Oriented Crystallization of Vaterite in Collagenous Matrices

Giuseppe Falini, Simona Fermani, Massimo Gazzano, and Alberto Ripamonti*

Abstract: The influence of high supersaturation on kinetic control and the importance of the polypeptide structure in the crystallization of calcium carbonate polymorphs were studied in cross-linked gelatin films containing high concentrations of the polypeptides poly-L-aspartate and poly-L-glutamate. Oriented crystallization of vaterite occurs in uniaxially deformed gelatin films containing poly-L-aspartate at concentations greater than 100 mg per gram of gelatin. The fact that no orientation of the mineral phase was observed with entrapped poly-L-glutamate at the same concentrations suggests that the oriented crystallization is controlled by the β sheet structure assumed by poly-L-aspartate in the presence of calcium ions. These results indicate that local supersaturation in the microenvironment in which nucleation and growth occur plays an important role in controlling the deposition of vaterite in cross-linked gelatin films. However, collagen bundles and the ordered and oriented polypeptide chains of poly-L-aspartate can contribute to the control of polymorphism by inducing the formation of a specific phase by epitaxial crystallization, as suggested by the preferentially oriented deposition of vaterite and aragonite. This is of potential significance in biomineralization processes and in materials science.

Keywords: bioinorganic chemistry · biomineralization · calcium carbonate · gelatin · materials science

Introduction

Calcium carbonate can be precipitated as three distinct crystalline phases: calcite, aragonite, and vaterite. Calcite is the most stable, and vaterite the least stable polymorph.[1]

Polymorphism selectivity in an inorganic environment requires different initial conditions of temperature and pressure and the use of additives.[2] The most soluble polymorph, vaterite, is selectively precipitated only in the presence of specific additives. It does not show defined morphologies, tends to form spherical aggregates, and when wet it changes into more stable polymorphs.[3] The polymorphism and habitus of calcium carbonate crystals are difficult to control. However, these problems are easily overcome by organisms, which control the polymorphism, chemical composition, morphology, and texture of the deposited mineral.[4] Calcite and aragonite are widespread in marine organisms, and vaterite, monohydrocalcite, and amorphous calcium carbonate are formed and stabilized by some organisms.[5]

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Several studies suggest that calcium carbonate polymorphism selectivity in organisms is controlled by specific glycoproteins in the presence of particular substrates.[6] The possibility of inducing preferred nucleation of calcium carbonate polymorphs under Langmuir monolayers with an appropriate supramolecular motif has been reported.[7] Charged groups on a substrate can stabilize crystal clusters in a specific or nonspecific way. When the charge distribution and repeat distances of the charged groups are complementary to the crystal surface of a specific crystal plane, the activation energy is lowered. The result is not only the preferred crystallization of one polymorph, but also oriented crystal nucleation on a specific plane of recognition. Falini et al.[8] induced the oriented crystallization of calcite and aragonite by means of cross-linked gelatin films with entrapped poly-L-aspartate (poly-Asp). These results suggested that the control of calcium carbonate polymorphism and the architectural assembly of the crystals were related to the modeling of the nucleation sites in terms of poly-Asp concentration, local supersaturation, and the shape of the microenvironment.

To investigate the influence of higher supersaturation on kinetic control and the importance of the polypeptide conformation on the oriented nucleation of calcium carbonate polymorphs, we used cross-linked gelatin films with high concentrations of the entrapped polypeptides poly-Asp and poly-L-glutamate (poly-Glu). The different structures of poly-Asp and poly-Glu—the former has a β sheet structure in the presence of calcium ions and the latter a random conforma-

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tion[9]—should differ in their influence on mineral deposition in uniaxially deformed cross-linked gelatin films.

Results

In the presence of gelatin films without entrapped polypeptide, calcite precipitates at the air/water interface and on the film surface. At a concentration of 100 mg of poly-Asp per gram of gelatin, aragonite was the only calcium carbonate polymorph detected in the unstretched films by X-ray diffraction analysis, whereas vaterite was the sole crystalline phase in uniaxially deformed films (Table 1; Figures 1 and 2). Under the same conditions, poly-Glu induced the formation of aragonite in unstretched films, and a mixture of aragonite and vaterite in uniaxially stretched films. At higher concentrations of entrapped poly-Asp (300 mg per gram of gelatin) only vaterite was detected in both stretched and unstretched films. Under analogous conditions poly-Glu induced the precipitation of both vaterite and aragonite in the films (Table 1). The FT-IR spectra of deproteinated samples confirm the identity of the crystalline phases in showing characteristic maxima at 744 and 875 cm^{-1} for vaterite and at 712 and 860 cm^{-1} for aragonite.[10] The presence of a small amount of amorphous calcium carbonate can not be excluded on the basis of X-ray diffraction and FT-IR data because of

Table 1. Mineral phases deposited inside gelatin films containing polypeptides.

