another orientation, typically that of a neighboring crystal (e.g. orange patches are seen in the furrows of the magenta tablet). Regrettably, the views are not well aligned, but can be compared by the hole feature in the yellow tablet to the left (arrow). Note, the rotational offset (indicated by the arrow) has changed the retardation colors of some of the tablets .


50 µm

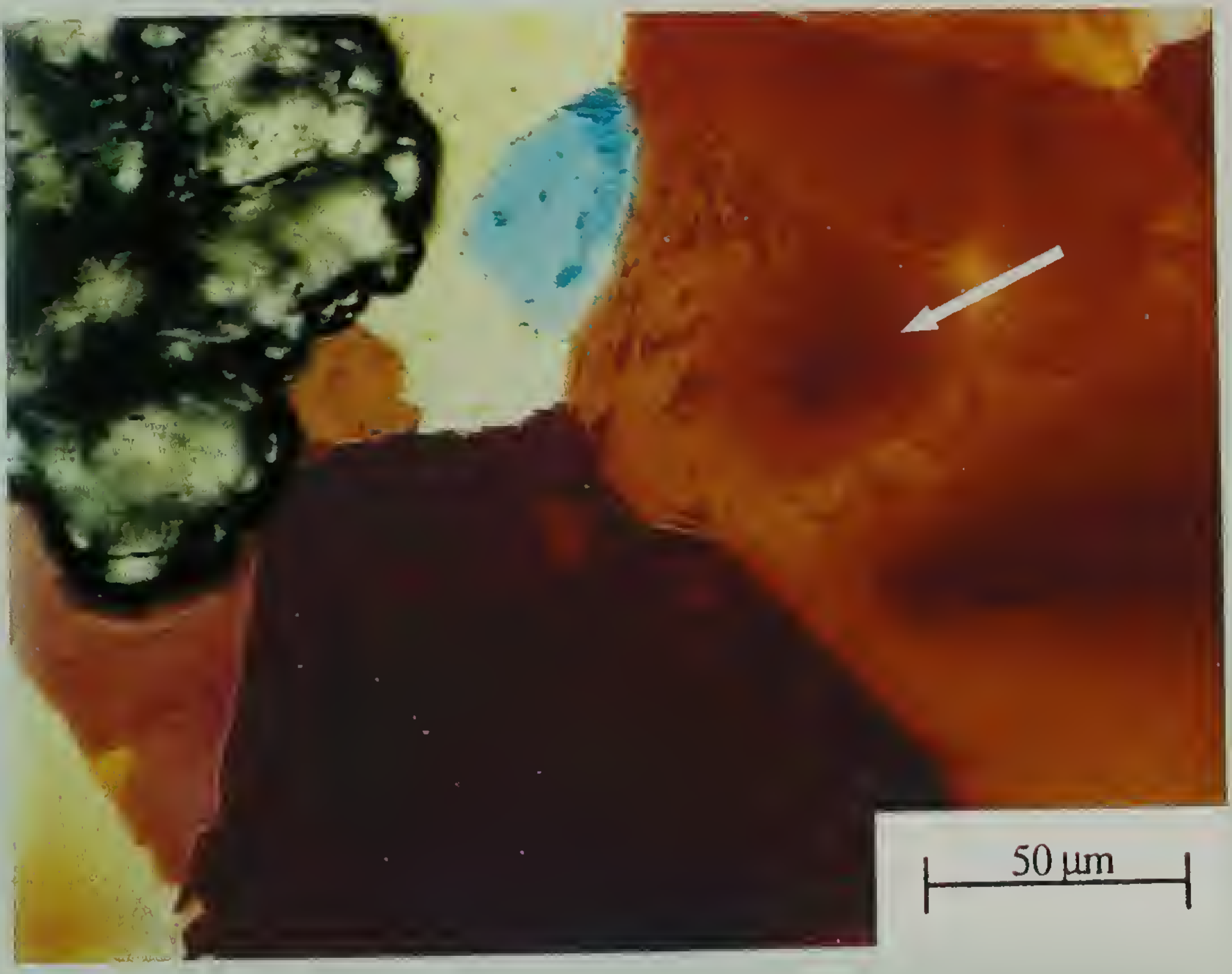
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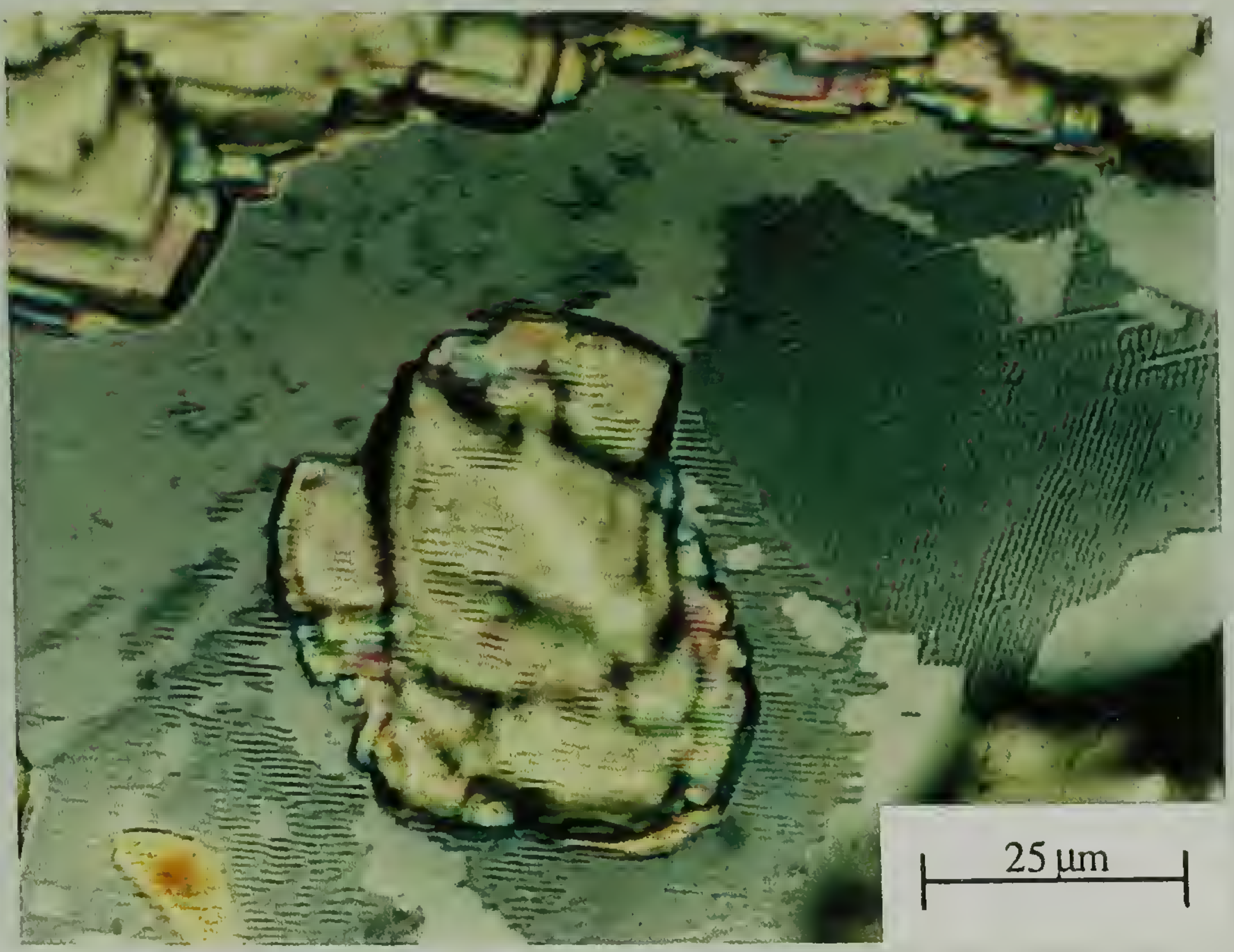
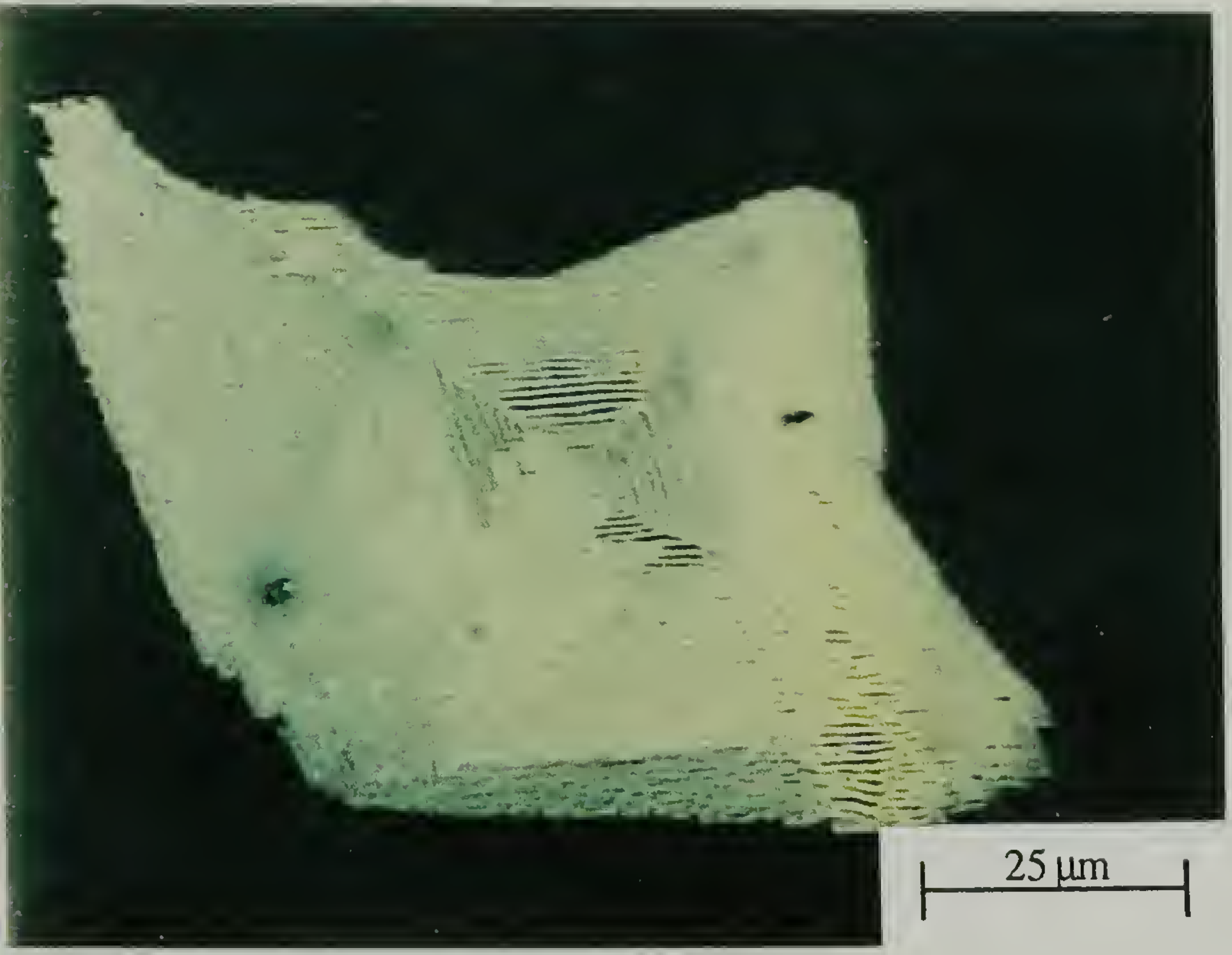
Fig. 3.6 Correlation of transition bars to lattice and transformation process.

(A:top) Small aggregates of calcite frequently grow epitaxially to the films, as seen in this example, where the two yellow aggregates exhibit the same extinction direction as the film. The facets on the crystal aggregates are aligned with the ridges in the film, demonstrating a correlation between the orientation of transition bars and crystallographic lattice (note of caution: the facets on the central aggregate are poorly defined; such distorted crystals are commonly produced by the polyaspartate additive). (B:bottom) Transition bars divide the single-crystal tablets composed of calcite into sectors, while spherulitic patches of film composed of vaterite display concentric growth rings. Both linear and wavy transition bars can be seen in the different sectors of the tablets. The similar incremental steps in the spherulitic film seem to indicate that the wrinkles are caused by aspects of the transformation process, and not by stereochemical interactions of the additive with specific crystallographic faces.


25 µm

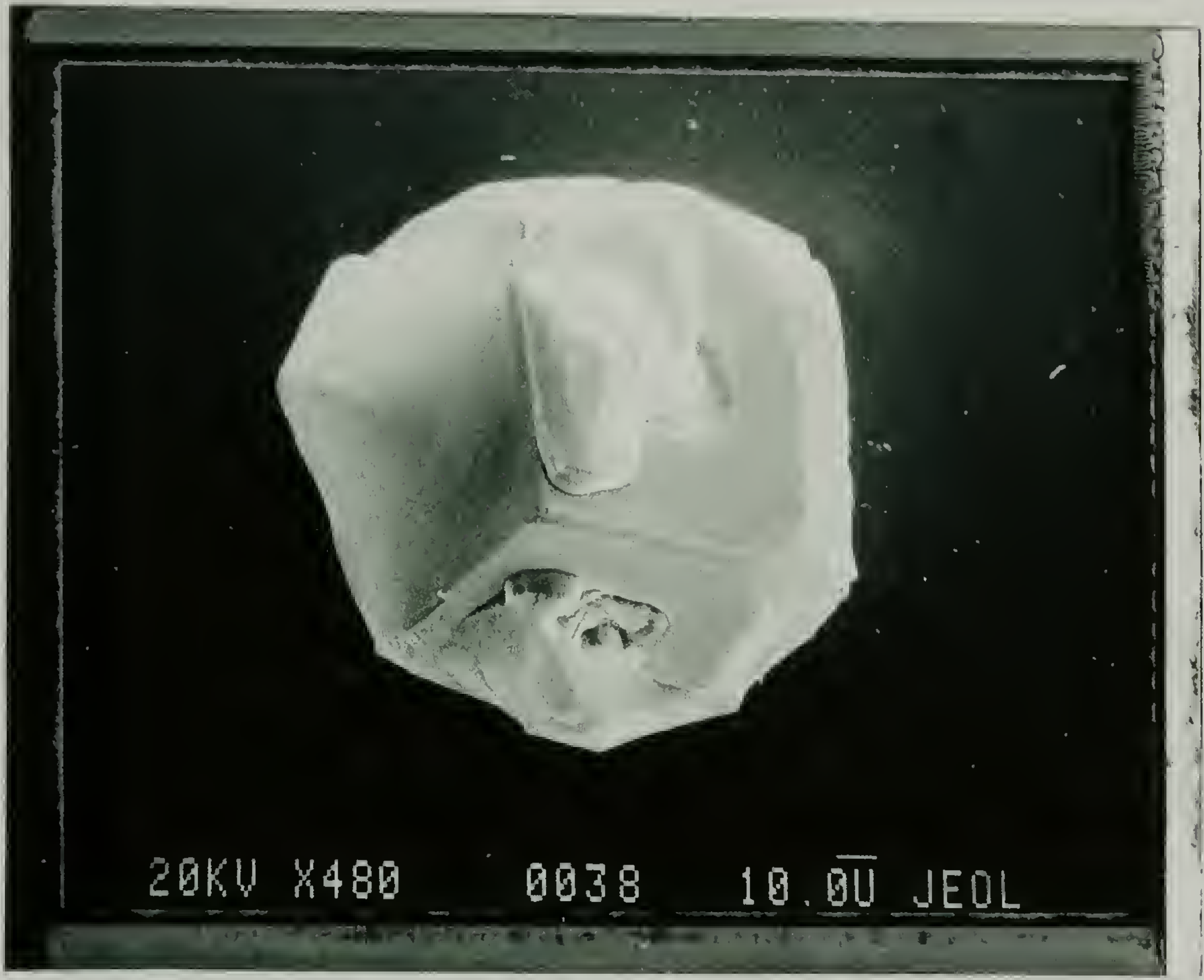

20 µm

Fig. 3.7 Sectorization of $CaCO_3$ tablets. (A:top) Sectorization is present in the tablets above, but is not as obvious as it is in this isolated rhombohedral-shaped tablet that is divided into four sectors by linear ridges. (B:bottom) This calcitic film was disturbed during the amorphous to crystalline transformation, and remnants of the originating tablets can be seen. Although the wrinkles have filled in, sectorization can still be detected by faint differences in the birefringence of alternate sectors of the tablets (although this is not the usual case).


