Biomimetic Crystallization of Calcium Carbonate Polymorphs by Means of Collagenous Matrices

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Abstract: Crosslinked gelatin films with entrapped poly-L-aspartate were used to induce the crystallization of calcite and aragonite polymorphs. Calcite with high degrees of isomorphous substitution (up to 12 mol% magnesium) has been obtained. The morphology and layered organization of magnesium calcite crystals grown inside the crosslinked gelatin films with entrapped poly-L-aspartate resemble some structural features of radial calcitic ooids. The concentration of entrapped poly-L-aspartate and the uniaxial deformation of the films control the growth of aragonite aggregates inside the films. Such aggregates grown inside uniaxially deformed films consist of parallel rods with an architectural assembly similar to that found in some marine organisms. The crystals' aggregation and the control of calcium carbonate polymorphism are related to the modelling of the nucleation sites by poly-L-aspartate structure and concentration, local supersaturation and microenvironment shape. The results indicate that the collagenous matrices with entrapped polyelectrolytes are versatile systems which can contribute to the elucidation of strategies for biomimetic materials chemistry.

Keywords
aragonite · biomineralization · calcite · crystal growth · peptides

Introduction

Many organisms are able to build organic–inorganic composite materials of elaborate morphology and texture. Furthermore, they can control the polymorphism and the chemical composition of the inorganic phase.[1] Calcite and aragonite, two polymorphs of calcium carbonate, can be selectively precipitated, and considerable amounts of magnesium calcite can be detected in the hard parts of many organisms.[1, 2]

Association between organic and inorganic substances has also been observed in the sedimentary environment. It has been suggested that in radial calcitic ooids organic concentric shells act as growth surfaces for carbonate.[3] Biogenic calcites and radial calcitic ooids can contain up to 40[4] and 12[5] mol% magnesium, respectively. However, in laboratory experiments at room pressure and temperature, it is not possible to obtain high-magnesium calcite from saturated solutions of calcium carbonate containing magnesium. Substitution of up to 6.5 mol% magnesium into calcite has been reported in conditions close to the biological ones.[6] Magnesium ions in solution induce the formation of aragonite, and at high magnesium/calcium molar ratios (>4) mainly aragonite is formed.[7]

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Model studies of crystallization at the organic–inorganic interface suggest that control of crystallization of inorganic compounds is affected either by the abilities of organic templates to mimic the lattice of a two-dimensional face or by the stereochemistry of the functional groups at the interface.[8] These studies offer valuable insights not only into natural processes but also for biomimetic materials chemistry.[1c]

Recently it has been found that aspartate-rich macromolecules extracted from aragonitic or calcitic mollusc shell layers are responsible for the selective precipitation of aragonite and calcite in vitro.[2b] Furthermore it has been shown that these soluble proteins alone are sufficient to control phase switching between calcite and aragonite.[9] Poly-L-aspartate (Pasp) has been considered an analogue of these proteins, which are in some way bonded to an insoluble protein matrix with gelling properties. It is difficult to attribute to the insoluble matrix a defined function besides that of mechanical support. However, its gelling properties could be related to the compartmental strategy used to control size, shape and aggregation of biological crystals.[8a, 8h, 10]

Although the structure of gelatin, which is constituted of denatured and degraded collagen molecules, is rather complex as a model, crosslinked gelatin with entrapped Pasp nevertheless has favourable properties for the simulation of the role of the mollusc organic matrix in the biological crystallization of calcium carbonate. In a previous communication we used crosslinked gelatin films with entrapped Pasp as a tool to obtain oriented crystallization of calcite.[11] Recently the growth of fluoroapatite aggregates by diffusion into a gelatin matrix has been reported.[12] Gels have been used to grow calcite crys-

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tals,[13] and the crystallization in gels has been thoroughly studied with the aim of growing inorganic single crystals.[14] Compared to noncrosslinked materials, gelatin films made rigid and insoluble by glutaraldehyde crosslinking are versatile systems in which to incorporate water-soluble polyelectrolytes. Thus the nature, density and structural geometry of the charged groups responsible for the crystallization of calcium carbonate can be easily regulated by changing the kind and amount of the polyelectrolyte entrapped in the film. Furthermore, the structure of the polyelectrolyte–gelatin supramolecular assembly can be changed by means of a simple mechanical deformation, which modifies the microenvironment of the nucleation and growth sites.