	Unstretched		Uniaxially deformed[a]	
	100[b]	300[b]	100[b]	300[b]
poly-Asp	unoriented aragonite	unoriented vaterite	oriented vaterite	oriented vaterite
poly-Glu	unoriented aragonite	unoriented vaterite and aragonite	unoriented vaterite and aragonite	unoriented vaterite and aragonite

[a] The films were deformed at 120%. [b] mg of polypeptide per gram of gelatin.

Abstract in Italian: *L'influenza della supersaturazione sul controllo cinetico e l'importanza della struttura del polipeptide sulla cristallizzazione dei polimorfi del carbonato di calcio sono stati studiati usando films di gelatina reticolata contenenti alte concentrazioni di due diversi polipeptidi: poli-*L*-aspartato e poli-*L*-glutamato. Cristalli orientati di vaterite si depositano all'interno di films di gelatina, uniassialmente deformati, contenenti poli-L-aspartato quando il contenuto del polipeptide è superiore a 100 mg per grammo di gelatina. Nessun effetto di orientamento è stato osservato nei films contenenti poli-*L*-glutamato. Ciò suggerisce che il controllo sulla cristallizzazione orientata è dovuto alla struttura ordinata assunta dal poli-*L*-aspartato in presenza di ioni calcio.*

*Questi risultati indicano che la supersaturazione locale nel microambiente, dove hanno luogo nucleazione e crescita, ha un ruolo importante nel controllo della deposizione di vaterite all'interno dei films di gelatina reticolata. Tuttavia, la matrice collagenosa e le catene polipeptidiche orientate e ordinate del poli-*L*-aspartato possono contribuire al controllo del polimorfismo, inducendo la formazione di una fase specifica mediante una cristallizzazione epitassiale, come suggerito dalla deposizione orientata di vaterite e aragonite. Ciò è perticolarmente significetivo per i processi di biomineralizzazione e per la scienza dei materiali.*

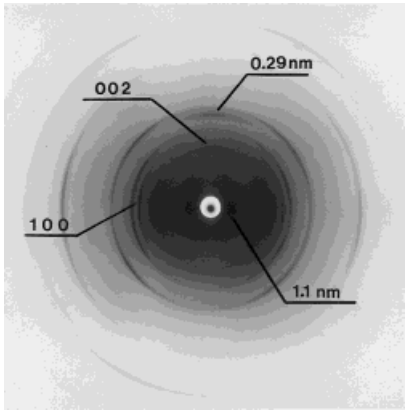

Figure 1. X-ray diffraction pattern of intact uniaxially deformed gelatin film with internally grown vaterite. The X-ray beam was perpendicular to the surface of the gelatin film. The characteristic collagen diffraction maxima, meridional at 0.29 nm and equatorial at 1.1 nm, are preferentially oriented parallel and perpendicular to the direction of elongation. The sample is inclined with respect to the X-ray beam to intensify the meridional reflection at 0.29 nm. The oriented vaterite reflections (002) and (100) are indicated.

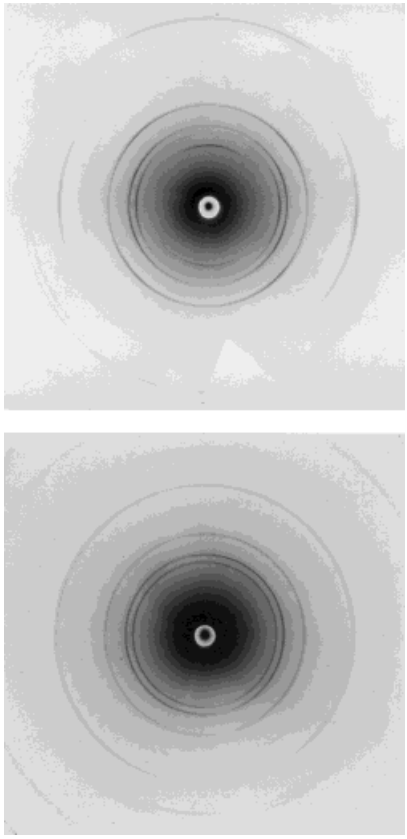

Figure 2. X-ray diffraction pattern of deproteinated vaterite fragments obtained with the X-ray beam perpendicular (top) and parallel (bottom) to the direction of elongation of the gelatin film.

