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Control of the architectural assembly of octacalcium phosphate crystals in denatured collagenous matrices

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Xerogels, obtained by evaporating a water solution of gelatin, were used as templates for the growth of crystal assemblies of calcium phosphate. Octacalcium phosphate (OCP) crystals were grown inside the gelatin xerogel by diffusion of solutions of calcium and phosphate ions into the gelatin films from opposite ends. The structure, morphology and orientation of the crystals grown on the surfaces and inside the gelatin films were studied by optical and scanning electron microscopy and X-ray diffraction. OCP was the unique detectable crystalline phase. The crystals preferentially grew as thin plates elongated along their *c* axes with large {100} faces parallel to the sheets of gelatin films. When the gelatin films were uniaxially deformed the OCP crystals grew with their *c* axes almost parallel to the direction of deformation. According to a mechanism of templated nucleation, a structural model for the ordered growth of OCP on the portions of triple helical collagen molecules has been proposed.

Introduction

The study of inorganic crystal assembly on or inside organic matrices to develop epitaxial growth or organic–inorganic composites has given rise to increasing interest in various fields from materials science to natural processes of biomineralization.[1]

Here we present a new method for the controlled architectural assembly of octacalcium phosphate (OCP) crystals in dry gels, xerogels, made from denatured and degraded collagen.

The OCP crystal nucleation, orientation, morphology and assembly are of interest because this salt has been found as a component of some calcified tissue,[2] and for a long time has been considered a precursor of the most biologically important hydroxyapatite (HA) because of their close structural similarities.[3,4] The crystallization tool in xerogels offers the possibility to control the organization of the crystalline units at various degrees of orientation. Moreover the peculiar flexible and adaptable structure of the gelatin xerogel allows the study of the microenvironment in which the nucleation takes place.

Several investigators, inspired by the role of collagen in bone and other calcified biological tissues, have used collagen gel systems for the study of deposition of calcium phosphates.[5] Hydroxyapatite[5] and fluoroapatite[6] formation has been investigated by means of dynamic and static collagen diffusion cells respectively. Epitaxial growth of OCP using ion diffusion through collagen fibrils of bovine Achilles tendon has been thoroughly studied by Iijima and co-workers.[7]

We have shown that xerogels obtained by evaporating a water solution of gelatin are versatile systems for the study of calcium carbonate crystallization.[8] Gelatin, formed from denatured and degraded collagen, has a poorly defined structure. However, in xerogels obtained from a careful casting process, portions of collagen molecules with the characteristic triple helical structure are still present and lie in parallel layers. It has been demonstrated by scanning electron microscopy (SEM) and X-ray diffraction that during uniaxial deformation the gelatin layers reorganize into bundles of segments of collagen molecules oriented along the direction of elongation.[8b] In these systems the importance of the microenvironment in the compartmentalized space for the control of nucleation, growth, morphology, crystal aggregation and polymorphism could be examined more easily than in native collagen fibers. The advantages of gelatin films over well studied systems, where native collagen matrices are directly involved,[5,7] are due both to the layered structure of collagen xerogels and to the possibility to control the degree of orientation of the collagen molecular portions in a medium more permeable to water solutions.

Experimental

High purity grade $Ca(CH_3COO)_2$, K_2HPO_4 and Tris buffer reagents (Merck Co.), type A gelatin from porcine skin (300 Bloom; Sigma Co.) and deionized water (2 μS, Millipore) were used.

Gelatin xerogels were obtained, as previously described,[8b] 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 0.01% sodium azide to prevent bacterial growth. The uniaxially deformed xerogels were prepared from xerogel strips 4 cm wide, 0.3 cm thick, and swelled in water–ethanol 2 : 3 (v/v) solution 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^{-1} up to a maximum elongation of 100%. The drawn samples were air dried at room temperature at constant elongation.