We have investigated the growth of calcium carbonate polymorphs in the presence of crosslinked gelatin films. Calcite with high degrees of isomorphous substitution, up to 12 mol% magnesium, has been obtained from solutions containing magnesium ions. The morphology and layered organization of magnesium calcite crystals grown into crosslinked gelatin films with entrapped Pasp resemble some structural features of radial calcitic ooids.[3] Moreover, we found that the concentration of entrapped Pasp and the macromolecular orientation induced by uniaxial deformation of the films controlled the formation of aragonite crystal aggregates to result in an architectural assembly similar to that found in the nacreous layers of marine organisms.[1]

Results

Characterization of gelatin films: Since the casting process has a pronounced effect on the structure of gelatin films,[15] the greatest care was taken in their preparation, and their ultrastructural organization was checked on dry samples by SEM and X-ray diffraction. SEM micrographies and X-ray diffraction patterns show that during the uniaxial deformation the gelatin layers reorganized into bundles parallel to the direction of elongation (Figure 1). These bundles contained oriented segments of collagen molecules, as indicated by the characteristic X-ray diffraction maxima at 0.29 and 1.1 nm, preferentially oriented parallel and perpendicular to the direction of the elongation, respectively.[16]

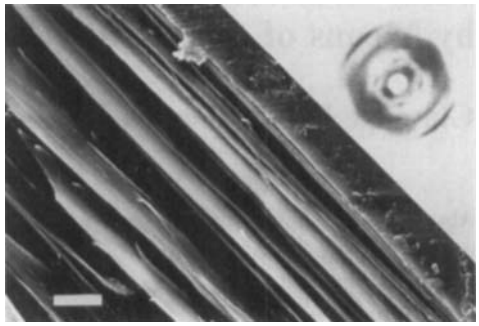

Figure 1. SEM image of a longitudinal section of uniaxially deformed gelatin film showing bundles of collagen molecule segments. Scale bar 10 μm. Inset: X-ray diffraction pattern of uniaxially deformed gelatin film with the X-ray beam perpendicular to the film surface.

About 30 μg of material per millilitre solution was released from the films at the end of crystallization experiments. The amino acid composition indicated that the films were made up of gelatin and contained only traces of Pasp. Swelling of the crosslinked films did not depend on the presence of Pasp, but was sensitive to stretching. In fact, when the unstretched films swelled they could increase in weight by up to 290(30)%, whereas the stretched films (200% deformed) could increase by 150(10) weight%. The standard error in parentheses was calculated from three independent experiments.

Crystallization in the presence of unstretched gelatin films: Calcite crystallization on crosslinked unstretched gelatin films with entrapped polymers containing carboxylate and/or sulphate groups has been previously described.[11] For all the polyelectrolytes considered, the induction times decrease and the nucleation density increases on increasing the polyelectrolyte content. Unlike the other polyelectrolytes, Pasp at low content induces preferential (001)-oriented crystallization of calcite. The highest frequency of oriented crystals was found for a content of 0.5 μg of Pasp per gram of gelatin.

When crystallization occurred from a solution of calcium and magnesium chloride at different molar ratios the crystalline product was mainly obtained on the gelatin film; just a few crystals were observed at the air–solution interface. The mineral phase was highly crystalline, as shown by the X-ray diffraction patterns. Magnesium calcite (Figure 2) was the sole calcium carbonate polymorph present on the gelatin films for a magnesium/calcium molar ratio less than 2.5 and entrapped Pasp concentration lower than about 0.5 mg per gram of gelatin. The nucleation density of magnesium calcite on the films decreased on increasing the relative amount of magnesium in solution.

The gelatin film allows a high degree of magnesium replacement of calcium in calcite independent of its Pasp content. The

Abstract in Italian: *Films di gelatina reticolata contenenti Poly-L-aspartato (Pasp) sono stati usati per indurre la cristallizzazione di calcite ed aragonite. Essi hanno permesso di ottenere magnesio calcite ad elevato grado di sostituzione isomorfa (12% in moli) e si è potuto dimostrare che la morfologia e la struttura a strati degli aggregati di magnesio calcite cresciuti all'interno dei films di gelatina contenenti Pasp hanno aspetti strutturali in comune con gli ooidi calcitici radiali. La concentrazione del Pasp e la deformazione uniassiale dei films controllano la crescita di aggregati di aragonite all'interno dei films. I cristalli di aragonite si aggregano a formare cilindri paralleli con un'architettura simile a quella trovata in alcuni organismi marini. L'aggregazione dei cristalli ed il controllo del polimorfismo del carbonato di calcio vengono messi in relazione con la forma dei siti di nucleazione, con la struttura e concentrazione del Pasp, con la sovrassaturazione locale e con il microambiente in cui avviene la crescita. I risultati indicano che la matrice collagenosa contenente polielettroliti rappresenta un sistema versatile che può contribuire a chiarire le strategie "biomimetiche" da applicare alla chimica dei materiali.*