25 µm


50 µm

Fig. 3.8 Products of the $CaCO_3$/polyaspartate system. While this chapter has focused on the thin tablets and films, the reader is reminded that a mixture of products is formed in this system, including aggregates of calcite and vaterite. (A:top) The aggregates of calcite are typically comprised of crystals that are severely distorted, each of which could be described as having a "molten" appearance, which is demonstrated here for this single crystal of calcite. Bar = 10 µm. (B:bottom) The lobed aggregates of vaterite with the helical protrusions are frequently surrounded by films (usually the spherulitic type as shown in Fig. 2.4). Fig. 2.2 shows a membrane surrounding the helices, which is of similar thickness to the films. Thus, it is likely that the membranes and films are generated by the same PILP process. The film surrounding this helix formed cracks when the sample was prepared for SEM, which seems to suggest that the film was not fully dehydrated when placed under vacuum. However, this is not the usual case. Most of the films remain intact after being dried if they have had sufficient time in solution to reach the crystalline state. Bar = 10 µm.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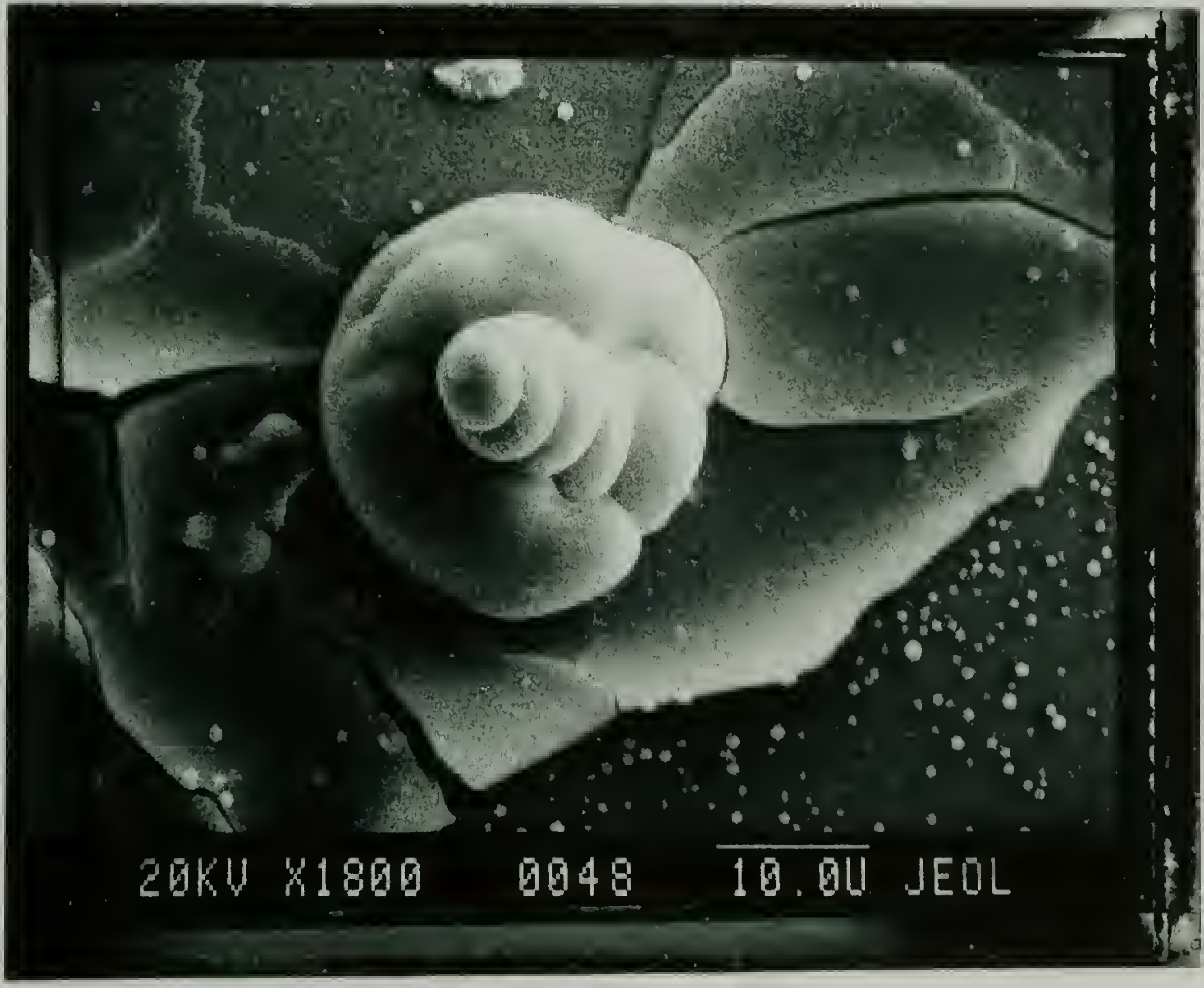
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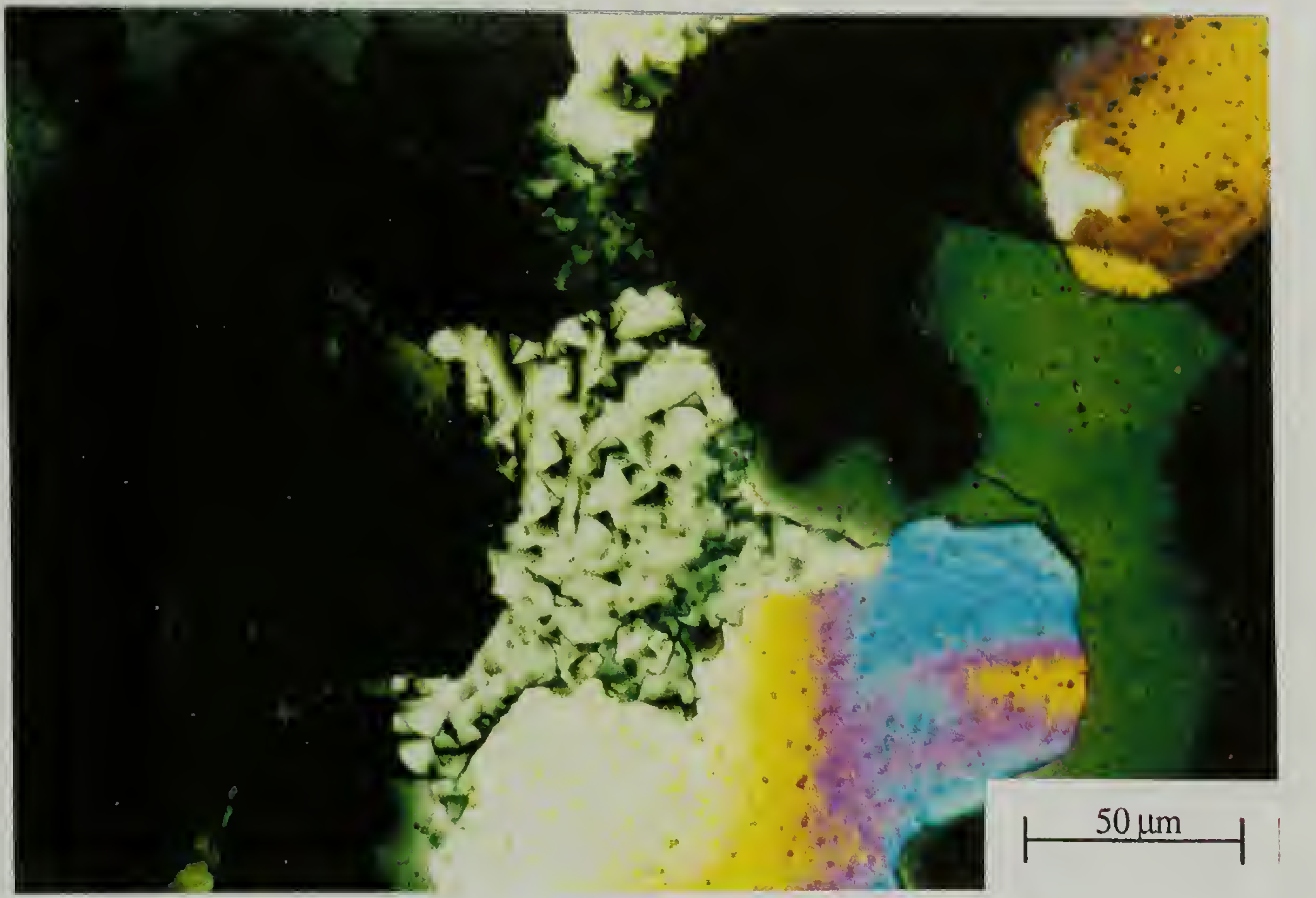
Fig. 3.9 More on the texture of the film products. (A:top) These thicker films, seen under crossed-polars, were formed at T= 4°C, and show both single-crystalline and spherulitic textures. The spherulitic texture seen here is different from that of the vaterite films described in Chapter 2 (see Fig. 2.4), and its polymorphic composition is, as of yet, undetermined because the film is too thick for electron diffraction. Note the "stepped-drop" appearance of the layers in the thick single-crystalline films (arrow). However, unlike the stepped-drops in liquid crystals (see section note 3.2), the layers here do not step down, but rather step up (the increasing order of retardation colors, from white to blue, indicates an increase in thickness of the wedge-shaped crystal). Evidently, the liquid-precursor had slid down the crystal wedge, leaving behind crystalline layers. (B:bottom) Epitaxial overgrowths, which occurred naturally on these tablets, demonstrate shifts in the crystallographic orientation of the underlying tablets. For example, the tablet marked with an arrow has crystal overgrowths with differing orientations on alternate sides, and is indicative of a biaxially-strained crystal.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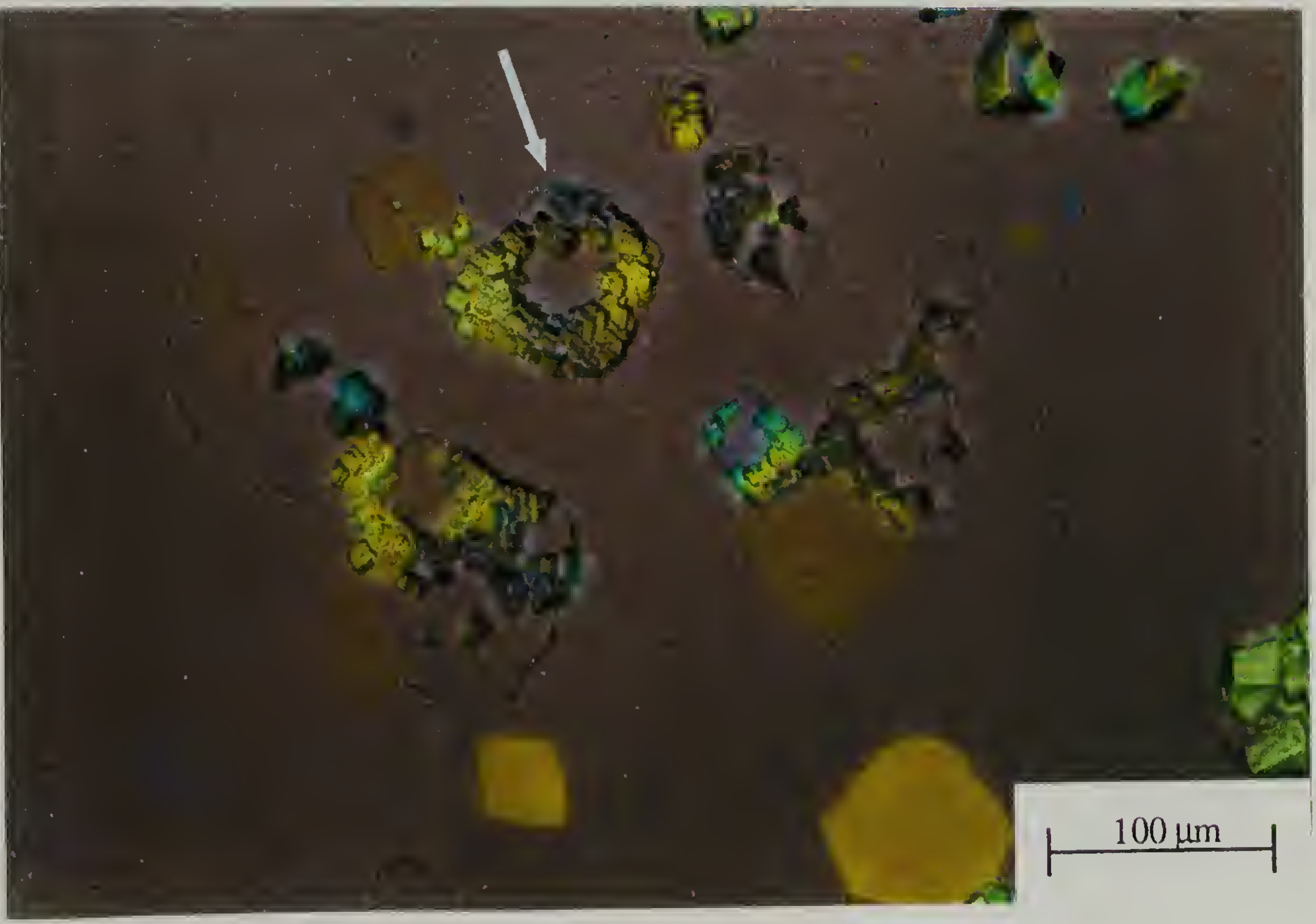
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CHAPTER 4

RELEVANCE OF A POLYMER-INDUCED LIQUID-PRECURSOR PROCESS TO CALCIUM CARBONATE BIOMINERALIZATION

Introduction and Review

Theories on biomineralization are based on *in situ* observations and *in vitro* experiments.[1-3] The latter case is especially useful because the basic interactions between organic and inorganic components can readily be investigated in a controlled and well-defined environment. *In vitro* systems typically use simple model polymers,[4, 5] or polymers that have been extracted from biogenic minerals, such as proteins[6-9] or sulfated polysaccharides,[10] to examine their interactions with the inorganic phase. It has been found that such polymers can interact with the ionic species of the forming mineral phase, to either inhibit or promote the nucleation and/or growth of the inorganic crystals, depending on whether the polymer is solubilized, or in the form of an insoluble matrix.[5] For example, the adsorption of solubilized polyelectrolytes onto the inorganic phase can be a means of inhibiting the growth of specific polymorphs,[2, 3] or of mediating the morphology through repression of the growth rates of specific crystallographic faces.[2, 3, 6, 7] Organic macromolecules in the form of a matrix may act as a template to promote nucleation of the inorganic crystals through either ionotropic interactions,[10] or more specifically, through stereochemical or epitaxial interactions to produce oriented growths.[2, 3, 5-7] Lastly, organisms appear to utilize the self-organizing ability of amphiphilic molecules to create compartments (e.g. vesicles) that enable control over the reaction medium and delineate space of the growing mineral phase.[1-3]

This work has examined an *in vitro* system that produces calcium carbonate ($CaCO_3$) films, tablets, and helical structures, by adding low levels of polyaspartate to a calcium chloride solution that is slowly saturated with ammonium carbonate vapor. The *in situ* observations presented in the last chapter showed that these unusual $CaCO_3$ morphologies result from the solidification of a phase separated hydrated $CaCO_3$/polymer liquid precursor. In this chapter, a comparison will be made between the features observed in this *in vitro* system and some of the puzzling features found in biominerals. This will lay the groundwork for an argument that suggests that a PILP type process might play a role, at a fundamental level, in the morphogenesis of $CaCO_3$ biominerals. Many of the principles upon which this argument is based have already been developed by others in the field, but it is believed the results of this *in vitro* model system help to demonstrate and clarify these ideas, as well as shed a new light on the subject of biomineralization.

Generality of the PILP Process

The ability of an *in vitro* system to accurately model biomineralization is limited, mainly because it lacks one of the most important ingredients, cellular control. In the polyaspartate system, only one aspect of the complicated processes of biomineralization is explored, and that is the influence of a solubilized acidic macromolecule on the morphology of $CaCO_3$ crystals. Other issues, such as the control of crystal orientation, location, and polymorph, are not dealt with herein. Oligomeric polyaspartate was chosen for this simple model system because the soluble proteins extracted from $CaCO_3$ biominerals frequently contain high levels (e.g. a third or more) of acidic residues, such as aspartic, glutamic, and phosphorylated amino acids,[1-3, 7] and some evidence suggests the presence of extended sequences of aspartate residues.[3, 7] There are other acidic macromolecules associated with biominerals, such as sulfated and carboxylated glycoproteins, glycosaminoglycans and polysaccharides,[3, 4, 10] and although they may have similar functions, further studies are needed before including them in this discussion.

Regarding cellular control, the carbonate vapor method used in this *in vitro* system is obviously not the means used by marine organisms to achieve supersaturation. Nevertheless, this technique was initially employed because the control reaction yields reproducible and pure crystal products (without other $CaCO_3$ polymorphs), which is desirable for morphological studies. In order to access the viability of the PILP process under differing synthetic conditions, the following four methods were tested: aqueous addition of $NaHCO_3$ and $CaCl_2$; KOCN decomposition method;[11] CO_2 escape method;[12] $(NH_4)_2CO_3$ vapor diffusion technique.[4] The products that resulted from addition of polyaspartate to these syntheses indicate that the PILP process was involved in each, to varying extents, as deduced by the formation of films and/or helices (see Table 4.1). Thus, it is concluded that the PILP process is not strictly a product of the vapor-diffusion synthetic technique, but is a general phenomenon that can occur under a variety of conditions in the presence of polyaspartate. It is particularly noteworthy that the addition of magnesium at a ratio of a $Mg^{2+}:Ca^{2+} = 5:1$, comparable to seawater,[11] or molluscan extrapallial fluid,[10, 13] dramatically enhanced the PILP process, to the point of depositing pure PILP film without solution crystallization byproducts (Table 4.1).

Comparison to Biominerals

Lateral growth of thin tablets. It was previously noted in Chapter 2 that the tablets and films deposited in this system are of similar thickness (≈ half a micron) and morphology (ranging from faceted to rounded platy tablets) to the aragonite and calcite tablets found in the nacre and semi-nacre of mollusk and bryozoan shells.[7, 14, 15] As Fig. 4.1 demonstrates (figures are at the end of the chapter), the films produced by the PILP process are composed of tablets that have grown laterally to produce a continuous mosaic of calcite crystals. In the nacreous layers in mollusk shells, lateral growth of tablets produces continuous lamellae of interconnected aragonite crystals.[2, 14] In semi-nacre,[15] although the calcite crystals are not organized into lamellar sheets, it is clear that

Table 4.1

Synthetic methods for the precipitation of $CaCO_3$

Synthetic Method*	Reactants†	Product Description‡
$(NH_4)_2CO_3$ vapor diffusion[4]	20 mM $CaCl_2$ $(NH_4)_2CO_3$§	aggs. of calcite and vaterite; helices; mosaic and spherulitic films and tablets; melded-droplets
	Temp. = 4°C	much more film, fewer aggs.
CO_2 escape[11, 16]	5 g/l $CaCO_3$ (dissolved by CO_2) = 20 mM $CaCO_3$	messy products, mostly on surface: aggs. of calcite and flattened spherulites, mosaic & dendritic film, a helix
KOCN decomposition[11]	20 mM $CaCl_2$ 2 g/l KOCN Temp. = 21°C (slow)	5 weeks: rubbery isotropic film with round birefringent patches dispersed throughout
	Temp. = 45°C (moderate)	2 days: streaks of film, both mosaic and spherulitic; arborescent aggregates
$NaHCO_3 + CaCl_2$ simple addition of reactants	20 mM $CaCl_2$ 20 mM $NaHCO_3$ 10 mM NaCl	aggs. of calcite and vaterite, many helices, melded-droplet film on solution surface (more film at a Temp. = 4°C)
Magnesium additive at $Mg^{2+}:Ca^{2+} = 3.5:1$	70mM $MgCl_2$ 20mM $CaCl_2$ $(NH_4)_2CO_3$§	thick films with windmill-type extinction around a centralized agg.; single-crystal patches w/ severe biaxial strain defects
	Temp. = 4°C	isotropic droplet film, crystallized after drying 4 days
Magnesium additive at $Mg^{2+}:Ca^{2+} = 3.5:1$	100mM $MgCl_2$ 20mM $CaCl_2$ $(NH_4)_2CO_3$§	isotropic droplet film, crystallizes after drying for extended times (depending on polymer conc.)

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*general methods were from the noted references, and modified as shown above.

†all experiments included 20 μg/ml P(D) additive unless otherwise noted.

‡control reactions yield calcite rhombohedrons, and sometimes vaterite spherulites.