Mineralization was conducted in a special double diffusion chamber, similar to that used very recently by Schwarz and Epple for the crystallization of HA and fluoroapatite in a polyglycolide matrix.[9] The chamber was formed by two separate ion reservoirs containing 500 ml of calcium and phosphate solutions respectively. The influence of the calcium and phosphate concentrations, as well as of the temperature, pH and foreign ions, on OCP nucleation and crystal growth in solution has been widely studied.[2b,4] On the basis of the results of these studies and after several double diffusion experiments carried out at different concentrations and pH values, we chose the following conditions in order to deposit sufficient crystals for morphological and structural characterization in an appropriate time at room temperature (20 ± 2 °C). One

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reservoir of the double diffusion chamber contained a solution of calcium acetate (7.3 mM) and the other one contained potassium monohydrogen phosphate (7.3 mM). Both solutions were buffered with Tris-HCl at pH 7.4. The two reservoirs were separated by a disk of the collagenous deformed or undeformed xerogel (3 cm in diameter). Mineral formation inside the collagenous matrix was observed after a few hours of diffusion of the calcium and phosphate ions from the opposite sides. The xerogel, which swells in contact with the water solutions, became opaque indicating that mineral deposition occurred inside the film. The diffusion was run for different times from six hours to two days. The variations of solute concentration were not monitored, because they were very small in comparison with the volume of solution and the quantity of the mineral formed. The mineralized xerogels were removed after the fixed time of diffusion, water rinsed and air dried. Deproteinated samples were obtained by treatment with hydroxylhydroxylamine at room temperature for 24 h. The samples were then rinsed with water and air dried.

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-Kα radiation was carried out with a flat camera or with a Siemens HI-STAR multiwire proportional counter. A crystalline phase could be identified with this method only if its content was greater than 5% of the crystals.

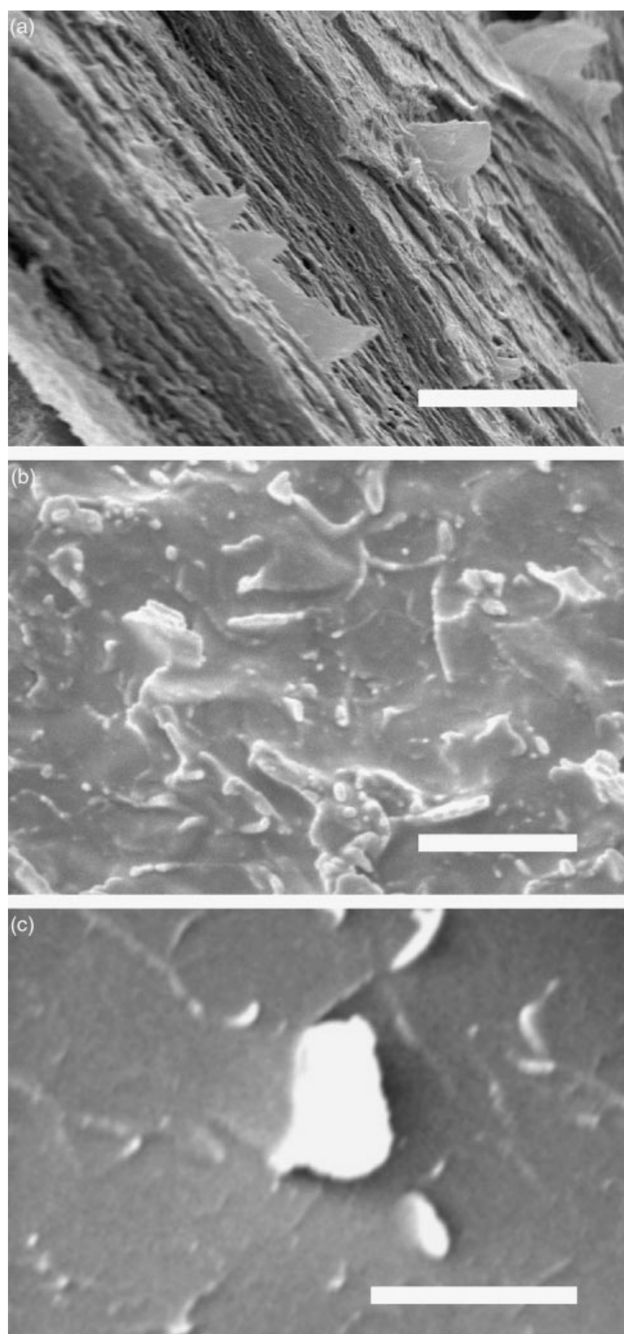

Fig. 1 SEM images of a gelatin film subjected to double diffusion for two days: (a) cross-section; (b) film surface of calcium side; (c) film surface of phosphate side. Scale bars: (a) 10 μm; (b) and (c) 50 μm.