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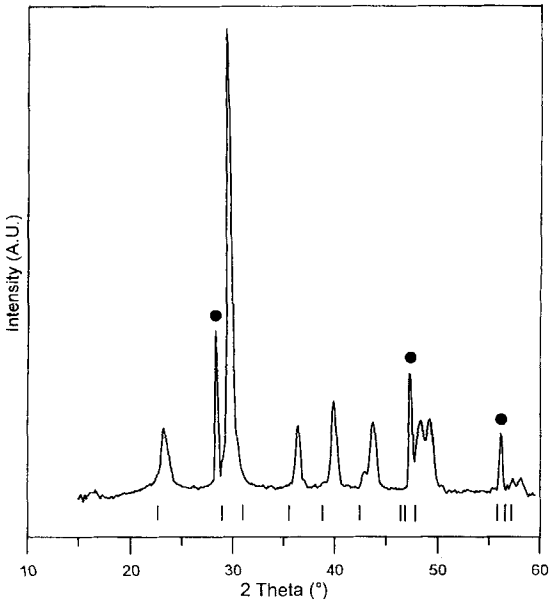

Figure 2. X-ray powder diffraction pattern of magnesium calcite containing 12 mol% magnesium. The peaks are shifted from those of pure calcite, which are indicated by lines beneath the spectrum. The black circles indicate the silicon peaks of the internal standard.

magnesium content of calcite, evaluated by comparison of the unit cell parameters (Table 1) with literature data,[17] reached 12(1) mol% when obtained from a solution with a magnesium/calcium molar ratio equal to 2.5. This value compares with that of 14.0(1) mol% magnesium obtained from atomic absorption spectroscopy. The substitution of magnesium in the calcitic structure was confirmed by the FTIR spectra, where the peak of pure calcite at 712 cm^{-1}[18] is shifted to 717 cm^{-1}. A shift in the same direction was observed for a sample of biogenic calcite containing 14 mol% magnesium, which has a peak at 719 cm^{-1}.

Table 1. Unit cell volume of calcite as a function of magnesium/calcium molar ratio in solution for different amounts of entrapped Pasp in the crosslinked gelatin film. The standard deviations are reported in parentheses.

Amount of Pasp	$[Mg^{2+}]/[Ca^{2+}] = 1.0$		$[Mg^{2+}]/[Ca^{2+}] = 2.5$	
	vol (Å^3)	% Mg [b]	vol (Å^3)	% Mg
0.0 [a]	364(1)	4.2(6)	358.4(2)	12(1)
0.5	364(1)	4.2(6)	358.3(2)	12(1)
500	363(1)	4.6(6)	358.3(1)	12(1)

[a] The amount of entrapped Pasp in μg per 1 g of gelatin. [b] Magnesium mole percent in calcite obtained by the volume reduction of the unit cell.[17]

The presence of magnesium in solution induced a substantial change in the crystal morphology. In the first stages of crystal growth, at a magnesium/calcium molar ratio equal to 1 and in the absence of entrapped Pasp, calcite crystals occurred as prisms elongated in the direction of the *c* axis, and capped with rhombohedral {104} faces. The single crystals aggregated with an angular spread of the hexagonal axis of a few degrees (Figure 3A). During growth the spread increased until the aggregate attained a globular shape (Figure 3B). The core of a fractured globular aggregate is shown in Figure 3C. In the presence of

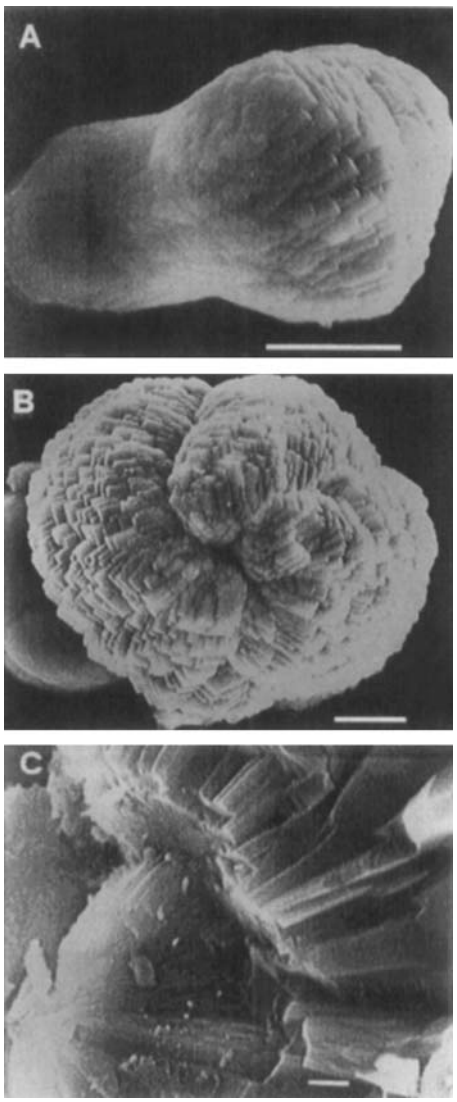

Figure 3. SEM images of magnesium calcite aggregates grown on the surface of a gelatin film without Pasp from a solution with a magnesium/calcium molar ratio of 1. A) Initial stage of growth; B) shape of an aggregate after four days; C) fractured section of an aggregate. Scale bars: A, 5 μm; B, 10 μm; C, 1 μm.

entrapped Pasp, the rhombohedral faces exhibited more rounded edges, which disappeared completely at a concentration of entrapped Pasp of about 0.5 mg per gram of gelatin.