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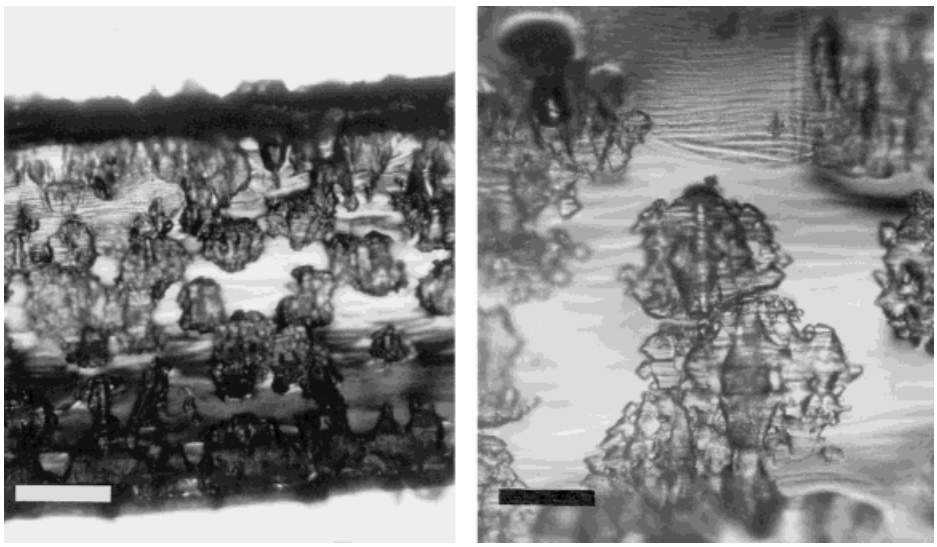

Figure 3. Optical micrographs of vaterite aggregates grown in oriented films at two different magnifications. Left: Scale bar = 500 μm; right: Scale bar = 200 μm.

the difficulties in detecting this form when it is associated with a crystalline phase.

Optical micrographs of vaterite deposits in uniaxially deformed films (120% elongation) are shown in Figure 3. The shapes of the vaterite aggregates are modulated by the film cavities formed by the collagen bundles. The shaping of the organic matrix and the orientation induced by stretching are more evident in the scanning electron micrographs of the deproteinated samples (Figure 4). The vaterite aggregates, which are randomly oriented in unstretched films, orient themselves inside the cavities of the stretched films.

The X-ray diffraction patterns (Figure 1) show that during the uniaxial deformation the gelatin layers reorganize in bundles parallel to the direction of elongation; they are composed of oriented segments of collagen molecules, as indicated by the orientation of the characteristic meridional and equatorial diffraction maxima at 0.29 and 1.1 nm, respectively. No diffraction effect due to poly-Asp chains in an oriented β sheet structure could be detected. This does not exclude the possible orientation of poly-Asp chains in the β sheet structure that is expected in the presence of calcium ions. In fact the strongest reflections of the X-ray fiber diffraction pattern of a β pleated sheet structure are hidden by those of the oriented collagen bundles.

The vaterite crystals grown in the uniaxially deformed films containing poly-Asp are preferentially oriented with the *c* axis parallel to the collagen fiber axis, which in turn is parallel to the direction of elongation (Figure 1). The arched vaterite reflections are on layer lines, as expected for a crystal rotating around the *c* axis. Indeed, the diffraction pattern did not change on rotation of the sample around the direction of elongation. Clearer diffraction patterns, obtained from deproteinated vaterite fragments with the X-ray beam perpendicular and parallel to the direction of film elongation, are shown in Figure 2. The *d* spacings, orientation, and indexes of the reflections for the hexagonal subcell[11] with $a = 0.413$ and $c = 0.849$ nm are listed in Table 2. No orientation was observed when the X-ray beam was parallel to the direction of elongation. The Debye rings were continuous, and the intensities of the (00*l*) reflections were not observed; this in agreement with a preferential orientation of vaterite crystals with the *c* axis parallel to the direction of elongation, but randomly oriented in all the other respects.