§exact concentration unknown because it diffuses into the reaction from the vapor phase.

they are also restricted to lateral growth. It has been suggested that compartmentalization is responsible for the thin dimensions of the nacreous layers,[14] but Erben has argued that a pre-formed compartment does not exist.[17]

Based on the findings presented in Chapter 3, which were the result of an *in vitro* model system with polyaspartate additive, an alternative (and demonstrable) hypothesis for the formation of the thin $CaCO_3$ structures in nacre can be brought forth: The deposition of a precursor film generated by a PILP type process could yield the morphological dimensions found in these nacreous structures. One might argue that the pictures in the literature of the surface of nacre show pyramids of isolated tablets,[3, 17, 18] rather than continuous precursor films as seen in this system. This is countered by pointing out that any amorphous $CaCO_3$ film surrounding the growing tablets would be dissolved away by the cleaning procedures used for biomineral sample preparation. Alternatively, localization of the precursor (such as by cellular secretion of a PILP phase) could be used in the complex and dynamic processes of biomineralization.

Sectorization of calcite tablets. The nacreous tablets in mollusk shells are arranged into three-dimensional hierarchical structures, while the tablets and films produced in my *in vitro* system using polyaspartate are deposited in a random fashion. Nevertheless, optical micrographs of the tablets (e.g. Fig. 2.5B) bear a resemblance to pictures in the literature showing a plan view of the growing surface of nacre.[2, 7, 14, 19] Even more striking is a comparison between the sectored calcite tablets shown in Fig. 4.2A, and the sectored calcite tablets of cyclostome bryozoan semi-nacre.[15] The six-sided calcite tablets in bryozoan semi-nacre are described by Weedon and Taylor[15] as being differentially etched in alternating triangular sectors: This etching (presumably caused by deterioration or the cleaning procedure) is seen as a pimpled surface in the three more soluble sectors, whereas the less-soluble sectors have smoother surfaces, but with well-defined growth lines. They also note that, in some of the six-sided tablets, three of the growing edges are slightly longer than the other three, giving alternately narrow and broad sectors, where the narrower sectors are more soluble. This description is consistent with the sectorization patterns in the PILP tablets, where "squiggly wrinkles" are frequently found in narrower sectors, while linear transition bars are found in broad sectors (Fig. 4.1B and 4.2A). In fact, if it is assumed that a PILP type process contributes to the formation of these biomineral tablets, it could explain the preferential etching patterns. For example, in the PILP system, the apparent correlation between transition bars and lattice suggests that the "squiggly" wrinkles arise from microdefects (Fig. 4.2B). Thus, it follows that the narrower sectors would be more soluble if they are less ordered.

Expression of unstable crystallographic faces. The epitaxial overgrowth technique, a method used for determining the crystallographic orientation of facet-less biomineral crystals,[20] was used in my study to determine the orientation of the calcite tablets composing the PILP deposited films. It was found that many of these tablets had expressed relatively unstable crystallographic faces. For example, the epitaxial overgrowths shown in Fig. 4.3A grew on an isotropic patch of film, demonstrating that it was not amorphous film, but rather, was calcite oriented perpendicular to the isotropic *c*-axis. This example was chosen because it is the (001) faces that are expressed in nacreous and semi-nacreous tablets; but in my system, a variety of other crystal faces are expressed as well (Fig. 4.3B). Therefore, it is deduced that the expression of the various crystallographic faces (in my system) is a result of physical constraints by the precursor phase itself, and is not due to cessation of growth by a compartment or stereospecific interaction. For example, the crystals that grow from the precursor phase are limited to planar dimensions, therefore, the surfaces that are expressed correspond to the planes that nucleated on the glass substrate.

It is seen that, while the PILP process can provide a morphogenic mechanism for the formation of thin tablets, and allows the expression of unstable crystallographic faces, other, more specific interactions would be required during the nucleation event for orientational control. Work by Addadi and Weiner[3-6] has shown that nucleation from the (001) planes of calcite can be promoted through a cooperative effect of polyaspartate, in the β-sheet conformation, bound to sulfonated polystyrene film. In their *in vitro* studies, it was found that an increase in the sulfonation of polystyrene films, alone, causes an increase in (001) oriented crystals until a maximum is reached (60%), at which point the films loose their structural integrity and decline to about 7% (001) orientation (this decline is thought to arise from the inhibitory action of the fluid-like disorganized surface);[4, 5] but adsorption of the structured polyaspartate (infrared shows about 50% β-sheet conformation) to the highly sulfonated films, led to a three-fold increase in the number of (001) oriented crystals (25%).

It is not hard to imagine that deposition of the PILP phase in combination with a specific nucleating substrate could produce a sheet of interconnected basal tablets, as in the nacreous layers. In some species, the orientation of the nacreous tablets is mediated in the *a,b*-directions as well,[5] thus even more specific interactions would be required during the nucleation event.

Formation of curved crystal surfaces. Thus far, this discussion has focused on a comparison between the tablets/films produced by the PILP process and the nacreous or semi-nacreous biomineral tablets. But the argument concerning the expression of unstable crystallographic faces can be extended to include single crystals that expose curved surfaces, and spatially-delineated single crystals (discussed in the next section). One of the hallmarks of the PILP process is that non-faceted crystal morphologies are generated by physical constraints of the precursor phase. A manifestation of this effect can be seen in the swirl-type film in Fig. 4.4A, in which the edges of the crystals are non-faceted and have continuous curvature.

In the above example, one might argue that the curved edges of the thin crystals expose little surface area, so that the minimizing surface energies that lead to faceting are negligible. However, it is noted that calcitic coatings deposited onto curved substrates, such as the metal wire in Fig 4.4, can also form crystals with curved surfaces, but in this case, the curvature extends over a large surface area. Thus, once again, it is seen that the physical constraints of the precursor phase can override the crystallochemical driving forces for minimization of surface energy, and can lead to crystals that express unstable faces, including those with curvature. In retrospect, these observations may seem trivial since we now know that these products are formed by a precursor process; but issues such as these (i.e. the expression of unstable crystal surfaces) have been the subject of many discussions on biomineralization, in which it has been assumed that $CaCO_3$ crystals are grown from a solution.

Spatial delineation of crystals. Along these same lines, glass beads were also tested as curved substrates for $CaCO_3$ coatings. In a few instances, coatings were deposited on these tightly curved substrates, however, most of the material deposited in the cracks and interstices of the overlapping beads (Fig. 4.5). The beads became "cemented" together and to the glass substrate by $CaCO_3$ crystals. These crystals are non-faceted and fill the space delineated by the beads; they are also of a single crystalline nature (as opposed to spherulitic), as judged by their uniform extinction under crossed-polars. The control reaction yielded characteristic rhombohedrons, with no preferential growth in the cracks (Fig. 4.5A). Although not an elaborate experiment, these spatially-delineated single crystals demonstrate an important point: A PILP process, used in conjunction with compartmentalization, can provide a viable means of morphological control.

The hallmark of biomineralization is that crystals are formed within compartments to generate unusual morphologies.[1-3] A classic example is the sea urchin spicule, which has a highly convoluted structure, yet is composed of a single crystal of magnesium-bearing calcite.[2] Of course, it is presumed that this elaborate morphology results from spatial delineation provided by the vesicle within which the spicule grows.[1-3] However, there seems to be very little evidence to show that a pre-formed compartment alone is sufficient for overriding the strong crystal driving forces that lead to faceted crystals in a solution crystallization environment. It has been suggested that transformation of an amorphous material to a crystalline phase could be a means for filling space with crystals, although it was predicted that such an energetically unfavorable state would rapidly nucleate and form spherulitic type growths.[2] The results presented here demonstrate that a polymer can stabilize or induce an amorphous precursor, and as a liquid, this precursor can readily fill a pre-formed space. The fluidity of a liquid type precursor would be advantageous for allowing dynamic morphological control, such as by mechanical fields applied to vesicular membranes by intracellular fibers.[3] Regarding the metastability of the precursor, both spatially-delimited single crystals and spherulitic growths have been produced by the PILP process, presumably because the polymer imposes kinetic limitations on the nucleation event.

Influence on composition. It is intriguing that both the nacreous and semi-nacreous tablets, found in very different organisms and composed of different $CaCO_3$ polymorphs, have such similar morphological features (e.g. thin platy habits, anhedral to pseudo-hexagonal shapes, sectorization, screw dislocation steps, etc.).[3, 7, 11, 14] Although there are most certainly differing levels of control in the formation of the various types of nacre, it seems likely that there is some commonalty in the processes involved. It has been shown that both calcite and vaterite films (and possibly calcium carbonate hexahydrate) can be deposited by the PILP process, therefore this process does exhibit some generality for different $CaCO_3$ polymorphs. However, my experiments thus far have been unsuccessful at producing aragonite tablets or films. This was attempted by adding high levels of magnesium ion (Mg-ion) to the solution, which is known to promote metastable aragonite through inhibition of calcite growth.[11, 12] Although the magnesium dramatically enhanced the PILP process, it mainly produced magnesian-calcite films (Fig. 4.6A). In retrospect, this failure to produce aragonite is not surprising considering that two independent groups have recently shown that certain soluble polyanionic proteins, extracted from nacreous biominerals, act to promote the growth of aragonite over calcite (and in the absence of Mg-ion).[8, 9]

High levels of Mg-ions are incorporated into these films, as shown by x-ray micro-analysis in Fig. 4.7 and Table 4.2. This supports the argument that a PILP type process may be involved in biomineralization, because it is not uncommon to find magnesian-calcite in biominerals,[1, 3, 11] yet calcite grown in solution does not incorporate significant levels of Mg-ions.[11, 21] Presumably, this difference arises because the Mg-ions are sequestered into the precursor phase along with the Ca-ions, and subsequently entrapped by the solidification process (see section note 4.1). It is notable, that these mixed compositions, as well as the "molten" morphologies described in Chapter 3, are the result of a solidification process that occurs at room temperature, and is even enhanced at cold temperatures (see Fig. 3.9A in Chapter 3). This finding may have geological implications because it is seen that very low levels of organic matter (barely detectable) can have considerable impact on the incorporation of impurities into what is presumed to be a solution-grown mineral.

General Discussion on Relevance to Biomineralization

The previous chapter showed that the polyaspartate additive can convert a solution crystallization to a solidification process, whereby non-equilibrium $CaCO_3$ morphologies are generated. To my knowledge, this non-specific but influential role of a polyelectrolyte has not been described before in regards to biomineralization. However, many related concepts have been proposed by others, such as space-filling amorphous precursors,[2] gelatinous viscosity effects (organic gels),[22-24] stabilization of amorphous phases[2] and precursor hydrated crystals,[6, 25] ionotropic[10] and cooperative nucleation.[7]

Table 4.2

Composition of magnesium-bearing films by EDS*

	Mg:Ca = 5:1†		Mg:Ca = 3.5:1†	
Element	Norm. wt. %	Norm. atom. %	Norm. wt. %	Norm. atom. %
calcium	22.13 ± 1.85	10.71 ± 1.02	44.88 ± 2.27	24.10 ± 1.56
magnesium	21.15 ± 1.18	16.87 ± 0.91	4.11 ± 1.31	3.63 ± 0.95
carbon	9.27 ± 0.54	14.95 ± 0.69	8.29 ± 0.35	14.84 ± 0.42
oxygen	47.45 ± 0.86	57.47 ± 0.34	42.72 ± 0.78	57.45 ± 0.05

*percentages were normalized with respect to a dolomite standard using an average of three sample areas ± standard deviation (n weighting); ZAF chi-squared values were as follows: $\chi^2 = 12.40 \pm 1.49$ for 5:1 and $\chi^2 = 29.05 \pm 1.26$ for 3.5:1

†reaction conditions given in Table 4.1.

It is my belief that the PILP process is the missing piece of the puzzle that brings these ideas together to form a workable hypothesis for the morphogenesis of $CaCO_3$ biominerals.

Most introductions to the field of biomineralization begin with a general introduction on the theory of solution crystallization, but if a PILP type process is involved, this classical description would not be appropriate, or certainly not complete, for explaining the morphological aspects of a solidification process. For example, non-equilibrium crystal morphologies result from physical constraints upon the precursor phase, such as the filling of space, or the deposition of a coating; the crystal driving forces still play a role during the nucleation event and transformation, thereby influencing the polymorphic type, orientation, and defect texture of the crystals.

The presence of intracrystalline proteins in biominerals is well documented,[3] and most discussions on biomineralization, which hitherto are based on solution crystallization theory, seem to implicitly assume that, if these polyanionic proteins play a role in mediating the crystal morphology, "specific" interactions would be involved. Certainly, interactions of this type have been demonstrated *in vitro*,[6] and the occlusion of proteins along certain crystallographic planes in the spicule of the sea urchin suggests that such interactions occur *in vivo* as well.[3] However, my results show that "specific" structural-type interactions with a growing crystal are not a necessity for morphological control; instead, a means of control can be accomplished prior to crystal nucleation, through "non-specific" chemical interactions. This aspect of my argument is appealing because it suggests that the morphological similarities between biominerals, in organisms that diverged and evolved separately (i.e. nacre and semi-nacre), could be founded on relatively simple chemical interactions. To recap what was said earlier, this argument is not intended to oversimplify the many processes that are involved in biomineralization; no doubt more specific interactions have evolved to build in the various levels of complexity that are observed in biominerals.

The details of the PILP process still remain to be elucidated, but it is hoped that this research will stimulate the interest of those working in the field of biomineralization. It is anticipated that direct observations of a PILP type process *in vivo* will be difficult to discern. An easier approach to this problem, but more direct than the experiments reported here, would be to test the acidic proteins extracted from biominerals to see if they exhibit a PILP process when placed in an *in vitro* environment that simulates sea water (or biomineralizing conditions). In one respect, this experiment has already been carried out by two different groups, Falini et al.[8] and Belcher et al.,[9] although they were not aware of, or looking for, the PILP process. Nevertheless, it is noted that the "tablets" pictured in their papers, deposited *in vitro* using soluble polyanionic proteins extracted from *Haliotis rufescens* (red abalone, gastropod mollusk)[9] and *Mytilus californianus*

(Bivalve),[8] are very similar in appearance to the ones seen in my studies, and in particular those pictured in Fig. 4.6B. Also, Belcher et al.[9] discuss a layering effect in their tablets that arises solely from the soluble protein additive, and a similar phenomenon occurs in my system, and can readily be understood (see section note 4.2)!

Conclusion

Based on the following features exhibited by a $CaCO_3$/polyaspartate *in vitro* model system, an argument has been presented which suggests that a PILP type process may be involved in the morphogenesis of $CaCO_3$ biominerals: lateral growth of thin $CaCO_3$ tablets to form a continuous sheet; expression of unstable crystallographic faces, formation of non-faceted single crystals with curved surfaces; spatial delineation of single crystals; sectorization of tablets; formation of $CaCO_3$ cement; formation of magnesium-bearing calcite. This argument is consistent with general considerations based on the pre-existing theories of biomineralization, aside from the fact that classical solution crystallization behavior is no longer applicable.

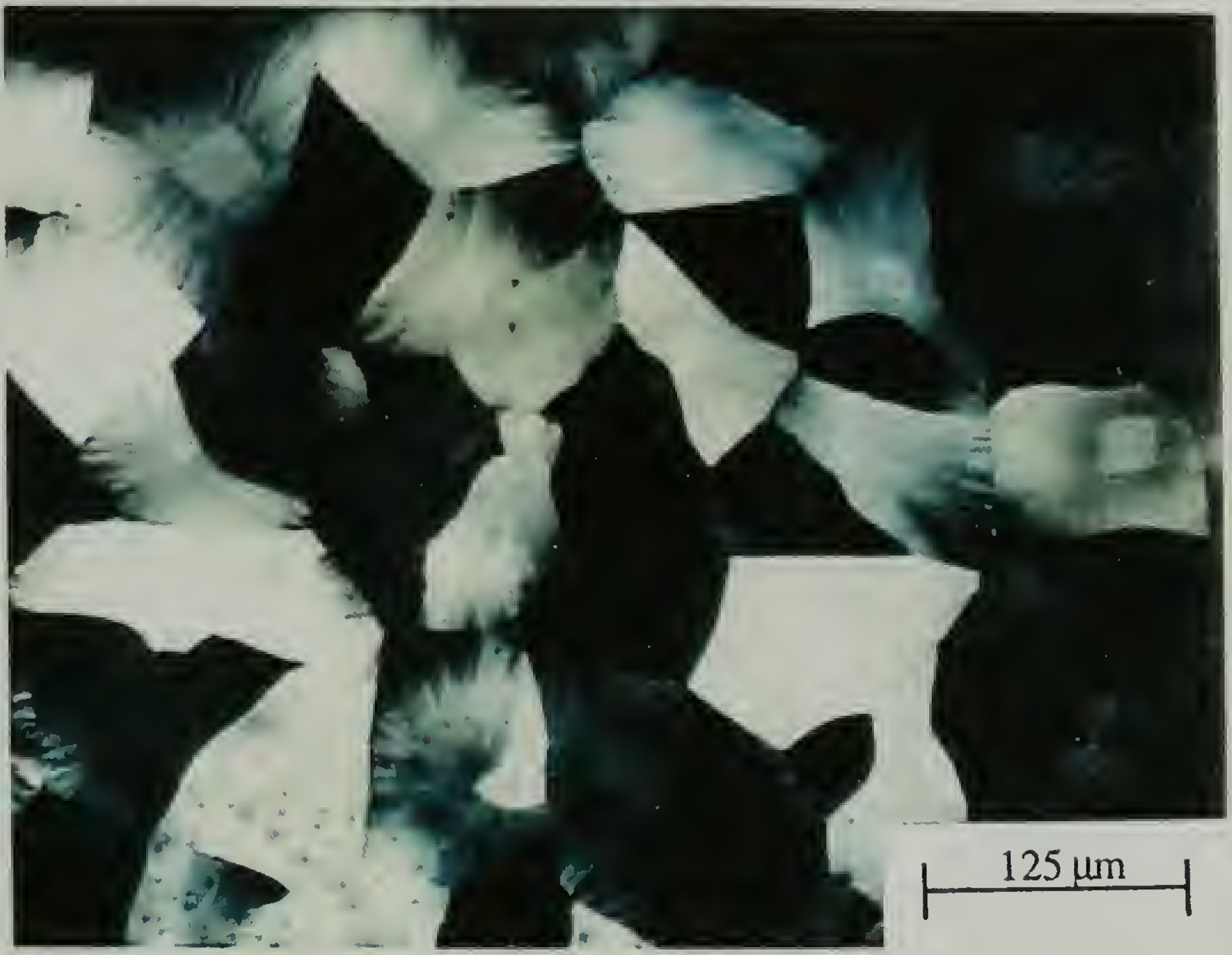
In the classic book On Biomineralization,[1] it is stated that "even though the final product is unique, the specific processes involved at each stage may be common to many different taxonomic groups. An important objective of research on biomineralization should be to identify and understand these underlying processes." A PILP solidification process could conceivably be at the foundation of spatially-delineated mineral formation, and should be considered in the cases where the mineral phase is found to contain soluble polyanionic proteins. At this point, one can only speculate about proteins that contain high levels of aspartate residues, but future work will determine if other acidic macromolecules exhibit similar behavior, and if the PILP process is applicable to different inorganic systems. With visions in mind of beautifully sculpted biomineral structures, one can start to imagine the potential of such a process.