At a magnesium/calcium molar ratio of 2.5, the aggregates of acicular magnesium calcite crystals, elongated in the *c* axis direction and radially oriented, consisted of a smaller hemisphere grown inside the film joined with a larger one grown towards the solution. Under the microscope between crossed polarizers they exhibited a pseudouniaxial black cross. In the presence of Pasp, at a concentration in the range 0.5 μg–0.5 mg g^{-1} gelatin, the smaller hemisphere was formed by a central core surrounded by concentric layers (generally two) of radially oriented crystals. The diameter of the inner core was about 40–60 μm and the average thickness of an external concentric layer was about 1.5 μm. These concentric layers were not observed in the larger hemisphere, which was formed of radially oriented acicular magnesium calcite crystals. A typical aggregate is shown in Figure 4, together with the mark left on the surface of the gelatin

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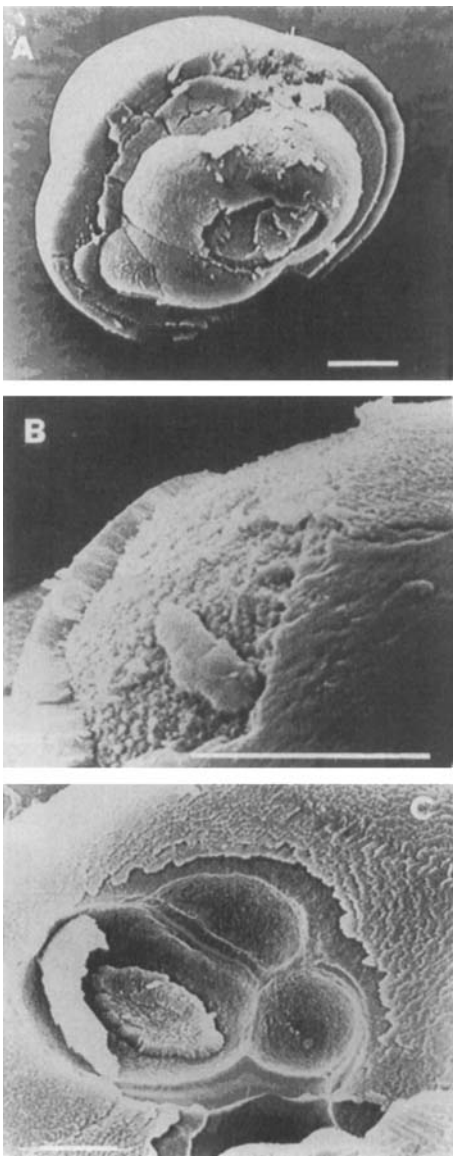

Figure 4. A) SEM image of an aggregate of magnesium calcite crystals grown from a solution with a magnesium/calcium molar ratio of 2.5. The aggregate was removed from the surface of a gelatin film containing 0.5 mg of Pasp per gram of gelatin. B) View of the broken surface of the smallest hemisphere in A) at higher magnification, showing the characteristic layered structure. C) View of the mark left on the gelatin film surface by the aggregate in A). Scale bars: 10 μm.

film. It must be noted that no influence on the calcite morphology and on magnesium substitution was observed in control experiments with an amount of gelatin in solution equal to that released from the crosslinked films.

Concentrations of entrapped Pasp higher than about 0.5 mg and up to the highest used concentration of 1.0 mg per gram of gelatin provoked a polymorphic switch from calcite to aragonite independent of the presence of magnesium ions in solution. This mineral phase nucleated inside the gelatin films and was organized in globular aggregates with a diameter in the range of 30–70 μm. Also, in this case, the density of nucleation of aragonite aggregates increased with the relative amount of entrapped Pasp in the gelatin film.

Crystallization in the presence of uniaxially deformed gelatin films: The presence of entrapped Pasp in uniaxially deformed films controls the deposition of calcium carbonate. In the absence of Pasp, calcite crystals with typical rhombohedral morphology were observed outside the film whereas no mineral phase was found in the film (Figure 5A). In the presence of Pasp, even at a concentration lower than 0.5 mg per gram of gelatin, the inorganic phase was mainly deposited inside the film, as can clearly be seen in Figure 5B. Only a few rhombohedral calcite crystals were formed outside the film. The amount of the inorganic phase deposited inside the film increased as the Pasp content increased.