Discussion

The results summarized in Table 1 indicate that poly-Asp exerts a more severe control on polymorphism than poly-Glu, and that uniaxial deformation favors the formation of the most soluble crystalline phase of calcium carbonate. With the increased diffusion of calcium ions in the film with a high concentration of polypeptide, local supersaturation can become so large that the product of the activities of the ions in the microenvironment in which crystallization occurs can greatly exceed the

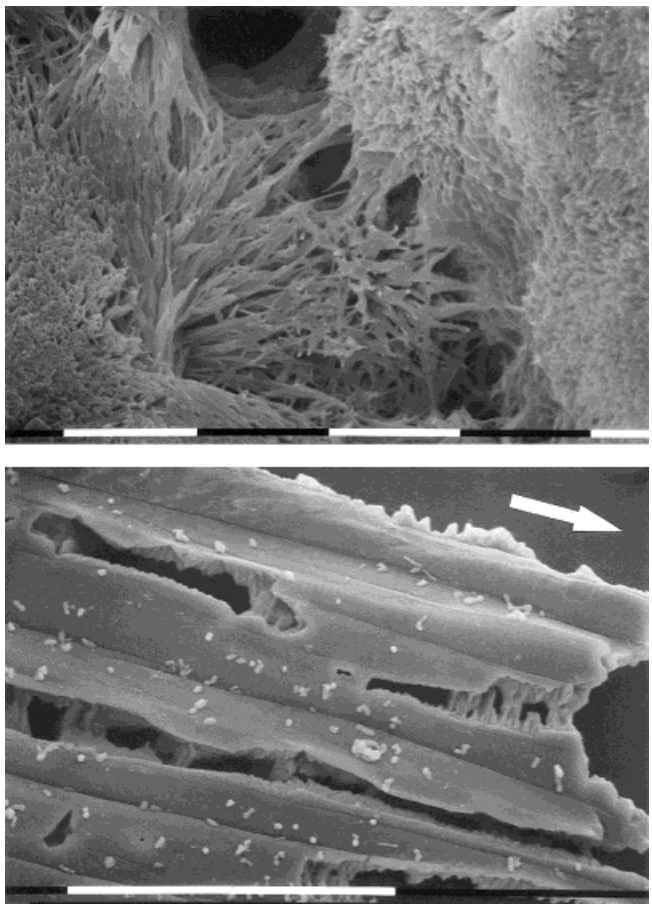

Figure 4. Scanning electron micrographs of deproteinated vaterite aggregates. Top: Grown in unstretched films (scale bar = 10μm); bottom: Grown in uniaxially deformed films (scale bar = 100μm). The arrow indicates the direction of elongation.

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Table 2. X-ray diffraction data obtained from diffraction pattern of Figure 2 top.

d [nm]	Orientation[a]	Intensity[b]	*hkl*[c]
0.426	M	m	002
0.359	E	s	100
0.328	F	s	101
0.2750	F	vs	102
0.2323		vw	[d]
0.2217		w	103
0.2124	M	w	004
0.2073	E	s	110
0.1856		w	112
0.1823	F	s	104
0.1797	E	vw	200
0.1651	F	w	202

[a] The reflections orientations are indicated as: M = meridional, that is parallel to film elongation; E = equatorial, that is perpendicular to film elongation; F = four arcs arranged symmetrically with respect to the meridional and equatorial directions. [b] vw = very weak; w = weak; m = medium; s = strong; vs = very strong. [c] Relative to the vaterite subcell with $a = 0.413$ nm $c = 0.849$ nm.[11] [d] This reflection can be indexed as (211) for the hexagonal cell parameters $a = 0.7169$ nm $c = 1.698$ nm.[3d]

thermodynamic solubility product. In accordance with Ostwald's rule,[12] which predicts that the least stable phase with the highest solubility is formed preferentially under kinetically controlled conditions of sequential precipitation, vaterite precipitates in cross-linked gelatin films when the polypeptide content is high. The average dimensions of the film cavities prior to mineral deposition decrease on uniaxial deformation, as indicated by swelling data.[8] This could imply a higher calcium content in a smaller volume and hence an increase in the local supersaturation, which could explain the preferred precipitation of the less stable form at the same polypeptide concentration. However, an orientation of the polypeptide chains in which the carboxylate groups protrude into the cavities of the uniaxially deformed films not only can contribute to the increase in concentration of calcium ions, but can also stabilize and/or induce an oriented crystalline form by specific interactions with a particular crystal plane.