Section Notes

Note 4.1 The atomic % values in Table 4.2 do not sum to a stoichiometric composition expected for an alkaline earth carbonate. The x-ray diffraction pattern of the 5:1 film shown in Fig. 4.6A matches calcite, but has a reduction in the spacings: 3.79, 2.99, 2.46, 2.25, 2.06, 1.88, 1.84. Reduced spacings are expected for incorporation of the smaller Mg-ions, however, this reduction is considerably less than would be predicted from the compositional analysis. For example, with the high level of magnesium in the 5:1 film, one might expect a dolomitic structure, which is the thermodynamically stable form at this concentration (dolomite mineral is a rhombohedral double carbonate, $CaMg(CO_3)_2$), as opposed to a solid-solution crystal); however, the spacings were not reduced to the point of matching dolomite, and no superstructure reflections were observed.[11] Interpolation between the hexagonal lattice constants plotted for the range of calcite to dolomite (as given by Lippmann),[11] calculated from the strong (10.4) reflection, yields an estimate of only 17 mol% $MgCO_3$. Lippmann points out that discrepancies between chemical analysis and diffraction spacings may arise from lattice disorder, such as tilting of the planar carbonate groups. It is also possible that the magnesium ions are excluded to defect sites and grain boundaries. Indeed, a high degree of microdefects are seen in the crystals composing these Mg-bearing films.

Note 4.2 Uniform layers are understandable in the PILP process since the layers are built of coalesced droplets of uniform dimensions (typically 2-4 μm), where a second round of accumulated precursor could deposit onto a pre-solidified layer. It has been observed that films preferentially grow onto certain substrates, with the most favorable substrate being calcium carbonate (relative to glass substrates, and then polystyrene dishes). This may be a consequence of the different wetting properties of the substrates, with a preferential adsorption occuring on the ionic surface of calcium carbonates, or alternatively, it may be a kinetic effect. For example, the amorphous precursor films sometimes dissolve rather than crystallize, thus epitaxial nucleation on calcitic substrates may speed up the crystallization to stabilize these overlayers relative to deposits on other substrates, where only heterogeneous nucleation is available.

Fig. 4.1 Lateral growth of $CaCO_3$ tablets. (A:top) This calcitic film was disrupted at an intermediate stage of the synthesis, and shows outlines of the originating tablets, before lateral growth produced a continuous sheet of interconnected tablets. Note, crystal defects are prevalent, especially towards the periphery of each single-crystal patch. (B:bottom) This film comprises a triangular tablet that is easily detected by the wrinkle patterns that have occurred during the incremental steps of lateral growth (polars are not fully crossed). Note, the wrinkles are being filled in, or covered, with a smooth layer of film, and would not normally be detected in the final film products.


125 µm

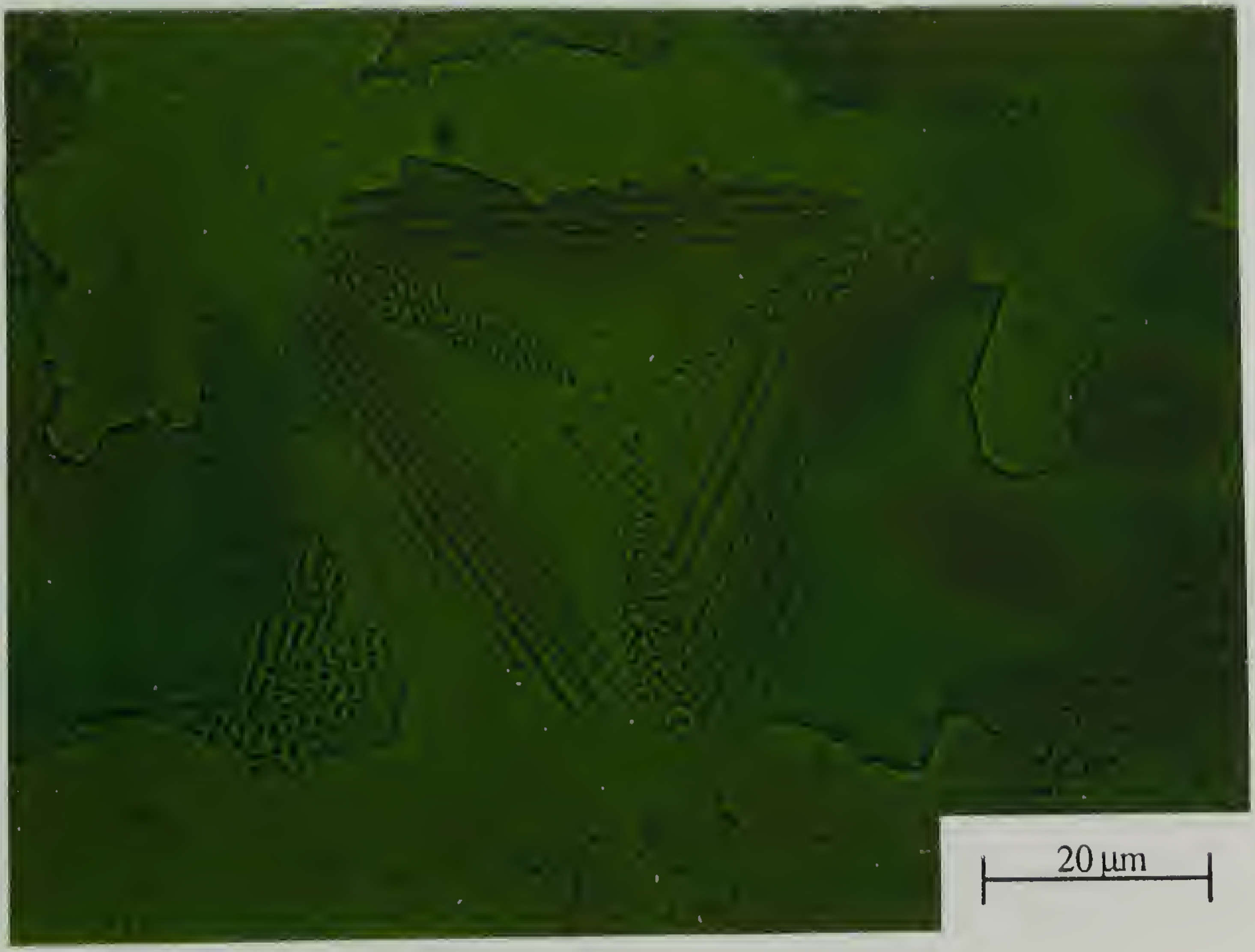
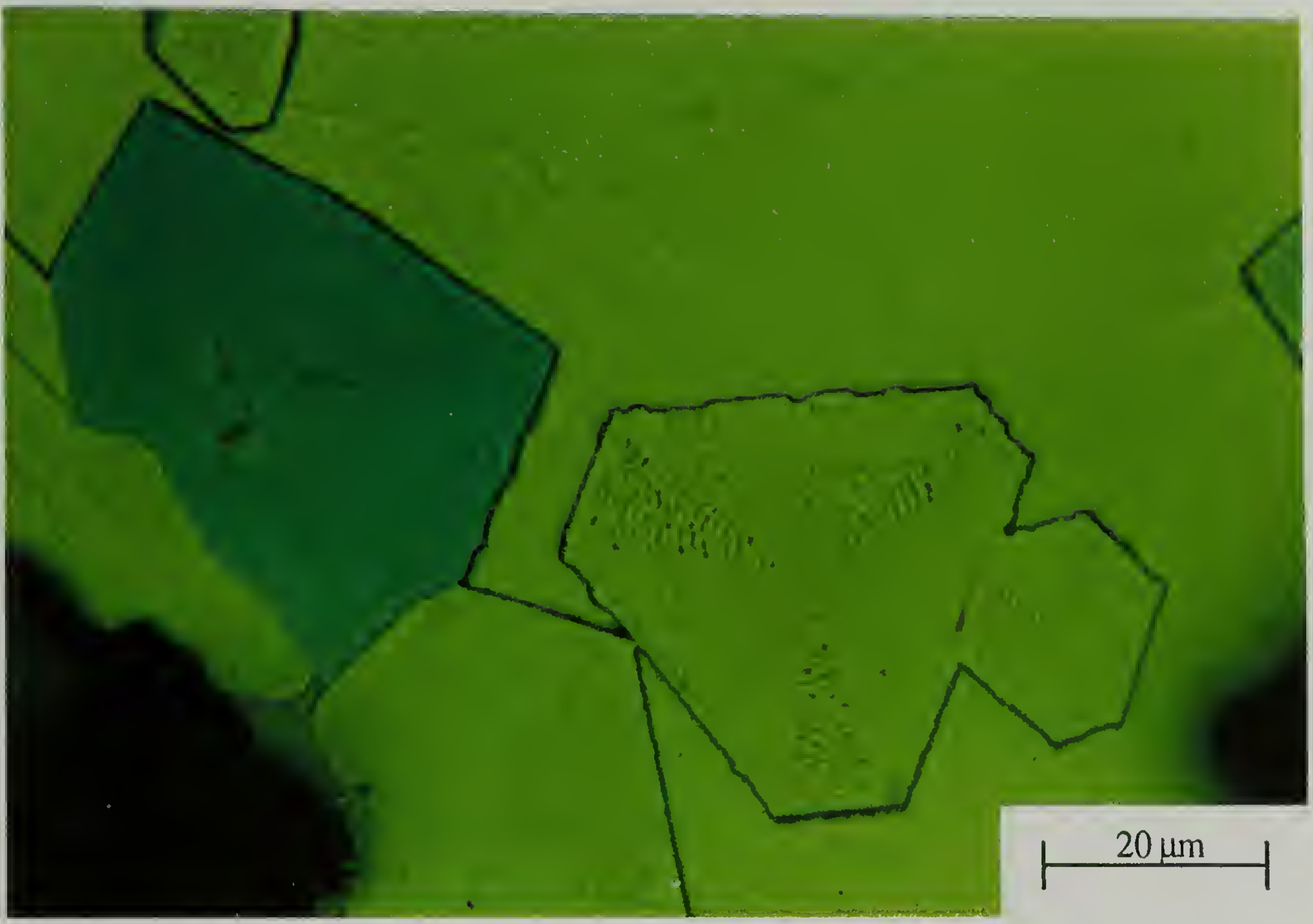

20 µm

Fig. 4.2 Sectored tablets with differential wrinkle patterns. (A:top) Hexagonal tablets have “squiggly wrinkles” in alternate, and frequently narrower, sectors (also seen in the pseudo-hexagonal tablet of Fig. 4.1). Note the similarity in these hexagonal tablets to the semi-nacreous tablets pictured in the literature,[15] which are sectored and have a similar differential pattern, but which are observed by partial etching rather than transition bars. (B:bottom) The wavering birefringence (crossed-polars with 1st-order red plate) of the disordered sector in this distorted tablet is suggestive of microdefects, consistent with the premise that the transition bars are orientationally correlated with the crystallographic lattice.


20 µm

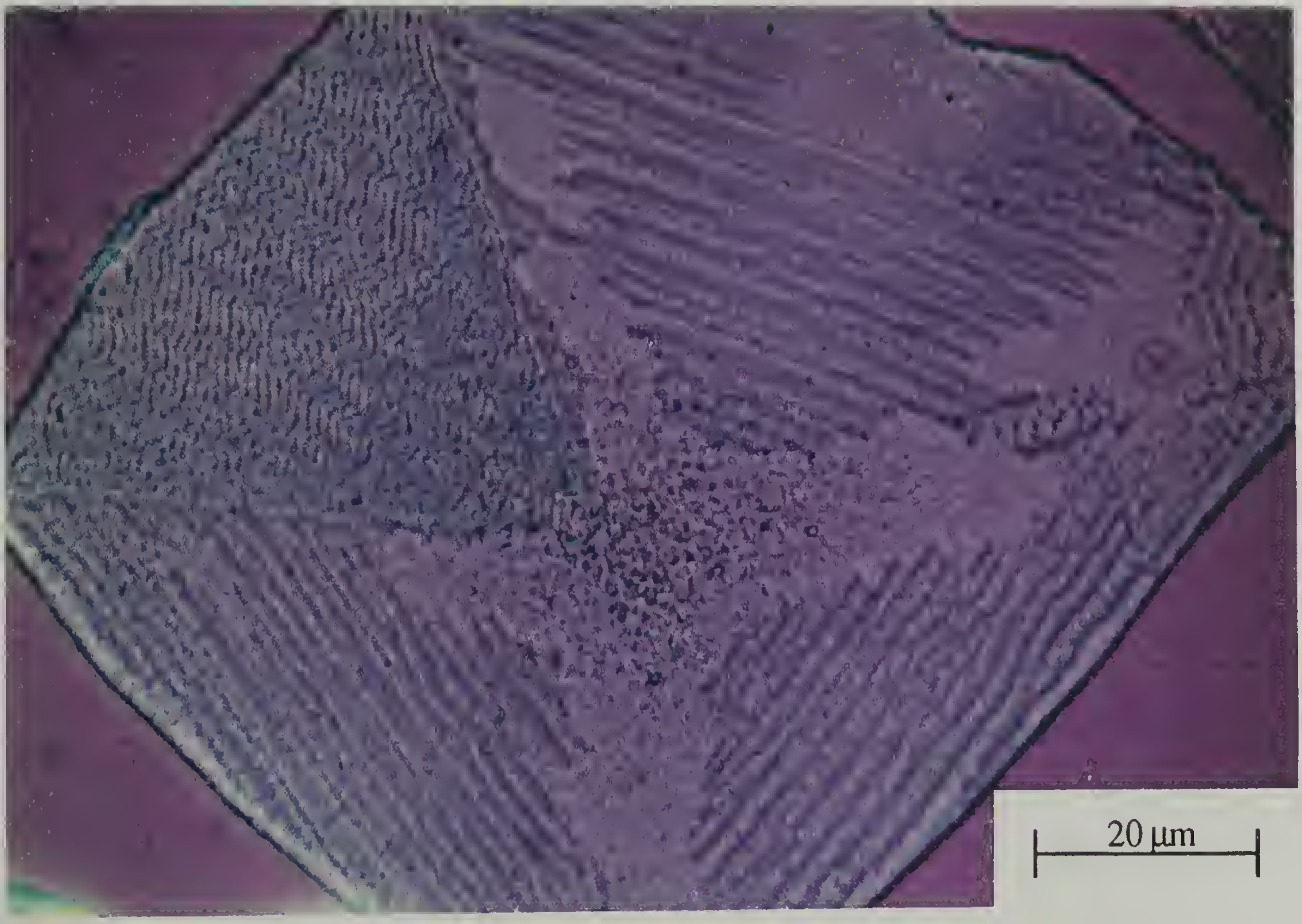
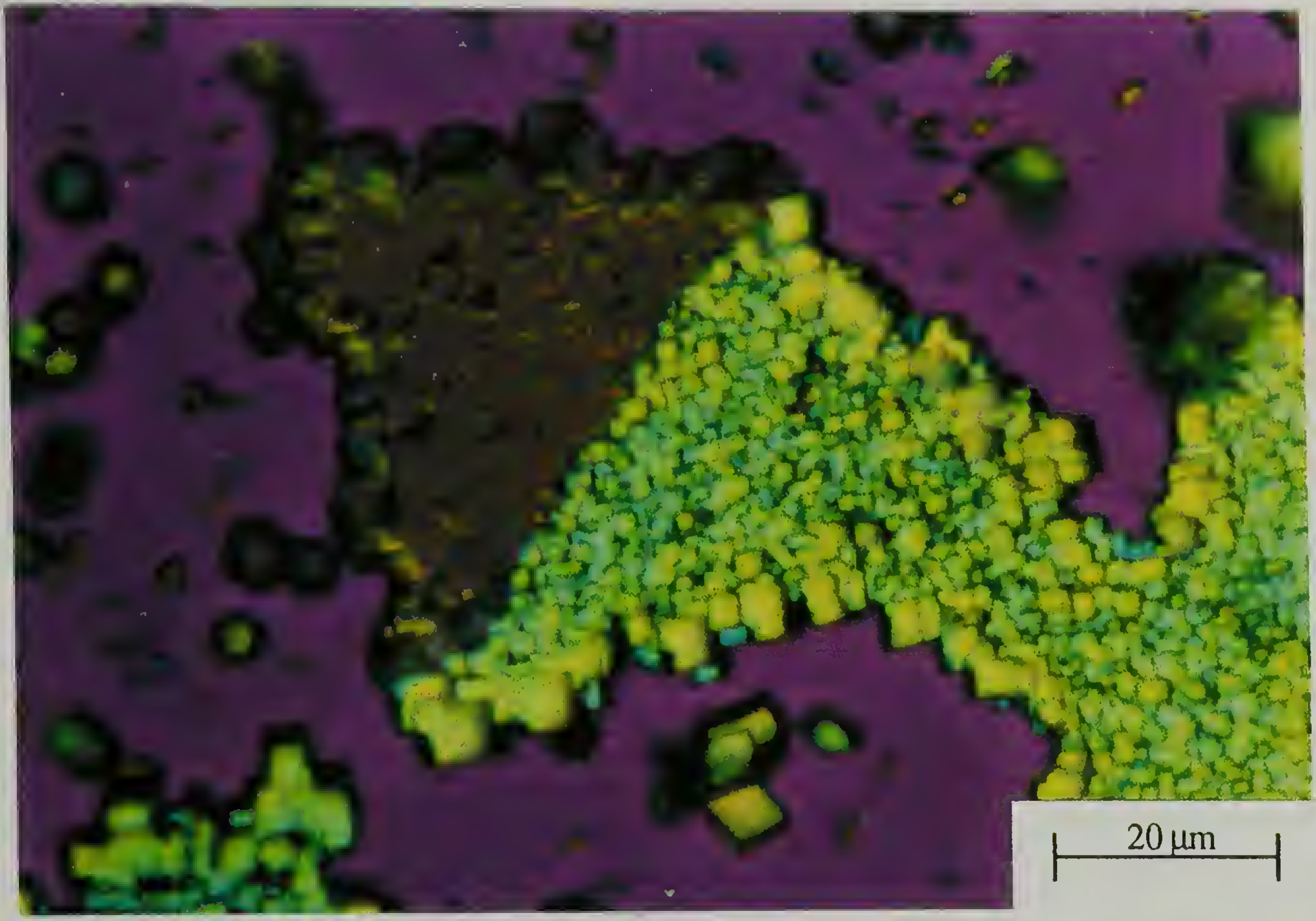

20 µm

Fig. 4.3 Expression of unstable crystallographic faces. The orientation of the underlying tablets can be seen by the epitaxial overgrowths. These optical micrographs were taken using the 1st-order red plate, so that both birefringent and isotropic material can be viewed in cross-polarized light (amorphous and isotropic crystals are the same magenta color as the background). (A:top) The secondary overgrowths on the magneta tablet to the left are oriented along their isotropic c-axes, thus the underlying tablet had expressed the relatively unstable (001) face of calcite. The highly birefringent overgrowths on the yellow tablet to the right express the typical {104} faces of calcite. The random, larger crystals, are from solution growths that precipitated in a non-epitaxial fashion. (B:bottom) A variety of crystallographic orientations are seen in the overgrowths on different tablets, but in this example, a gradual shift in orientation is seen within individual single-crystal patches. Once again, such strains in the crystallographic lattice are likely caused by dehydration shrinkage stresses.