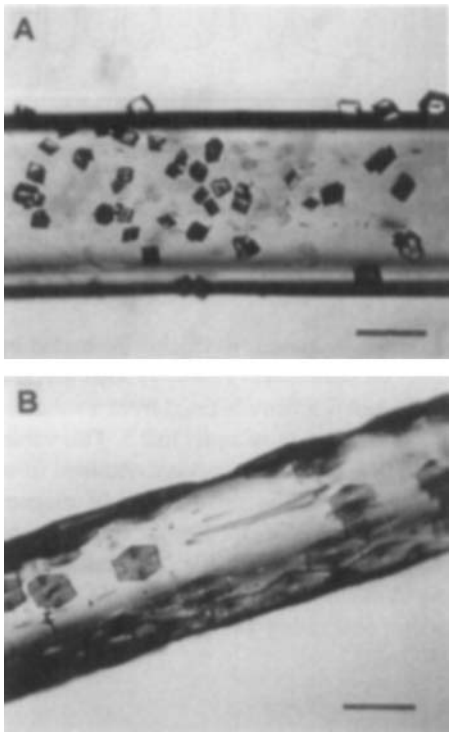

Figure 5. Optical micrographs of calcium carbonate deposits grown in the presence of uniaxially deformed film (200% elongation). A) Rhombohedral calcite crystals formed on film without entrapped Pasp. B) Aragonite aggregates formed inside the film with entrapped Pasp. Scale bars: 100 μm.

Under the microscope between crossed polarizers the inorganic deposits inside the film exhibited a pseudouniaxial black cross. The X-ray diffraction patterns obtained with the X-ray beam perpendicular or parallel to the surface of the film indicated that the deposits consisted of aggregates of aragonite crystals independent of the presence of magnesium ions in solution; aragonite was the only calcium carbonate polymorph revealed inside the gelatin films by X-ray diffraction analysis. X-ray fibre diffraction of collagen is easily recognizable and the typical meridional and equatorial reflections at 0.29 nm and 1.1 nm, respectively, indicated the direction of the film elongation parallel to the fibre axis (Figure 6). The aragonite crystals showed a slight preferential orientation with the *c* axis perpendicular to the direction of elongation. The aragonite diffraction maxima (012), (112) and (013) were spread into two symmetric long arcs when the X-ray beam was parallel to the surface of the film and

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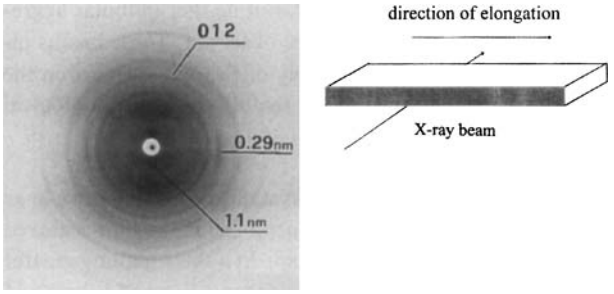

Figure 6. X-ray diffraction pattern of intact uniaxially deformed gelatin film with aragonite grown inside. The X-ray beam is parallel to the gelatin film surface and perpendicular to the direction of elongation as shown. The reflection at 0.29 nm is due to collagen bundles oriented along the direction of the film elongation.

perpendicular to the direction of elongation (Figure 6). The arc lengths increased with increasing Bragg angle; the centres of greatest darkness were aligned perpendicular to the fibre axis. The Debye ring of the maximum (111) formed four arcs arranged symmetrically with respect to the meridional and equatorial directions. A similar, even if much smaller, orientation effect of the aragonite crystals was observed when the X-ray beam was perpendicular to the surface of the gelatin film. This crystal orientation was checked in fragments of deproteinated aragonite deposits, which give clearer diffraction patterns. Although the degree of orientation was low, the diffraction data suggested that the aragonite crystals were oriented radially to the direction of elongation, so the crystals oriented with the *c* axis perpendicular to the X-ray beam contributed more to the diffraction than the oblique ones. The aggregates appeared as flat lozenges with contours and lengths depending on the elongation of the films and on the presence of magnesium ions in solution, but they were invariably aggregates of aragonite crystals. The presence of magnesium ions in solution induced the formation of more rounded lozenges, which were shaped by the bundles of the denatured and degraded collagen molecules. The observed correlation between the degree of elongation and the form of lozenges indicates that their form reflects the initial shape of the sites where they grow. The modelling of the organic matrix was more evident in the deproteinated samples. The aggregates of aragonite crystals formed rods parallel to the direction of elongation (cross-section 2–3 μm in diameter, Figure 7). The slight orientation effect observed in the diffraction patterns suggests that the crystals that form each rod are preferentially oriented with the *c* axis perpendicular to the rod length.

Discussion

The results of this study, summarized in Table 2 and Figure 8, show that the collagenous matrices with entrapped Pasp are able to control aragonite or calcite polymorph deposition. Aragonite invariably crystallizes inside the crosslinked gelatin films, independently of the presence of magnesium ions in solution. However, magnesium ions as well as the stretching of the film influence the architectural assemblies of the crystals.