A stronger electric field due to higher concentration and more closely spaced three-dimensional distribution of the negatively charged carboxylate groups should favor the interaction with the most positively charged crystalline plane. Vaterite has two homocharged calcium planes (001) and (100) with a charge density of about 6.7 calcium ions per square nanometer, similar to that of the {012} calcium planes of calcite, but higher than those of the (001) calcium planes of calcite (4.5 calcium ions per nm^2) and aragonite (5.0 calcium ions per nm^2). In biological environments[4] calcite and aragonite often crystallize from their (001) planes, in which the calcium ion positions are practically identical.[1] Oriented nucleation of calcite from the homocharged (012) plane in calcitic sponge[13] and under monolayers of polydiacetylene carboxylates has been described.[14] Crystallization of calcite or aragonite from the (001) plane in the presence of poly-Asp with the β pleated-sheet structure will result in orientation of the crystallographic *c* axis perpendicular to the polypeptide sheet according to energy-optimization calculations,[15] which revealed that the strongest binding of poly-Asp in the β conformation occurs with the (001) basal plane of calcite. This interaction between the polypeptide and the (001) plane of calcite was invoked to explain the (001)-oriented crystallization of calcite on the surface of the gelatin film at concentrations of entrapped poly-Asp of less than 0.5 μg per gram gelatin.[8a] At poly-Asp concentrations between 0.5 μg and 1.0 mg per gram gelatin, crystalline deposits of aragonite partially oriented with the *c* axis perpendicular to the direction of elongation were formed in uniaxially deformed cross-linked gelatin films.[8] Recently, crystallization of aragonite preferentially oriented with the (010) plane parallel to the monolayer surface of 5-hexadecyloxyisophtalic acid was reported.[7b] Ionic interactions of calcium ions with hydroxyethyl cellulose was considered to be one of the possible factors that influences the precipitation of organized vaterite platelets from aqueous solutions of the cellulose ether.[16] Oriented crystallization of vaterite from homocharged (001) calcium ion planes takes place under stearate monolayers, whereas under positively charged octadecylamine monolayers, vaterite crystallizes from (001) and (110) planes.[17] The orientation of vaterite crystals formed in uniaxially deformed cross-linked gelatin films containing poly-Asp suggests that poly-Asp with the β structure interacts with the most positively charged crystal plane (100), which is parallel to the *c* axis.

It is difficult to deduce in a complete way from these results the factors that control the oriented crystallization of calcium carbonate polymorphs. However, there is no doubt that the density and geometrical arrangement of the negative charges on the surface or in the cavities of the organic matrix in which nucleation occurs control the oriented nucleation and influence the polymorphism selectivity. A plausible mechanism of aragonite and vaterite growth inside collagenous matrices must involve both thermodynamic and kinetic control. The sequential precipitation of aragonite and then vaterite when the local supersaturation is increased by a higher concentration of poly-Asp or poly-Glu, or by the smaller size of the cavities in the stretched films, suggests kinetic control of polymorphism. However, the orientation of both aragonite[8b] and vaterite crystals grown in uniaxially deformed films containing poly-Asp suggests thermodynamic control by specific recognition of homocharged calcium-ion crystal planes by the β sheet structure of the polypeptide. Thus the deposition of vaterite inside uniaxially deformed gelatin films with entrapped poly-Asp can be schematically represented as shown in Figure 5.