20 μm

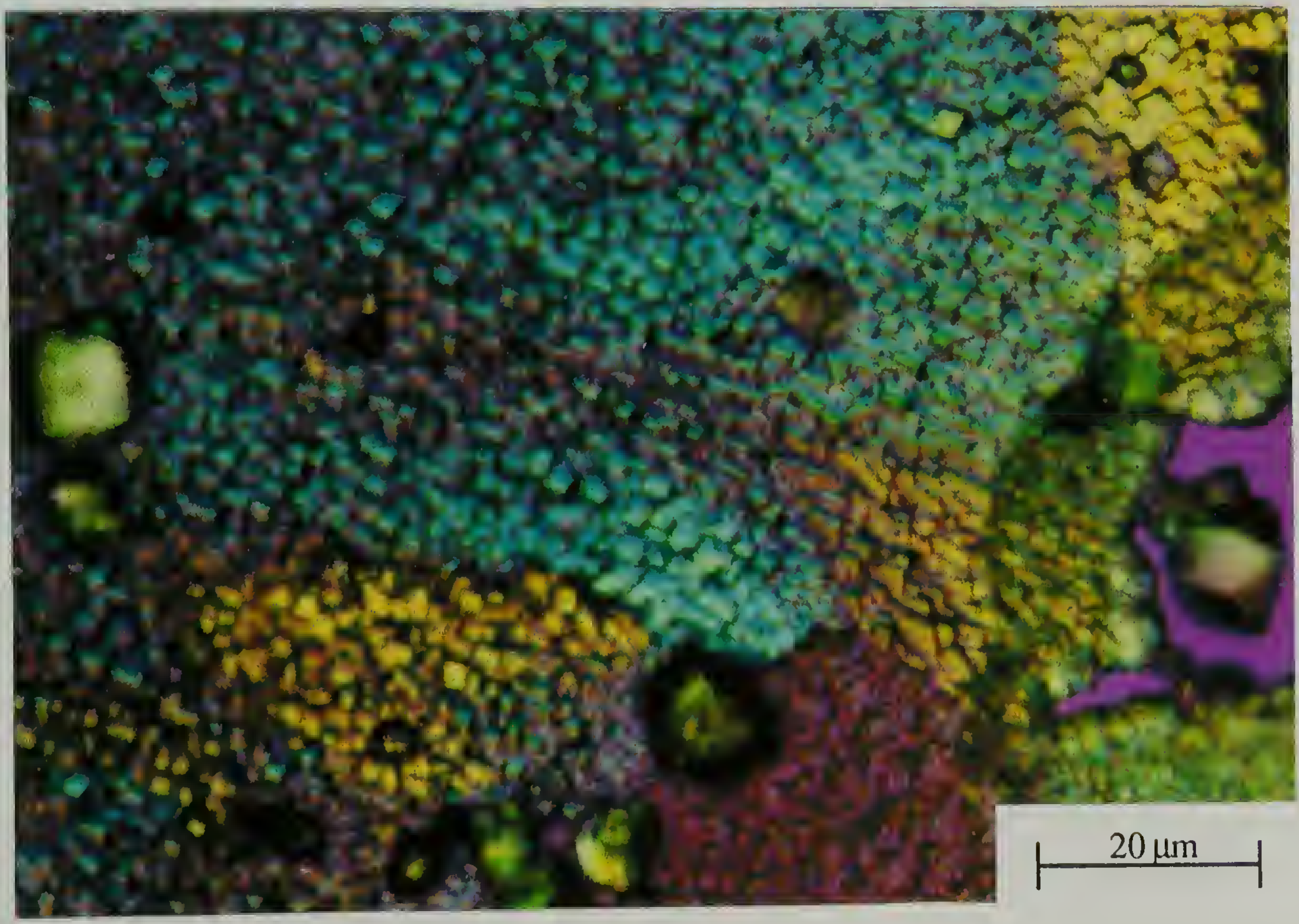
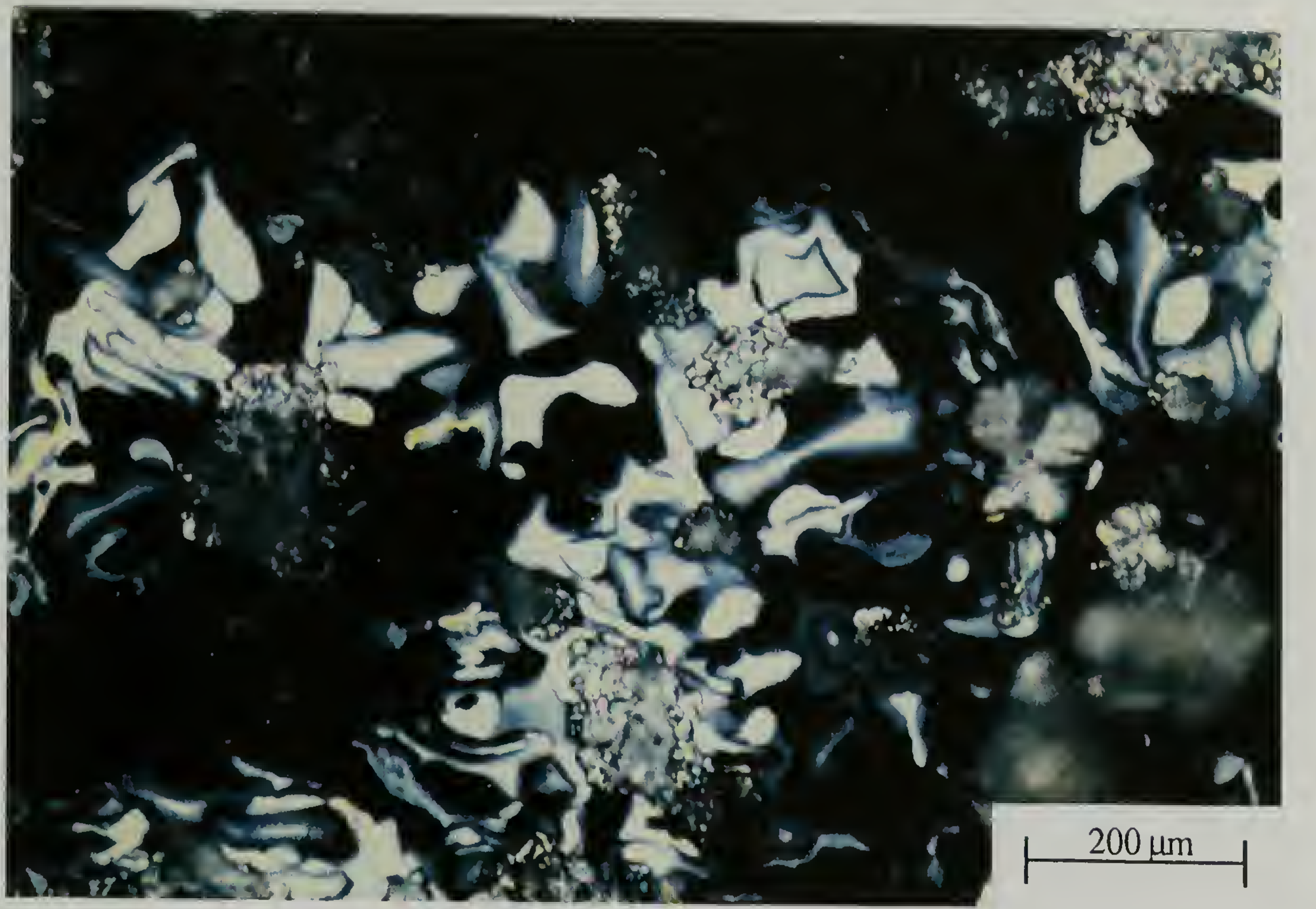

20 μm

Fig. 4.4 Non-faceted single crystals. (A:top) This swirl-type film has non-faceted, smoothly-curved edges, unlike typical crystals of calcite grown from solution. Each bright patch exhibits a uniform, single-crystal extinction as the stage is rotated under cross-polars. (B:bottom) A mosaic film was deposited onto a Pt/Pd wire (200 μm D.), and contains single crystal patches that have large curved surfaces (reflected light with cross-polars and the 1st-order red plate).


200 µm

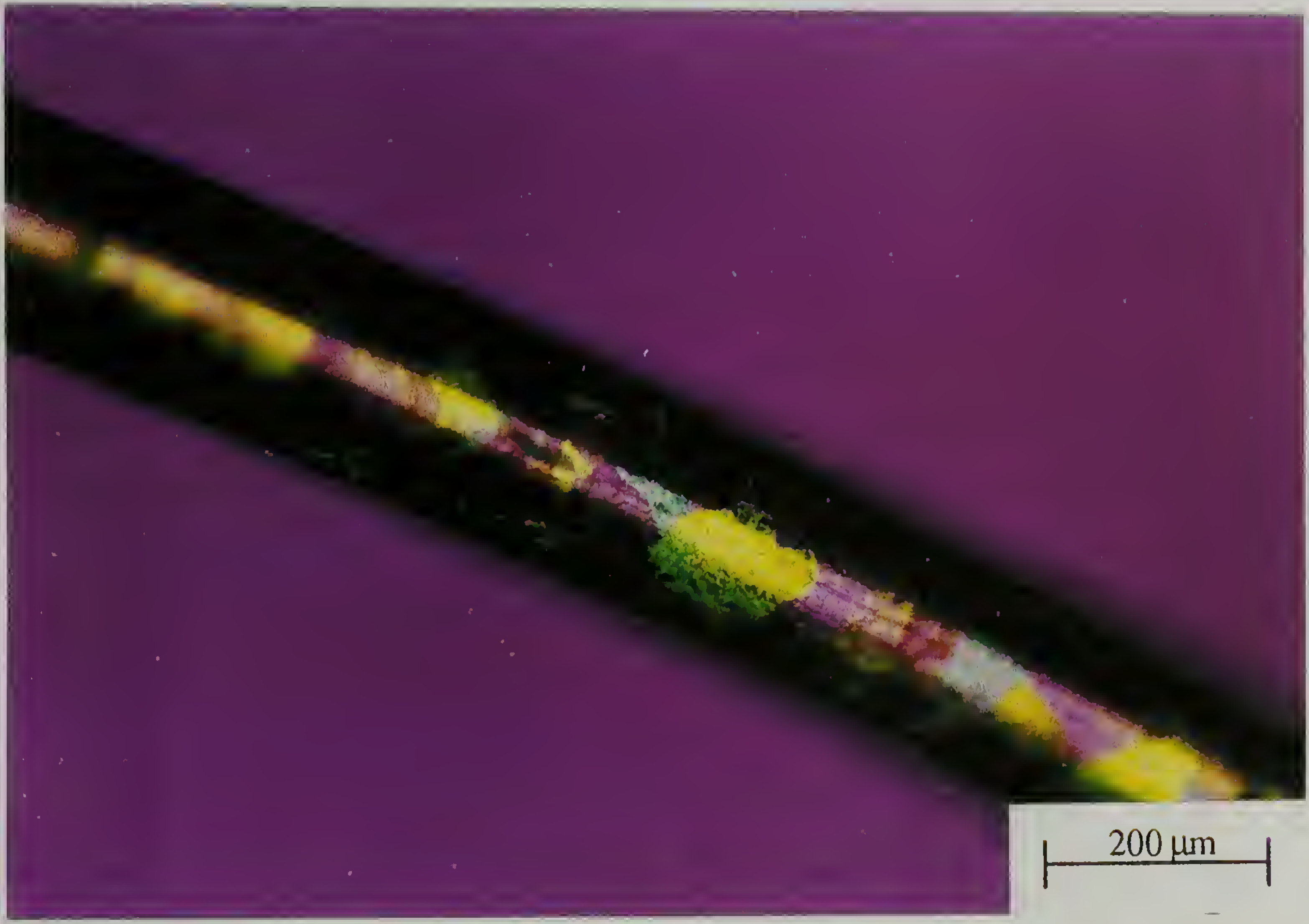
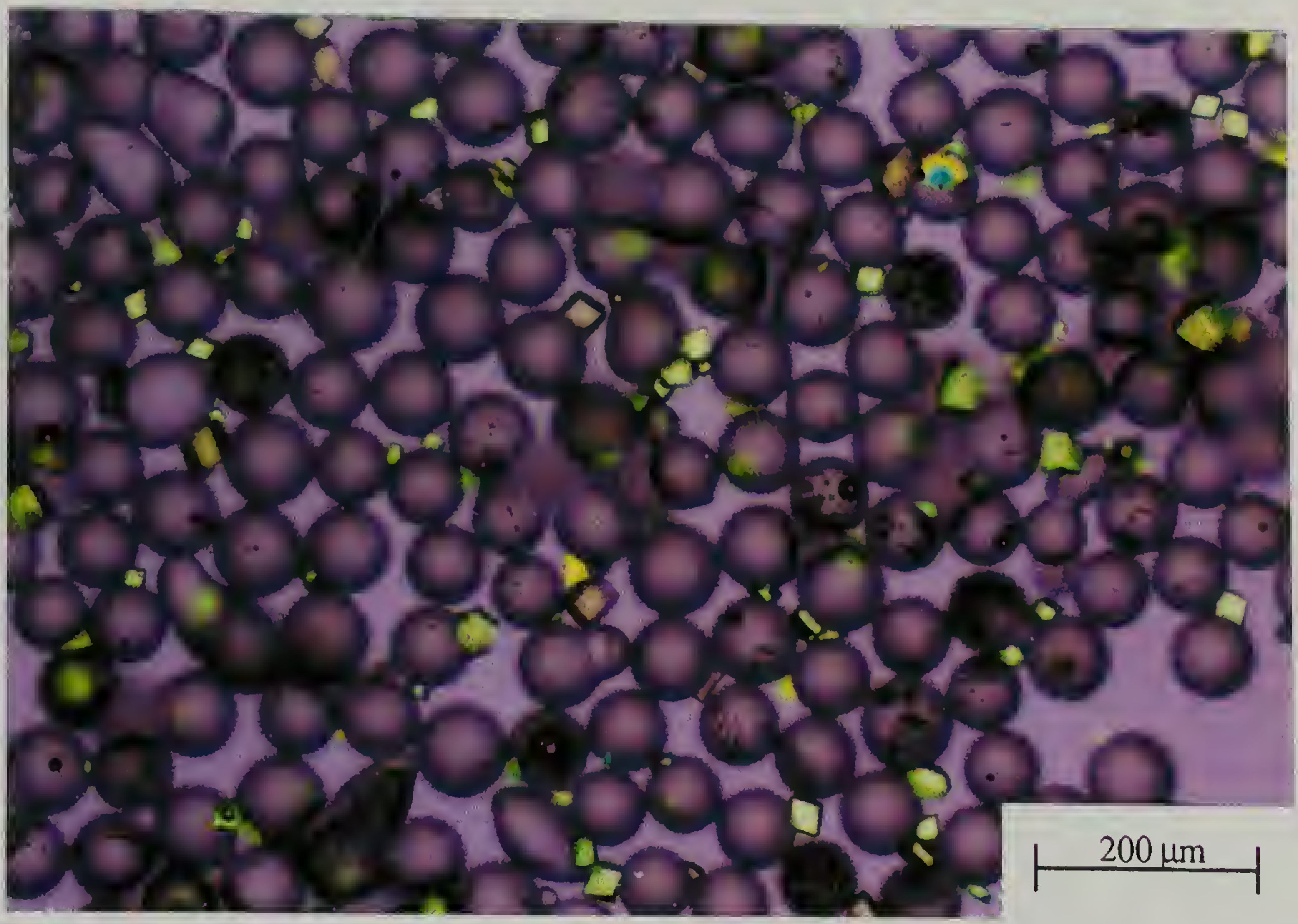

200 µm

Fig. 4.5 Spatially-delineated single crystals. (A:top) Supelco post column beads, 70 μm in diameter, were added to the crystallizing dish. The control reaction (without additive) produced calcite crystals of rhombohedral habit. (B:bottom) With the addition of polyaspartate, the PILP phase accumulated in the interstices of the glass beads and created spatially-delineated single crystals. The arrow shows an example of a large single crystal (uniform extinction under crossed-polars) that fills the space between the beads, as judged by the lack of crystal facets and wedge-like interference fringes. The large interface created by filling the interstitial space with $CaCO_3$ causes the beads to become "cemented" to the glass substrate.