Crystallization of calcite: In the absence of magnesium ions in solution and of entrapped Pasp in the films the crystallization of rhombohedral calcite occurred on the film surface.

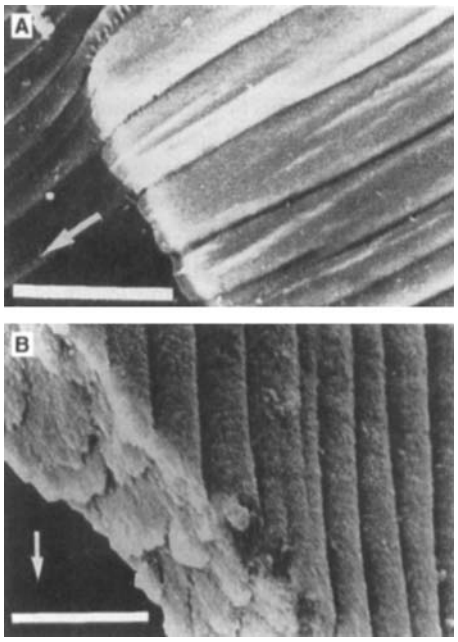

Figure 7. A) SEM image of aragonite aggregates formed inside the uniaxially deformed gelatin film with entrapped Pasp; B) view of fractured deproteinated aragonite aggregates. The arrows indicate the direction of the film elongation. Scale bars: 10 μm.

Table 2. Crystallization of calcium carbonate polymorphs.

Crosslinked gelatin films	Pasp concentration [a] 0.0	<0.5 μg	0.5 μg–0.5 mg	0.5–1 mg
Unstretched	unoriented calcite [b]	oriented calcite [b]	calcite [c] aggregates	aragonite [d] aggregates
Uniaxially deformed (200% elongation)	unoriented calcite [b]	oriented calcite [b]	aragonite [d] rods	aragonite [d] rods

[a] The amount of entrapped Pasp in 1 g of gelatin. [b] Calcite containing up to 12 mol% of magnesium with $[Mg^{2+}]/[Ca^{2+}] = 2.5$. [c] Ooid-like aggregates of magnesium calcite were obtained from solution with $[Mg^{2+}]/[Ca^{2+}] = 2.5$. [d] Aragonite was always found inside the film.

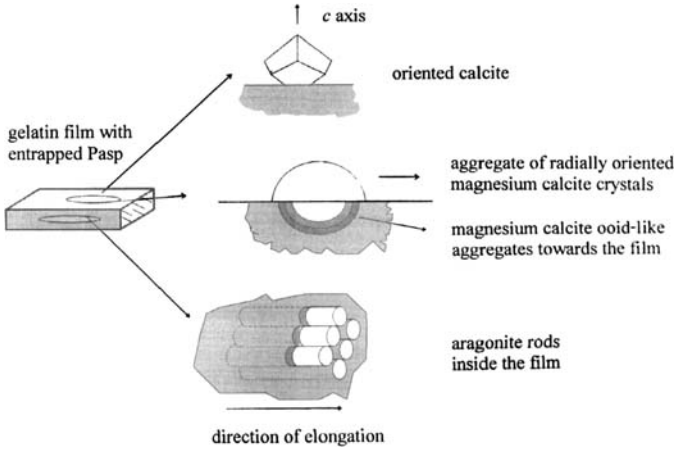

Figure 8. Schematic illustration of calcium carbonate crystallization on and inside the crosslinked gelatin films.

Entrapped Pasp induced the highest frequency of (001)-oriented crystallization of calcite on the films surface when its content did not exceed 0.5 μg per gram of gelatin.[11] Many studies have attempted to define the interplay of polyanionic

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proteins and other biomolecules with specific crystal planes of biogenic calcium carbonate.[8] The binding of Pasp to calcite has also been studied by atomic force microscopy and molecular modelling.[10c] Energy optimizations revealed that the polypeptide assumed the most energetically favourable beta conformation when the local electrostatic interactions did not prevail, and that the strongest binding was with the calcite (001) basal plane. On this basis we postulated a beta structure for entrapped Pasp. Carboxylate groups protruding from the surface of calcium-preloaded crosslinked gelatin films provide the stereochemical interaction with the (001) planes and, as a consequence, the oriented crystallization.[11]

When magnesium ions were present in solution (magnesium/calcium molar ratio equal to 2.5), calcite containing up to 12 mol% magnesium crystallized in the presence of unstretched gelatin films. The presence of entrapped Pasp had no effect on the magnesium substitution in the calcite (Table 1). The mechanism which leads to the formation of magnesium calcite with high degrees of magnesium substitution for calcium is still unknown. The difficulty of accommodating the magnesium ion in calcite is probably related to its high charge density, which makes the hydration enthalpy and the radius of the hydrated ions higher for magnesium than for calcium. According to this hypothesis, the reduction of magnesium hydration energy in water–alcohol mixtures leads to the crystallization of calcite with a magnesium content up to 14 mol%, unusual for a synthetic calcite obtained at room temperature.[19] Since the presence of gelatin in solution did not influence the magnesium substitution for calcium, it seems likely that the crosslinked gelatin films may make the incorporation of magnesium in the calcite easier by reduction of the hydration sphere on nucleation.