Conclusions

In conclusion, the local supersaturation in the microenvironment in which nucleation and growth occur plays an important role in controlling the deposition of aragonite or vaterite in cross-linked gelatin films. However, the collagen bundles and oriented β chains of poly-Asp can contribute to the control of polymorphism by inducing the formation of a specific phase by epitaxial crystallization, as suggested by the preferential oriented deposition of vaterite and aragonite only in the presence of poly-Asp. The fact that mixtures of the

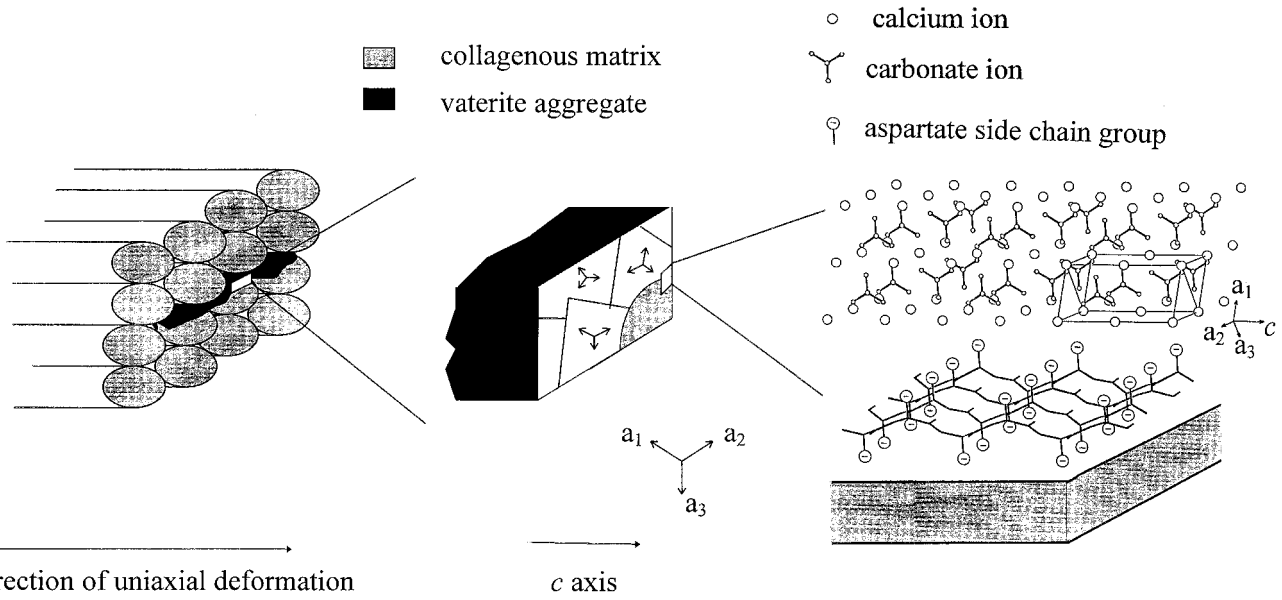

Figure 5. Schematic illustration of the orientation of vaterite in a uniaxially deformed cross-linked gelatin film containing entrapped poly-Asp. The subcell of vaterite is shown with one of the three possible orientations of the carbonate ions.[11]

different forms of calcium carbonate were not found in the films containing poly-Asp indicates that the supramolecular assembly exerts strict control over polymorphism. These results on the oriented crystallization of a definite calcium carbonate polymorph are of potential significance in crystal engineering as well as in biomineralization.

Experimental Section

High-purity $(NH_4)_2CO_3$ and $CaCl_2 \cdot 2H_2O$ were purchased from Merck; type A gelatin from pigskin (300 Bloom); poly-L-aspartic acid (sodium salt, M_r 9600) and poly-L-glutamic acid (sodium salt, M_r 9600) were purchased from Sigma; deionized water (2 μS, Millipore).

Gelatin films containing polypeptides were prepared as previously described.[8b] The cross-linked gelatin films become increasingly brittle with increasing concentration of entrapped polypeptide. Therefore, the highest polypeptide concentration used was 300 mg of polypeptide per gram of gelatin. At this concentration the films can be uniaxially deformed only up to 120% elongation.

Calcium carbonate crystals were grown in the films at 18 °C by slow diffusion of $(NH_4)_2CO_3$ vapor into solution of calcium chloride(10 mM), as described by Addadi et al.[9] Although it is difficult to reproduce the nucleation density, the same mineral forms were observed in repeated experiments. Deproteinated samples were obtained by treatment with sodium hypochlorite.

Morphological investigations were carried out with an optical microscope and a Philips XL-20 scanning electron microscope. High-angle X-ray diffraction analysis with $Cu_{K\alpha}$ radiation was carried out with a flat camera. Fourier transform infrared spectra were measured on KBr pellets in a Nicolet 250 FT-IR spectrometer.

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