200 µm

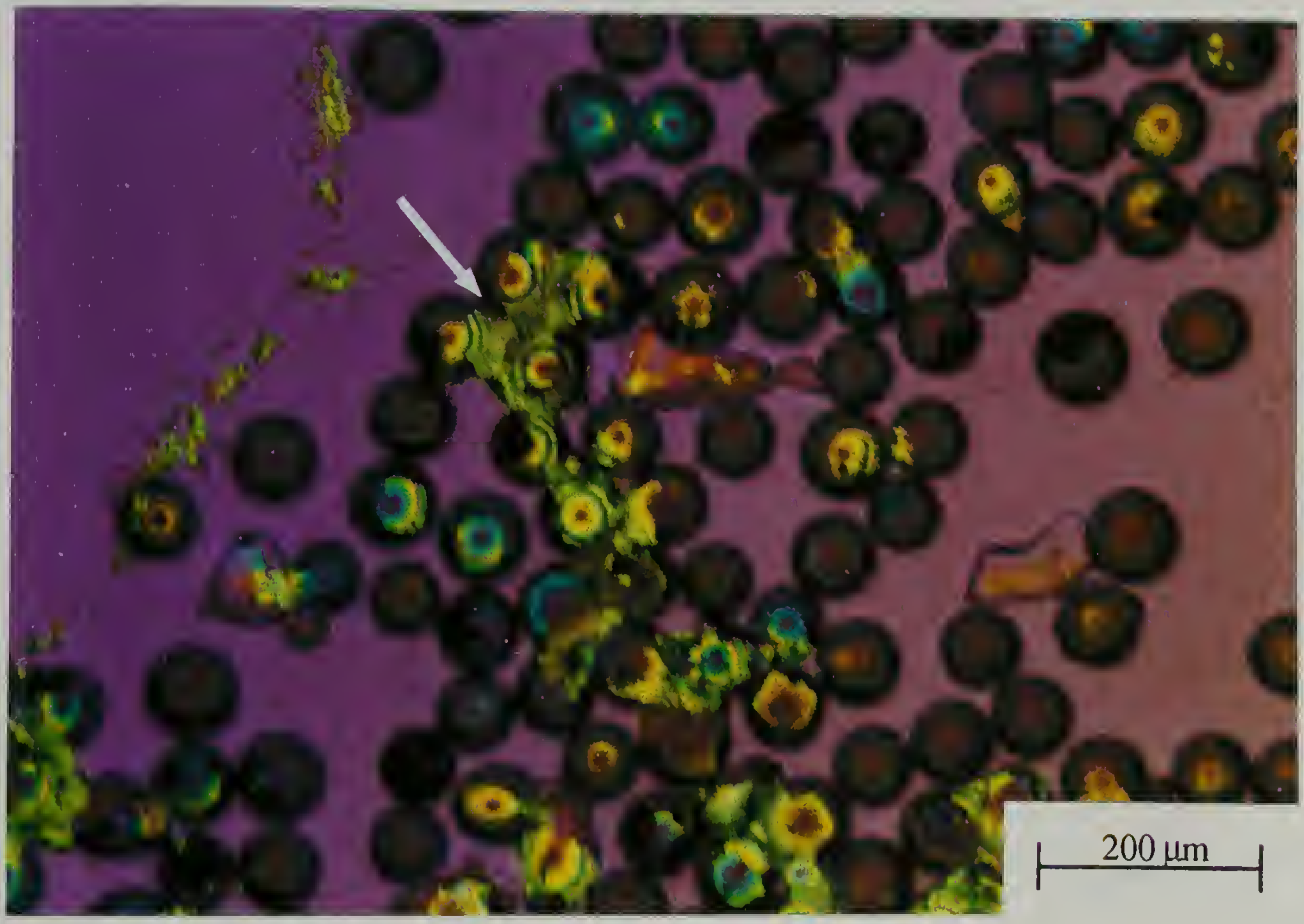

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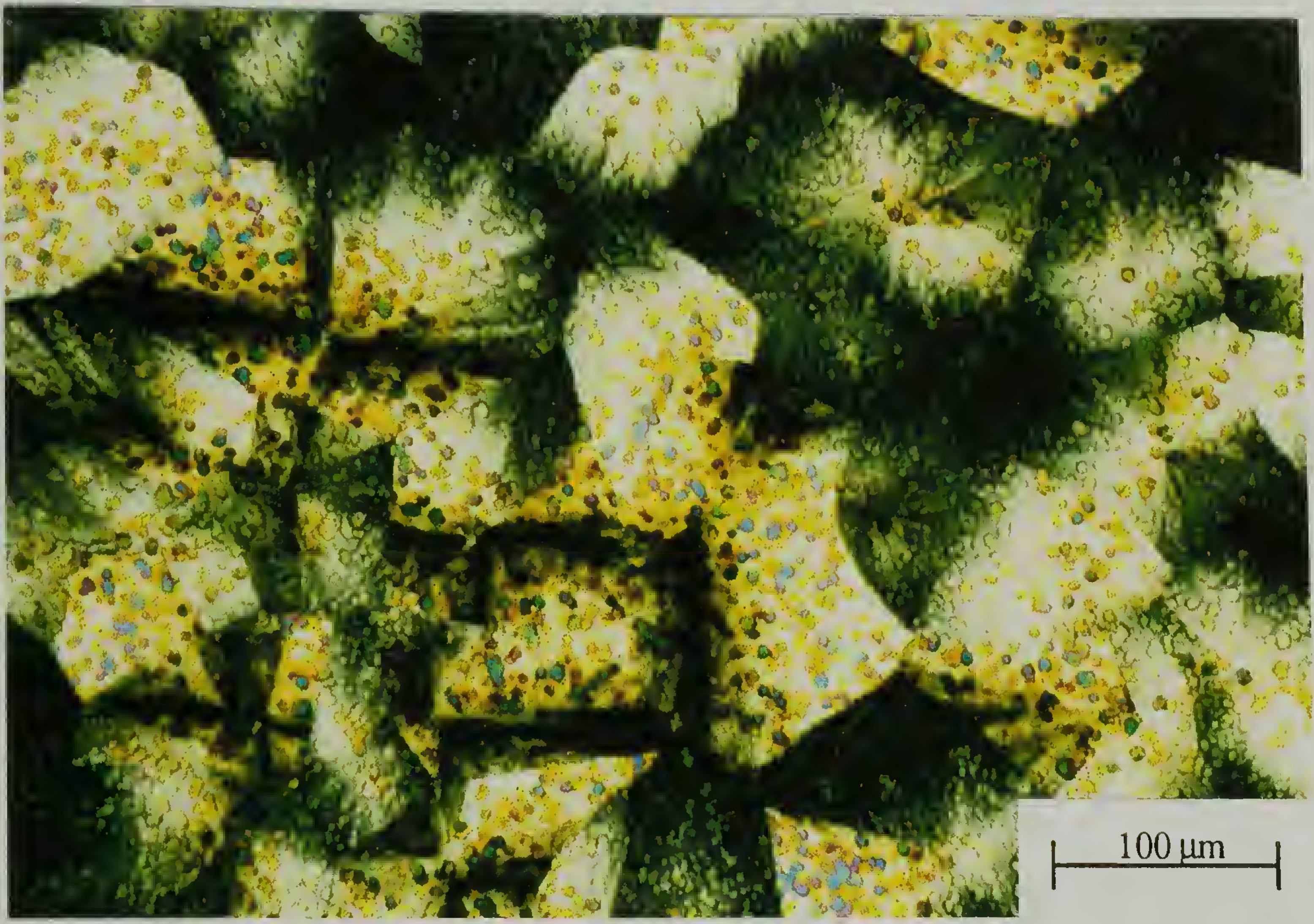
Fig. 4.6 Enhancement of PILP process by magnesium ion. Due to the additional inhibitory action of Mg^{2+}, a lower level of polymer can be used to induce the PILP process, and pure film can be produced. However, the induction time for nucleation and the rate of crystal growth are reduced. (A:top) For example, this film was still amorphous when it was removed from the solution after 5 days, and it took about two months to crystallize in the dried state, as judged by the birefringence under cross-polars. The polyaspartate concentration was only 5 µg/ml, with a ratio of Mg:Ca = 5:1. (B:bottom) At a lower ratio of Mg:Ca = 3.5:1, a mixture of products is formed, including thick tablets/films and crystal aggregates. As the stage is rotated under crossed-polars, tablets that emanate from a centralized rosette-type aggregate extinguish in a consecutive manner, resembling a windmill. Note, pure film can be produced at this concentration under the lower temperature conditions listed in Table 4.1.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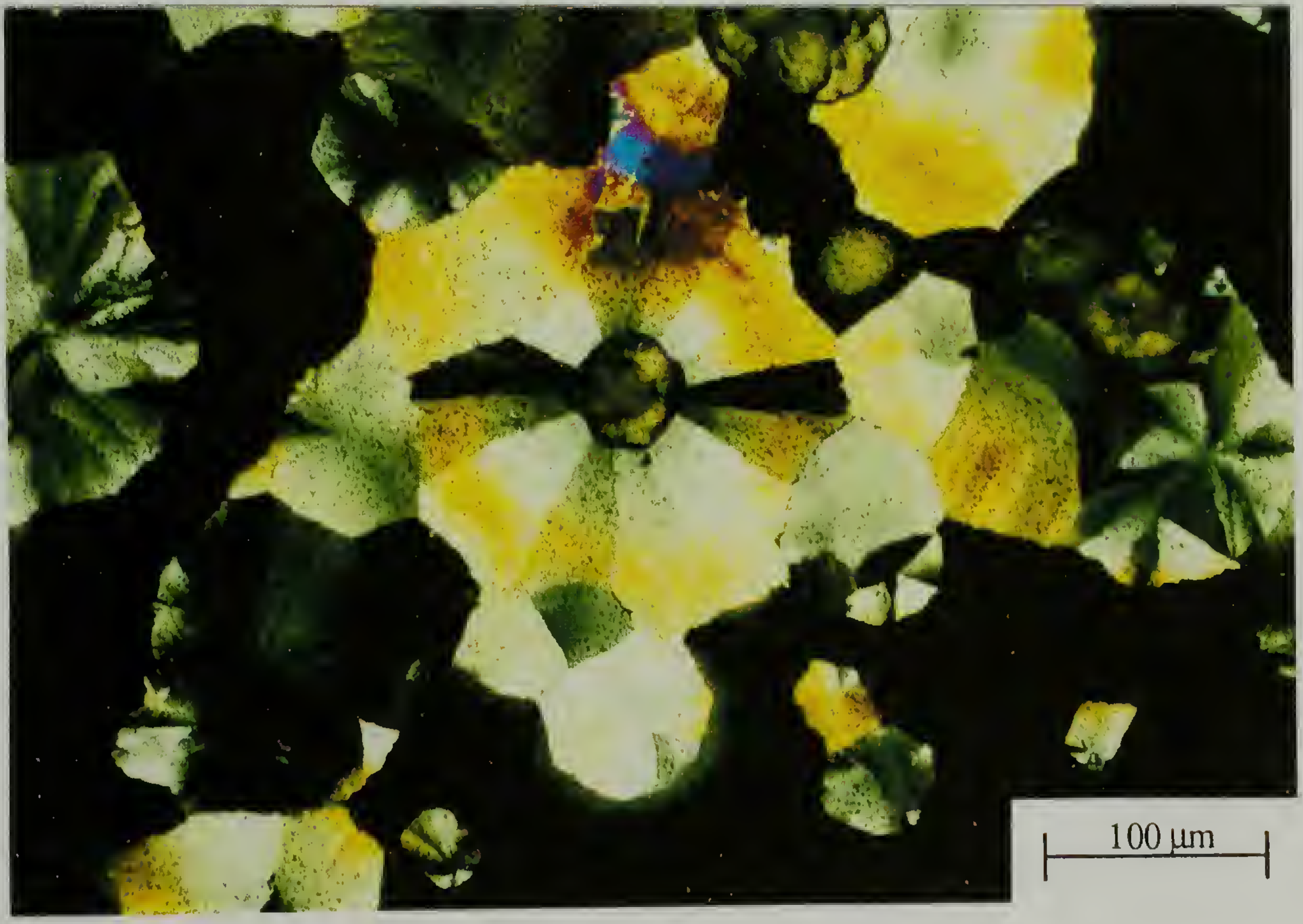
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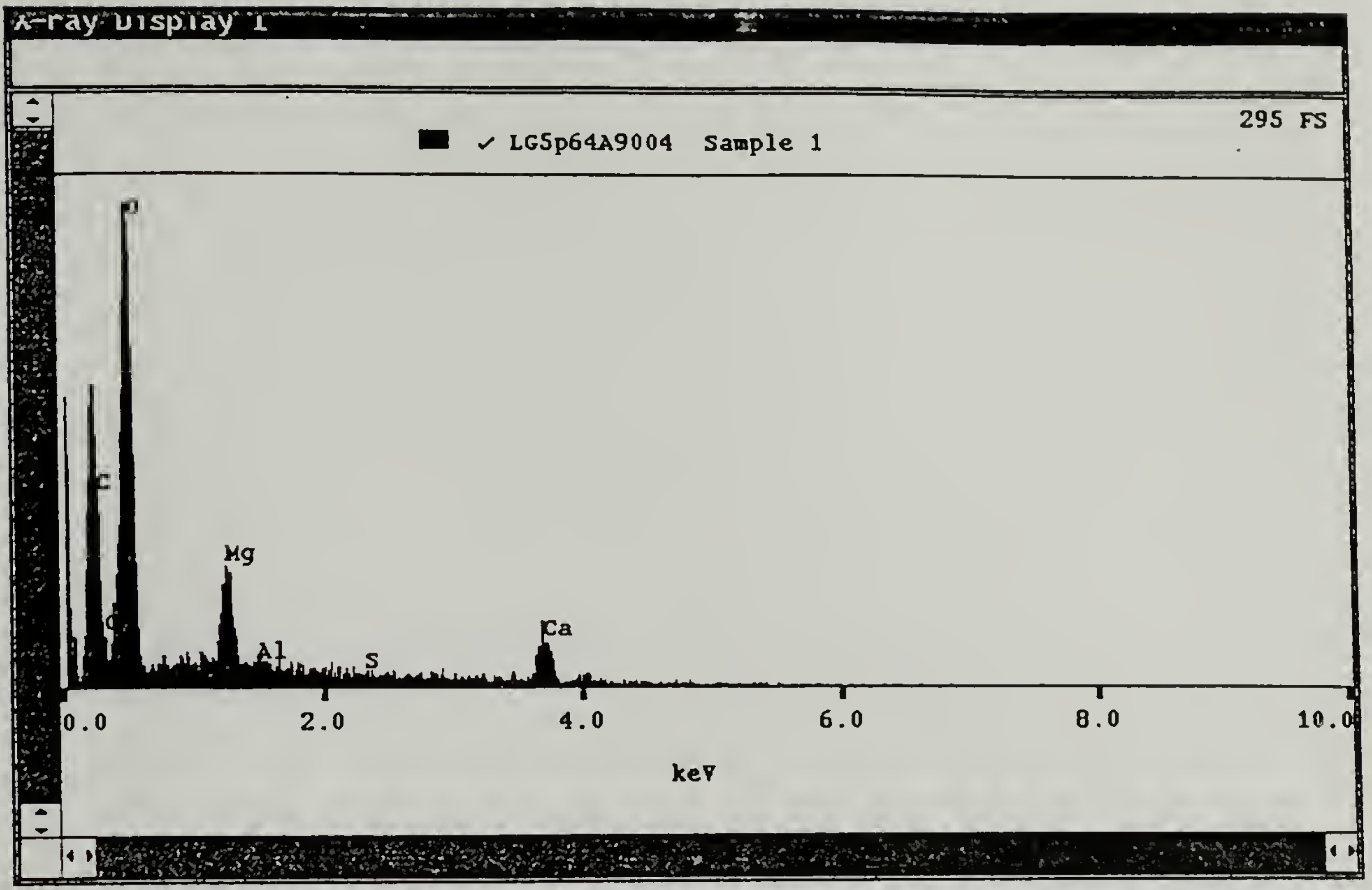
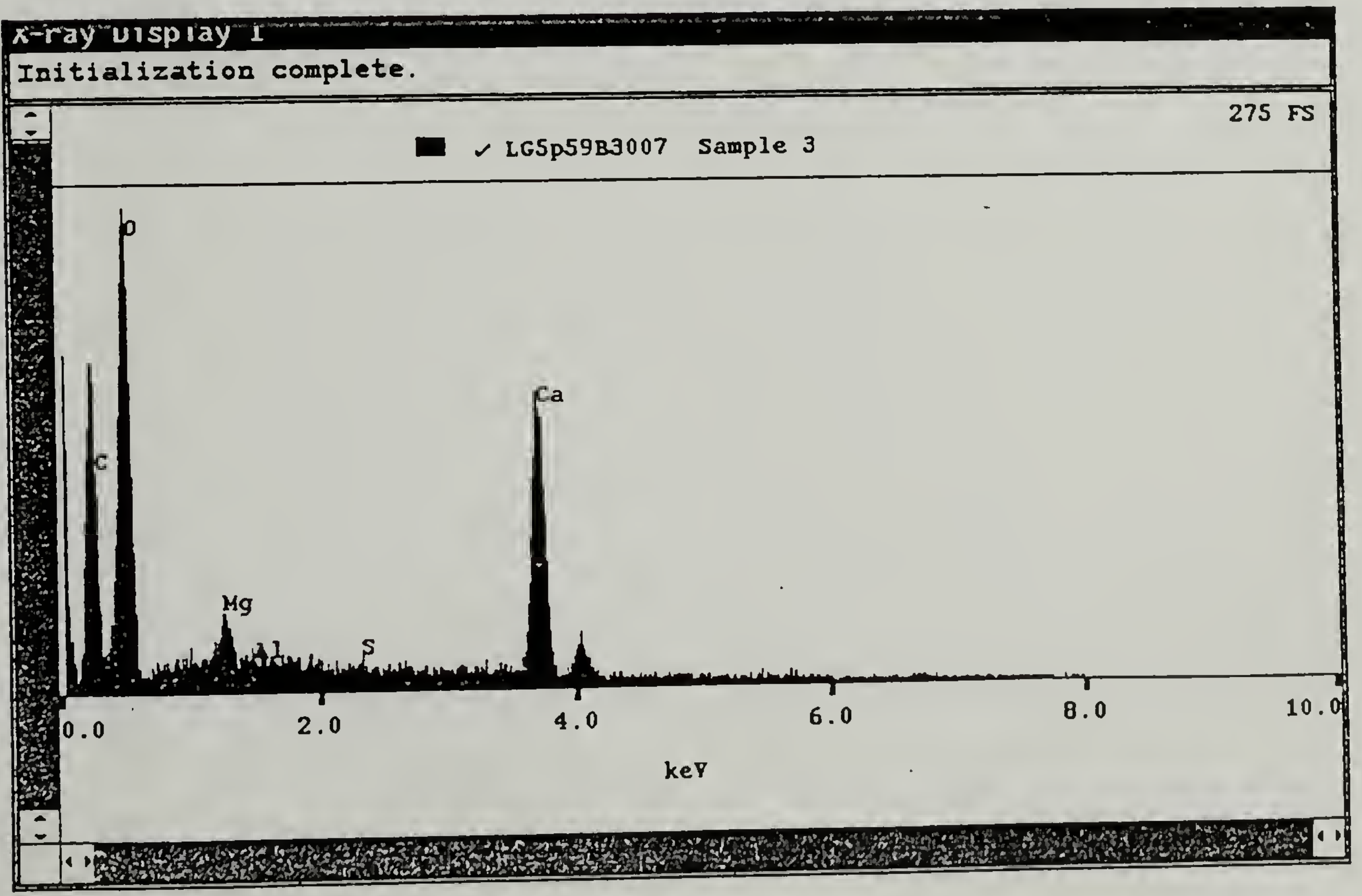
Fig. 4.7 Magnesium incorporation into calcitic films. X-ray microanalysis was used to determine the chemical composition of some of the magnesium-bearing films. The energy dispersive spectra (EDS) were obtained on a JEOL JSM-6320F semi-in-lens FE-SEM, at 8KV accelerating voltage and 45° take-off angle: ZAF (atomic absorption frequency) analysis results, shown in Table 4.2, were obtained using a dolomite standard (Geller Microanalytical Lab). (A:top) EDS spectrum of a film produced at the ratio of Mg:Ca = 5:1 under the conditions listed in Table 4.1. (B:bottom) EDS spectrum of a film produced at the ratio of Mg:Ca = 3.5:1. The magnesium incorporation was considerably lower under these conditions (using the same polymer concentration of 20 µg/ml, but at 4°C to avoid mixed products, as described in Fig. 4.6C).