The presence of magnesium in solution caused considerable changes in the crystal morphology.[6, 20] Globular aggregates of radially oriented magnesium calcite crystals elongated in the *c* axis direction were made up of two hemispheres. The smaller one, grown towards the film, was formed by a central core surrounded by concentric layers of radially oriented magnesium calcite crystals. The formation of these layers could be related to the compartmental structure of the unstretched gelatin films.[14] The adsorption of Pasp on the (001) plane of the calcite might interrupt the crystal growth along the *c* axis direction. The polymeric covering thus formed might serve as a substrate for further crystallization, giving rise to another shell, and so on with a mechanism similar to that proposed by Davies et al. for the formation of ooids in supersaturated seawater solution containing humic acids.[3b] The homogeneity of the layer thickness (about 1.5 μm) and the presence of the layered structures in the different stages of growth strongly suggest a constant mineralogy and exclude any recrystallization effect.

Habit modifications of growing crystals are the most obvious consequences of the effect of the additives on the crystal growth.[8b] Therefore control experiments on the influence of the macromolecular components released from the film were carried out. The presence of a small amount of gelatin in solution (30 $\mu g mL^{-1}$) did not influence the morphology of crystalline phases, whereas in the presence of Pasp in solution the crystals showed rounded edges and often completely lost their crystal morphology.[6] The simultaneous presence in solution of both additives enhanced this effect, but neither globular aggregates nor layered organization were observed. These results indicate that supramolecular assembly of Pasp and gelatin in the crosslinked films is responsible for the observed morphological effects.

Crystallization of aragonite: The crystallization of aragonite as a unique calcium carbonate polymorph inside the film occurred in the presence of the entrapped Pasp. At a Pasp loading greater than 0.5 mg up to the highest used concentration of 1.0 $mg g^{-1}$ gelatin, crystallization occurred almost exclusively inside the gelatin films, giving rise to aggregates of aragonite crystals independently of the presence of magnesium ions in solution. Aragonite formation in uniaxially deformed crosslinked gelatin films occurred at a lower Pasp content (0.1 $mg g^{-1}$ gelatin). Therefore, the control of mineralization into the film depends on the entrapped Pasp concentration and on the film deformation. As the content of Pasp increased, ionic diffusion into the films increased and more calcium ions could bind to Pasp in the microenvironments where nucleation and growth occur. This suggests that the crystallization of aragonite is related to the modelling of the nucleation sites by the structure and concentration of Pasp, local supersaturation and microenvironment shape. Control experiments carried out in the absence of gelatin films excluded any supersaturation or foreign-ion effect on the control of aragonite crystallization inside the films.

The X-ray diffraction patterns indicate that the triple helical structure of collagen at least partially survived the denaturation and degradation process into gelatin, and that molecular orientation can be induced by uniaxial deformation of the gelatin films. The reorganization of the molecular segments into bundles might be responsible for the variation of the permeability, as shown by swelling measurements, as well as for the change of the nucleation site shapes. Unfortunately, these possible changes in structure or ordering of entrapped Pasp on stretching cannot be detected either by X-ray diffraction or by spectroscopic techniques because of its low content compared to gelatin. However, it seems likely that the mechanical strain not only orders the molecular segments of collagen but also induces the orientation of the polypeptide chains along the direction of elongation. The crystalline deposits of aragonite formed inside the uniaxially deformed crosslinked films were partially oriented with the *c* axis perpendicular to the direction of elongation. No orientation was found in the aragonite aggregates grown into unstretched films.

The architectural assembly of the aragonite crystals is undoubtedly controlled by the molecular organization of the crosslinked gelatin films. When the organic matrix is dissolved by treatment with hydroxylhydrazine, the aggregates formed inside the stretched films clearly appeared to be composed of parallel rods circular in cross-section (Figure 7). These rods consisted of small aragonite crystals preferentially oriented radially to the rod length, and extended parallel to the bundles of crosslinked gelatin. Although greater in diameter, they resemble those found by Mutvei in the septal nacre of *Spirula spirula*.[21, 22] This finding suggests that the supramolecular organization of the Pasp chains and degraded collagen molecules in the stretched collagenous matrices might mimic the function of the organic matrix of the aragonitic layers in marine organisms.

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Recently Miyamoto et al.[23] suggested that the collagenous repeat domain Gly-X-Asn (where X = Asp, Asn, or Glu) found in nacrein, a soluble matrix protein of the nacreous layer in oyster pearls, could form a network like collagen molecules, capable of contributing to aragonite crystal growth. This hypothesis is very impressive in view of our findings on the aragonite crystallization inside collagenous matrices, even if at this stage it is difficult to formulate a persuasive detailed model for the mineralization process.

Concluding Remarks

Crosslinked gelatin films with entrapped Pasp represent a useful tool to mimic calcium carbonate deposition in natural processes such as the formation of calcitic ooids or the regulation of biological crystal growth by skeletal intracrystalline components of marine organisms. These films are versatile systems that offer valuable insights into the applications of important biomimetic principles relevant for the formation and design of inorganic materials:[8c, 24] a) the precipitation of high-magnesium calcite is regulated by the gelatin matrix; b) the immobilization of Pasp within the gelatin matrix provides a substrate that is capable of induced-oriented nucleation by molecular templating; c) the assembled polymeric matrix (Pasp and crosslinked gelatin) provides spatially constrained environments for control of calcite and aragonite polymorphism and for construction of organized aggregates of crystals.

Experimental Section

High-grade $(NH_4)_2CO_3$, $CaCl_2 \cdot 2H_2O$ and $MgCl_2 \cdot 6H_2O$ reagents (Merck), type A gelatin from porcine skin (300 Bloom) and poly-L-aspartic acid (sodium salt, M_r 9600; Sigma) were used. Deionized water (2 μS, Millipore) was used.

Noncrosslinked films were obtained on the bottom of a tissue culture Petri dish of diameter 5.5 cm after water evaporation at room temperature from 10.0 mL of 5% aqueous gelatin solution containing up to 1.0 mg of Pasp per gram of gelatin. The films were crosslinked by soaking for 24 hours at room temperature in 5.0 mL of a 5 wt% glutaraldehyde[25] solution. The film kept on the bottom of the dish was then washed three times with water, equilibrated for 24 h with 5 mL of 2 mM $CaCl_2$ solution and then washed again with water and dried. The air-dried films are about 100 μm thick.

The noncrosslinked films were cut into strips 0.3 cm wide and allowed to swell in water/ethanol solution (2/3 v/v) for 3 days. Each strip kept in the water/ethanol mixture was stretched by means of an Instron 4465 tensile tester instrument at a speed of 0.5 cm/min up to a maximum elongation of 300%. The drawn sample was air-dried at room temperature at constant elongation. The crosslinked films were washed three times with water, incubated for 24 h in a 2 mM $CaCl_2$ solution and then washed again with water and dried.

Crystallization solutions with different Mg^{2+}/Ca^{2+} molar ratios (0.0; 1.0; 2.5) were obtained by addition of the required mass of magnesium chloride to a known volume of 10 mM calcium chloride solution. Calcium carbonate crystals were grown as reported by Addadi et al.[26] at a temperature of 18 °C by very slow diffusion of $(NH_4)_2CO_3$ vapour onto Petri dishes containing 3 mL of crystallization solution over the gelatin films. Although it is difficult to reproduce the nucleation density, the same mineral forms and the same trend in the nucleation density as a function of Mg^{2+}/Ca^{2+} molar ratio was observed in repeated experiments. Control experiments without films were carried out with Pasp and gelatin added to the crystallization solution at about the same concentration of the material released from the films.

The swelling of the crosslinked gelatin films with or without entrapped Pasp in 10 mM $CaCl_2$ solution was followed gravimetrically. Square samples of 0.3 cm edge were cut. The weighed samples were then equilibrated in the $CaCl_2$ solution for 48 h at room temperature, dried with blotting paper and weighed.

Crystals on gelatin films were initially examined by optical microscopy. More detailed morphological investigations were carried out by means of a Philips XL-20 Scanning Electron Microscope. Samples were deposited on aluminium stubs and sputter-coated with a thin layer of gold. Inorganic phases crystallized inside the films were examined as such and after treatment with hydroxylhydrazine, which dissolves the organic matrix.[27]

High angle X-ray diffraction analysis employing $Cu_{K\alpha}$ radiation was carried out by means of a flat camera. X-ray powder diffraction patterns were recorded with a Philips PW 1050/81-PW 1710 system. The least-squares method was used to calculate the unit cell parameters from the well-determined positions of the three most intense reflections. The peak positions were corrected with silicon as internal reference.

Fourier transform infrared spectra (FTIR) were obtained from KBr pellets using a Nicolet 250 FTIR spectrometer. A biological sample of magnesium calcite (from sea urchin teeth) containing 14 mol% magnesium was used for comparison.

Magnesium and calcium contents of the crystals were determined with an atomic absorption spectrometer (Perkin-Elmer 373).

The amino acid composition of the material released from the films at the end of the crystallization experiments was determined after hydrolysis in 6 M HCl at 110 °C under nitrogen. The analyses were carried out by HPLC following the method of Galletti et al.[28]

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