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CHAPTER 5

CONCLUSIONS

It is believed that the data that has been presented in this dissertation, as well as its interpretation, will play a pivotal role in developing an understanding of biomineralization processes, and in particular, the regulation of morphology of $CaCO_3$ biominerals. While the PILP process may be just one aspect of the overall processes involved in biomineralization, its involvement at the foundation of the reaction would be expected to contribute to many of the other types of control as well. Thus, the role of the PILP process (as I envision it) is presented in the form of a flow diagram in Fig. 5.1, which summarizes the overall scheme of biomineralization from the viewpoint of how the various constituents contribute to the different types of control over biomineral properties.

As mentioned in the introduction, overall control over properties of the mineral phase is mainly accomplished through cellular regulation of its constituents (Fig. 5.1A). As can be seen in Fig. 5.1B, a new category has been included that was not discussed in the introduction, and that is the process-directing role of a soluble additive. Of course, this category is based on the PILP process discovered in this project. The interactions of this type of additive are considered to be non-specific, whereby the function is performed prior to the creation of a solid interface.

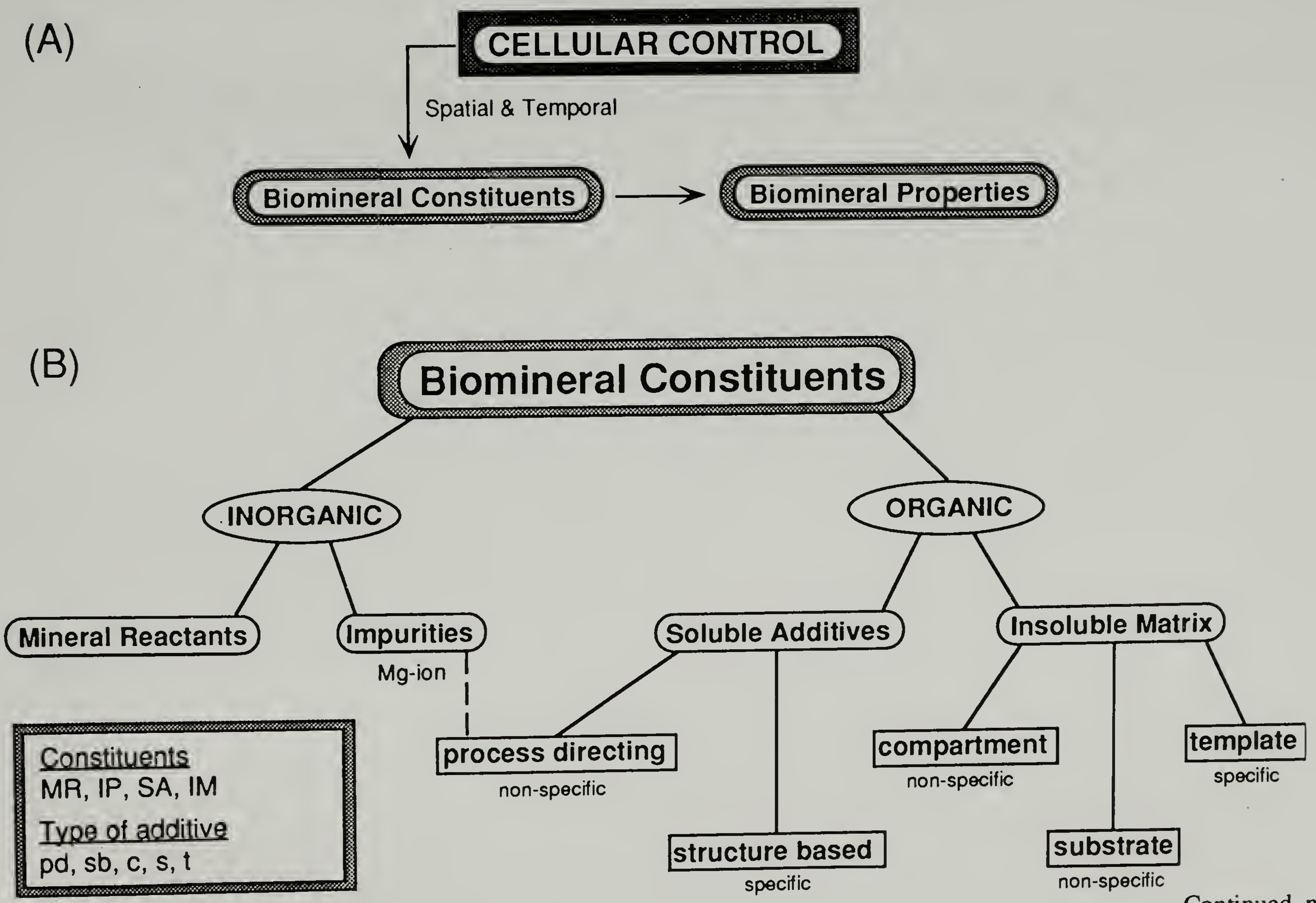
Fig. 5.1B is rather busy, and requires some explanation. The process-directing agent (encircled pd) is listed at some point under each of the six property divisions. However, its main function is likely in the regulation of shape, where it would be particularly influential in space-filling and coating type processes, as has been shown in this report. Experiments thus far suggest that the PILP process has little effect on the orientation of crystals, however, it is included under the orientation division because the liquid precursor is presumably a highly supersaturated phase, and as such, may promote epitaxial nucleation on insoluble matrices. In terms of size control, spatial delineation of the precursor phase can be a means of setting boundaries, or, since it is a separate phase, a compartment may not even be necessary (as in thin tablets that are limited by the precursor itself). For location control, it is envisioned that a precursor phase could easily be positioned (i.e. secreted from the cell), as opposed to a general concentration of ions in the solution growth process, in which control over the position for nucleation would be essential. In the texture division, which overlaps the composition division, it seems likely that the textures are a consequence of the process, rather than a regulated function. This is probably the case for the sectored tablets and the magnesian-calcites, but perhaps not for the spine of the sea urchin, which seems to derive benefits from the occluded proteins (and these presumably arise from specific interactions).

Under the polymorph subdivision of composition, a question mark is placed under the soluble additives lane (boxed SA). This is because of the recent reports that describe polymorphic control using the soluble proteins extracted from mollusk shells (references 8 and 9 in Chapter 4). This finding was quite unexpected because it had been implicitly assumed that this type of control was caused by structural interactions during nucleation on an insoluble matrix. It may be that specific interactions with the soluble additive lead to this type of control, such as by selective inhibition of one polymorph's nuclei over the other. However, there is another possibility that could arise from non-specific interactions, and that is control over the level of hydration of a precursor phase. It seems plausible that transformations of different hydrated phases could lead to different polymorphs, either through kinetic effects, or through structural similarities between hydrated crystals and the anhydrous forms. Therefore, the process-directing additive is included in this lane containing the question mark, but awaits further investigation to test this hypothesis.

This schematic is based on observations of the PILP process in the formation of calcium carbonates, and clearly more studies are needed to determine the generality of the process in terms of other mineral phases, and other types of polyanions, etc.. Moreover, *in vivo* studies on biomineral formation are needed to prove my argument, which is based solely on observations of an *in vitro* model system, but it is anticipated that continued work in this area will prove fruitful.

A better understanding of biomineralization processes may be expected to lead to advances in bone and dental care; or to methods of controlling undesirable mineralization, such as the hardening of biomedical implant materials (e.g. artificial hearts), or the formation of kidney stones, or the build-up of scale in industrial piping systems. The control of $CaCO_3$ morphology could be of value to the paint and paper industry because many of the properties of these materials are affected by the morphology of the $CaCO_3$ filler (e.g. brightness, opacity, strength, etc.). More generally, this work has contributed to the basic knowledge of organic/inorganic interactions. However, the significance of the polymer has not been fully realized, because details of the PILP process have only been elucidated at the physical level. Future studies, directed at determining molecular-level interactions, are needed to access the possibilities for other polymeric/inorganic systems, where the goal is to develop new biomimetic strategies for crystal and particle engineering.

Fig. 5.1 Generalized scheme for $CaCO_3$ biomineralization. (A) Cellular control of mineral properties can be accomplished in both the spatial and temporal domain through regulation of the reactive and additive constituents. (B) The consituents that are present in biominerals can be divided into categories that pertain to the role the species plays in controlling the formation and properties of the mineral phase. (C) A breakdown of the different types of properties that can be regulated in biominerals, and the mechanism believed to be responsible for the differing levels of control exerted over these properties. In reality, these pathways will overlap, and many constituents may be involved in each aspect of mineral formation, but only the major player(s) that contribute in some special way to each property are listed. The mechanisms that are deamed to be the most plausible are enclosed with solid lines, while other possibilities are enclosed with shaded lines. The abbreviations for the mechansims are based on the first letter(s) of the biomineral constituents or type of additive, as presented in 5.1B, and are as follows: For the constituents category, MR = Mineral Reactants, IP = Impurities, SA = Soluble Additives, IM = Insoluble Matrix; and for the type of additive, pd = process directing, sb = structure based, c = compartment, s = substrate, t = template.



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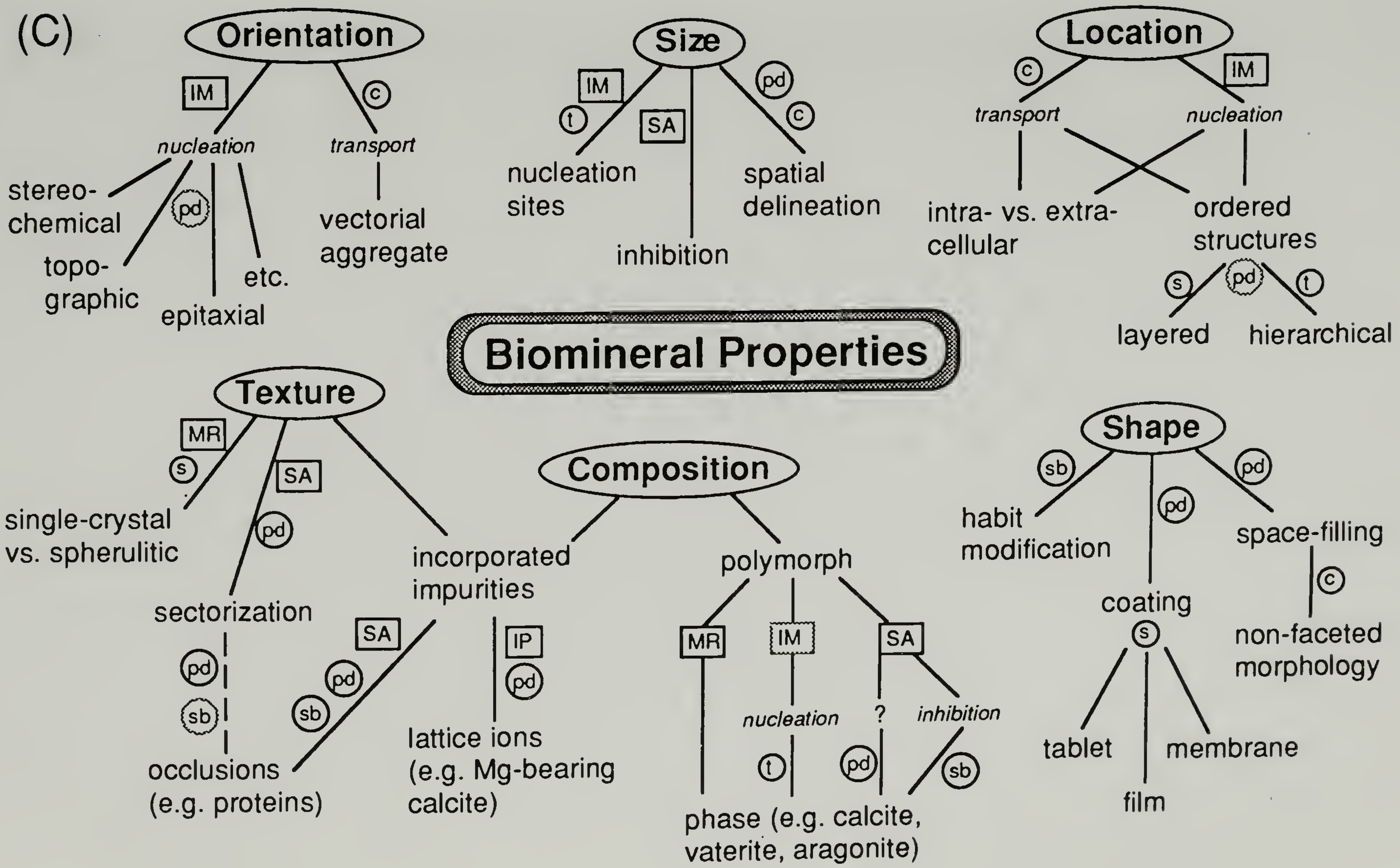


Fig. 5.1 continued.

APPENDIX A

INFLUENCE OF OTHER POLYMERIC ADDITIVES

This observational data is included to demonstrate the range of morphologies that result from interactions between polymers and growing calcite crystals. Almost all of these morphological alterations appear to be the result of non-specific interactions, with the possible exception of BSA and the periodic polypeptides, in which the expression of new faces was described (but this distinction was not easy to define). There appear to be a variety of types of non-specific interactions, where many additives seem to lead to edge-clipping and rounding of corners, while others seem to lead to crack defects, and finally the P(D), P(E), and PMAA lead to severely distorted crystals, and aggregation. Most likely, these distorted crystals resulted from some aspects of the PILP process (described in Chapter 3), but were influenced to a lesser degree than the pure PILP film products. These observations seem to suggest that a PILP type process might be found with other polyelectrolytes, such as PMAA and P(E), if more optimal conditions were obtained. Indeed, there is a rather narrow window for the reactant concentrations at which the PILP process occurs with polyaspartate. Certainly, many more experiments could be carried out in this area, with the hopes of learning by comparison, and of finding less expensive polymeric materials.

Table A.1

Morphological influence of various polymeric additives on $CaCO_3$ crystals

Polymer	Abbr.	M.W. (vis)	Conc. (μg/ml)	Description of Morphology
poly(vinylpyrrolidone)	PVP	360K	200	random crack type defects
poly(methacrylic acid)	PMAA	?	1-2	clipped edges, bulges
			10	aggs. of distorted rhombs
			370	spherulites w/ rough surfaces
poly(vinyl alcohol)	PVA	?	1800	cracks // to cleavage planes
poly(ethylene oxide)	PEO	200K	184	spherulites (60 μm)-stacked disks
			30	normal rhombs
poly(glutamic acid)	P(E)	3.3-	0.7	distorted, swirly rhombs
		10.6K	2	aggs. of distorted rhombs
			10	" and small spherulites (5 μm), and film coating on aggs.
			≈350	spherulites (15 μm)- radial type
poly(aspartic acid)	P(D)	14.4K	0.7	a few large rhombs, some with spiral pits
			1-5	aggs. of distorted rhombs
			5-30	aggs. of spherulites and rhombs, helical protrusions
		", 6.8K optimal	10-30	both types of aggs., helical protrusions, and films
			≈350	mesh of irreg. particles (0.2 μm)
L-glutamic acid	E	129	333	clipped edges, possibly new faces
bovine serum albumin	BSA	66K	30	clipped edges, possibly new faces (rough), spherulites
-[(AG)nEG]-cleaved		13.3K-20.5K	15-45	clipped edges (striated), layered and rounded corners (smooth)
-[(AG)nEG]-fusion		?	3-33	" and possibly new faces (rough)
			≈350	grotesque alterations (very rough)

APPENDIX B

POLYMORPHS OF CALCIUM CARBONATE

There are three anhydrous polymorphs of $CaCO_3$, calcite, vaterite, and aragonite; and two confirmed hydrates, the monohydrate and hexahydrate.[1] There are also some basic $CaCO_3$ polymorphs, but these will not be discussed here. Calcite is the thermodynamically stable form (some solubility constants are listed in Table B.1), but the metastable phases are frequently found as well. However, the hydrated polymorphs are very unstable, and usually transform to one of the three anhydrous forms, depending on the solution conditions.[1]

Table B.1

Properties of the five $CaCO_3$ polymorphs*

Polymorph	sp. gr. (g/cm^3)	Ksp (kmol^2/m^3)	Crystal Structure
calcite	2.71[2]	4.5[1] to 4.7[2] x 10^{-9}	rhombohedral
aragonite	2.94[2]	6.0[1] to 6.9[2] x 10^{-9}	orthorhombic
vaterite			hexagonal
$CaCO_3 \bullet H_2O$			hexagonal
$CaCO_3 \bullet 6H_2O$	1.82[3]	7.58 x 10^{-8} (-1.6°C)[4]	monoclinic

*values were obtained from indicated references.

Calcite, when grown from solution, tends to adopt a rhombohedral habit that expresses the (10.4) crystallographic faces (hexagonal indices are commonly used).[1, 5] This rhombohedral habit is not based on the rhombohedral unit cell (which is an acute rhombohedron at a different orientation), but rather is related to the cleavage cell.

Figure B.1A shows typical rhombohedral calcite crystals that are formed under the experimental conditions given in this report, in the absence of additives. In Fig. B.1B, the other two anhydrous forms of $CaCO_3$ can be seen as well, where they appear to have nucleated on a large strand of dust. To insure pure and reproducible crystal products, which is essential for morphological studies, the solutions must be extensively filtered to remove dust and other impurities to avoid the problem shown here. Cleaning the glass coverslips with Nochromix® solution is beneficial in this regard.

The metastable vaterite polymorph has a loosely-packed hexagonal structure, and usually grows in the form of spherulites (as seen in Fig. B.1B).[1, 5] The spherulites appear to be composed of thin disk-like crystals. This form tends to grow under conditions of high supersaturation, and especially with excess carbonate species.[1] Single-crystals of vaterite have been grown under compressed monolayers of stearate, where they had a lens-shaped disk morphology.[6]

The metastable aragonite polymorph has an orthorhombic unit cell, but is sometimes referred to as pseudo-hexagonal because its lattice is so close to being hexagonal. Aragonite is slightly more soluble than calcite, even though it is a denser phase (Table B.1). Like vaterite, aragonite also tends to grow as spherulites, but the individual crystallites have an acicular habit, and the spherulites are seen as a bundle of needle-like crystals (Fig. B.1B).[1, 5] High solution temperatures favor aragonite, as does the presence of certain impurities, in particular magnesium and strontium. This impurity effect is thought to arise from inhibition of the more stable polymorph, calcite.[1, 5] Aragonite crystals are commonly found in biominerals, presumably because of the high level of impurities in sea water, such as magnesium-ion.

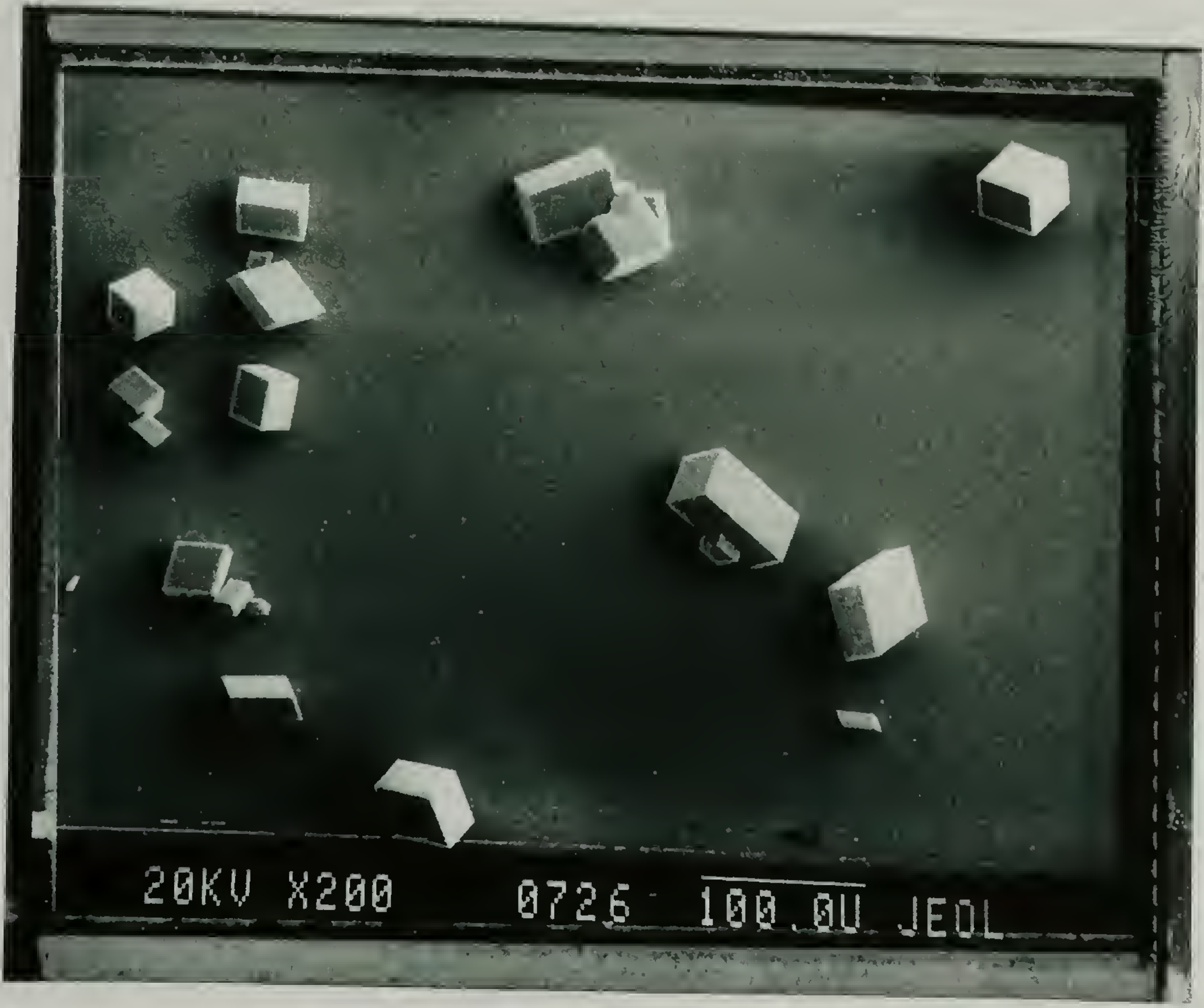
The monohydrate polymorph, which has a monoclinic unit cell, tends to grow *in vitro* in the form of small spherulites, but Lippman has produced monolithic trapezohedral crystals.[1] Magnesium-ion is reported to promote the formation of this polymorph, possibly for the same reasons as aragonite.

The hexahydrate polymorph, which has a hexagonal unit cell, typically forms a platy habit, although prismatic crystals have also been produced.[7] The geological name of this mineral is Ikaite.[4] The polymorph has mainly been produced using organic additives, such as aspartic acid[8] and polyphosphates (i.e. Calgon®),[3, 8-10] and especially at lower temperatures.[9] The hexahydrate has a rather unusual lattice structure in that it is composed of individual molecules of $CaCO_3 \bullet 6H_2O$ that are held together by an extensive network of hydrogen bonds.[8]

Although amorphous $CaCO_3$ is very unstable and not commonly found, it can be produced at low temperatures, with high concentrations (0.1 M), in the presence of base.[11] This amorphous material was used to investigate transformations to the more stable, crystalline forms.

This appendix has described the typical morphologies of these minerals as grown in solution, which may be quite different from geological and biogenic crystals of the same polymorph.

Fig. B.1 Examples of the three anhydrous $CaCO_3$ polymorphs. (A:top) This scanning electron micrograph shows typical products of the control reaction that was run with each set of experiments in this project; rhombohedral crystals of calcite were formed, with an average size of about 40 to 60 microns. (B:bottom) If care is not taken to remove dust and impurities, other crystal polymorphs can be formed as well. A single crystal of calcite is present in the middle of this large dust strand, and a spherulite of aragonite is seen at the far right end, while hexagonally-lobed donuts of vaterite appear to have wrapped around the dust strand. Take note of the acicular habit of the aragonite crystals, which is quite different from the platy habit of aragonite crystals found in the nacre of mollusk.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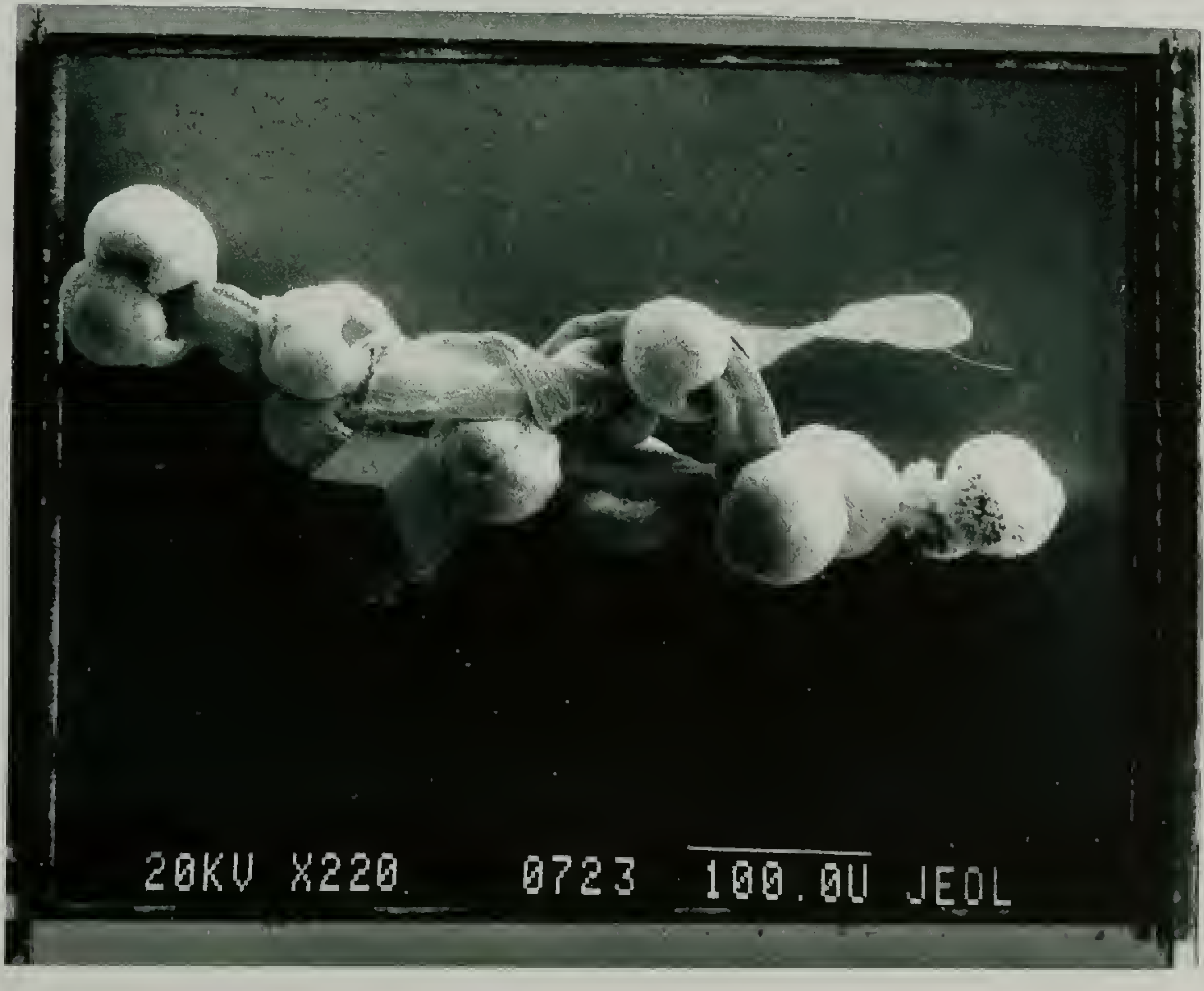
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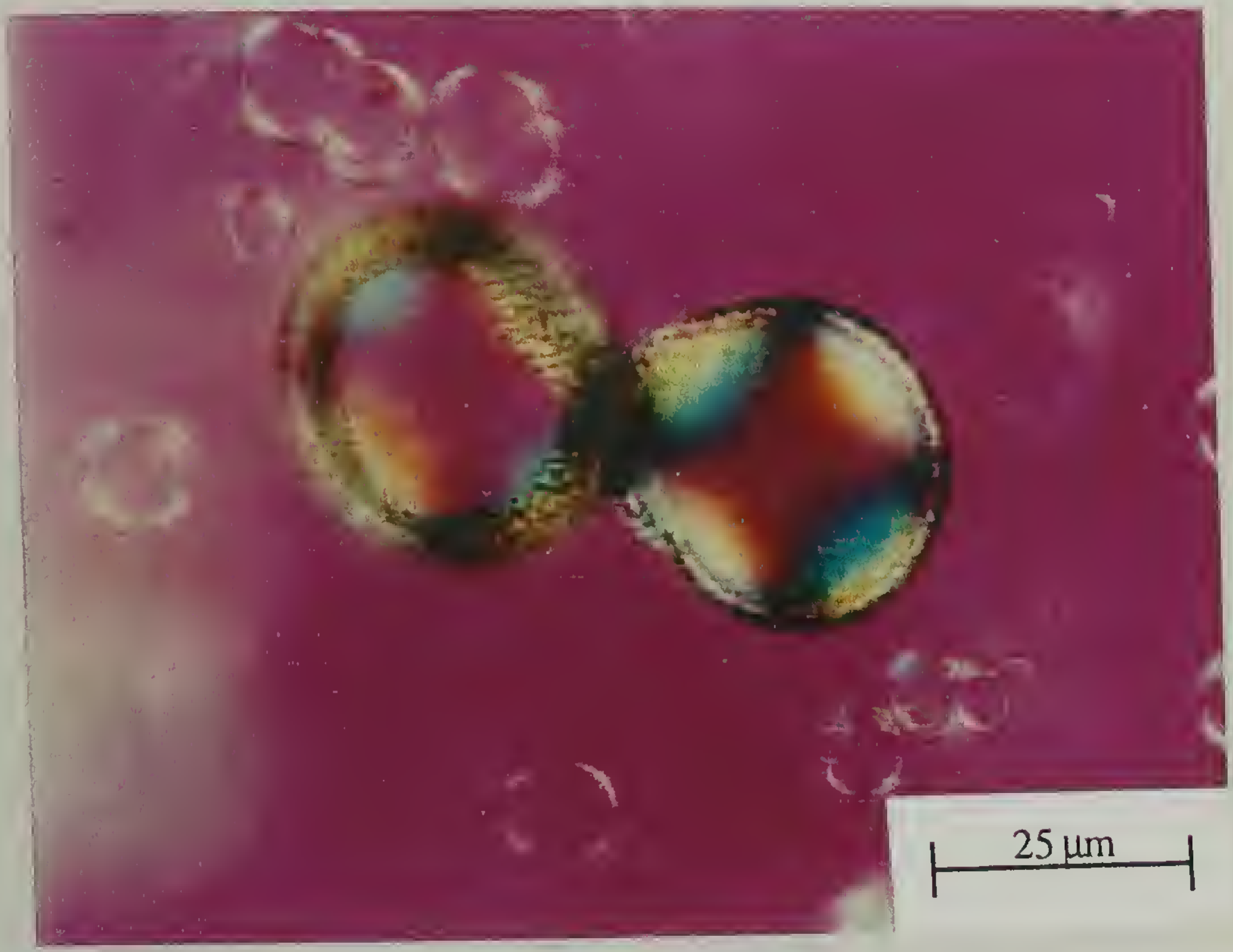
APPENDIX C

HOLLOW-SHELL SPHERES

Hollow-shell spheres were found in some crystallizing dishes that had been left sitting for several weeks (Fig. C.1). The conditions were reproduced and monitored to determine how the hollow particles were formed. It was observed that the initial particles were solid spherulites, whose interiors subsequently dissolved away with time (as the solutions slowly evaporated). This data is not included in the main body of the dissertation because these structures have not been well characterized at this point in time. Nevertheless, it is included here to make a comparison to biominerals, of which there is an example in the literature on "calcispheres",[12] which have a very similar hollow-shell morphology.

The polymorphic composition of these *in vitro* hollow spheres has not yet been determined, but it is likely that these structures are composed of a hydrated phase, being that they tend to collapse and loose their birefringence upon drying. This observation was also seen for some of the hollow-helices reported in Chapter 2, and it is possible that the two phenomena are related. For this reason, I felt it might be informative to include this brief appendix.

Fig. C.1 Hollow-shell spheres. These hollow spheres were found in some magnesium-ion reactions that were left sitting for a couple of weeks. It has since been determined that they come from solid spherulites in which the interior has preferentially dissolved away. (A:top) In this example, the spherical shell is broken and quite thick (brown), but depending on how long the spherulites have been dissolving, very thin shells that are practically transparent can also be produced. (B:bottom) These hollow-spheres are of similar size and structure to "calcispheres", biominerals from recent calcareous dinoflagellate cysts.[12] The interference colors using the 1st-order red plate are yellow in the 1st and 3rd quadrants, and blue in the 2nd and 4th quadrants, indicative of a negative optic sign. Although the polymorphic composition of these spheres has not yet been determined, they appear to be similar to the radial type calcispheres pictured in the literature.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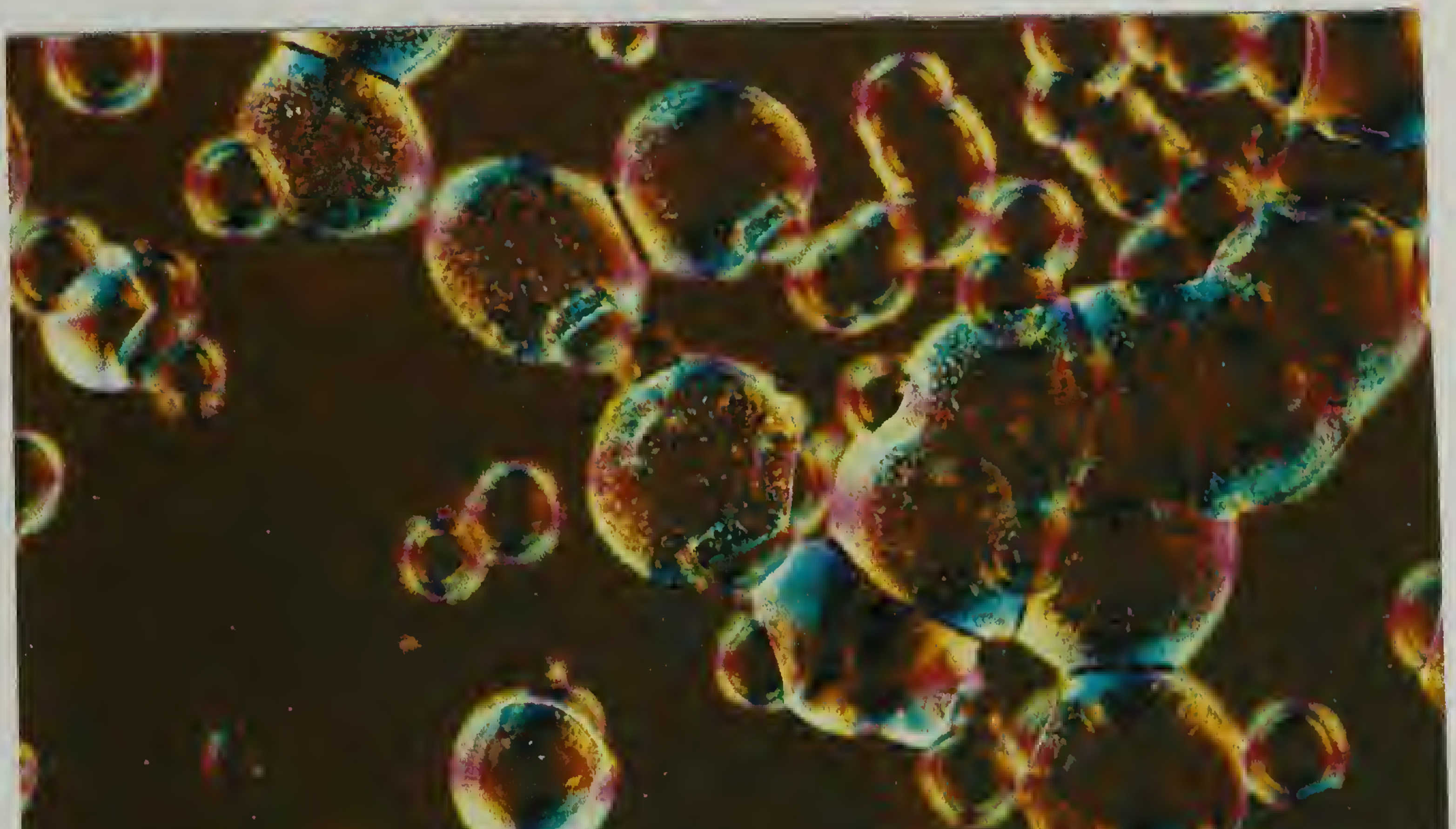
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