Results and discussion

SEM images of an undeformed gelatin film subjected to double diffusion for two days are shown in Fig. 1. The cross-section image (Fig. 1a) clearly shows a layered structure, which appears more marked than that of an unmineralized gelatin films.[8b] Figs. 1b and c show mineral deposition on or near the surface of the calcium side while the phosphate side appears flat with little or no deposition. The X-ray diffraction patterns of an undeformed mineralized gelatin film are shown in Fig. 2. The measured *d*-spacing of the Debye rings (Fig. 2a), which are those of the strongest reflections of OCP, and the absence of Debye rings or diffraction effects due to other crystalline phosphates, indicate that OCP is the unique or at least the main deposited crystalline phase. However, the presence of amorphous material can not be excluded. The weakness of the (*h*00) reflections with respect to the expected intensities for a random orientation of the OCP crystals is in agreement with the marked orientation of these reflections in the X-ray pattern obtained with the incident beam parallel to the film surface (Fig. 2b). This indicates that the crystals are preferentially oriented with their *a* axes perpendicular to the film surface. SEM images of deproteinated samples submitted to double diffusion for different times substantiate the results of the X-ray study. This is illustrated in Fig. 3, where SEM images of deproteinated samples of undeformed xerogel subject to double

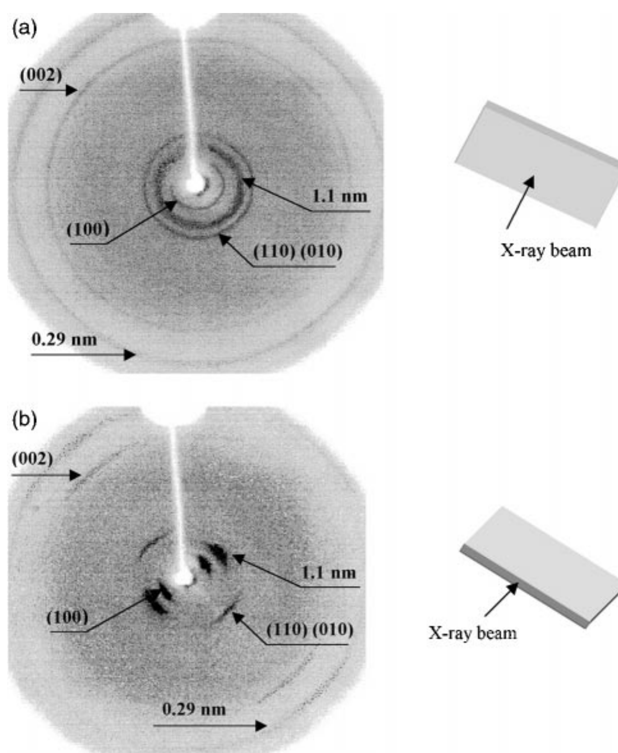

Fig. 2 X-Ray diffraction patterns of air-dried mineralized gelatin film with the X-ray beam perpendicular (a) and parallel (b) to the film surface. The directions of incidence, the indices of the strongest reflections of OCP and the spacings of the characteristic reflections for the triple helical structure of collagen are indicated.

diffusion for two days are shown. The sequence of deposition starts from the phosphate side on the bottom right of the sample cross-section shown in Fig. 3a. After a few hours only thin plates, which lie with the {100} faces parallel to each other and to the xerogel surface, are formed (Fig. 3c). When the time of diffusion increases, (100) blades grow towards the calcium side with the most developed faces almost perpendicular to the xerogel surface (Fig. 3b).

The uniaxial deformation of the xerogel induces a different aggregation of the OCP crystals. They envelop the oriented bundles of triple helices with the {100} faces parallel to the bundle surface and with their *c* axes parallel to the deformation direction (Fig. 4). This crystal orientation is confirmed by the orientation of the (002) reflection in the X-ray patterns, which are similar to that shown in Fig. 2b when the X-ray beam is both parallel and perpendicular to the gelatin film surface.

The results of this study demonstrate the ability of the gelatin xerogel to give rise to an ordered architectural assembly of OCP crystals and provide some insight into the underlying principles that govern OCP nucleation inside collagenous matrices. Iijima *et al.*,[7] using Achilles tendon consisting of collagen fibers oriented along the tendon axis, found the same results in regard to OCP crystallization inside the collagenous matrix and to the orientation of the crystals with their *c* axes parallel to the collagen fiber axis. However, unlike Achilles tendon the collagen xerogel has swelling properties that make the system more permeable to ionic water solution. This allows a more copious crystallization and an ordered assembly of the crystals governed by the compartmentalized space of the layered gelatin film. Furthermore, it is possible to control the degree of orientation of the OCP crystalline units as a function of the degree of orientation of the molecular portion of collagen in the uniaxially deformed xerogels.

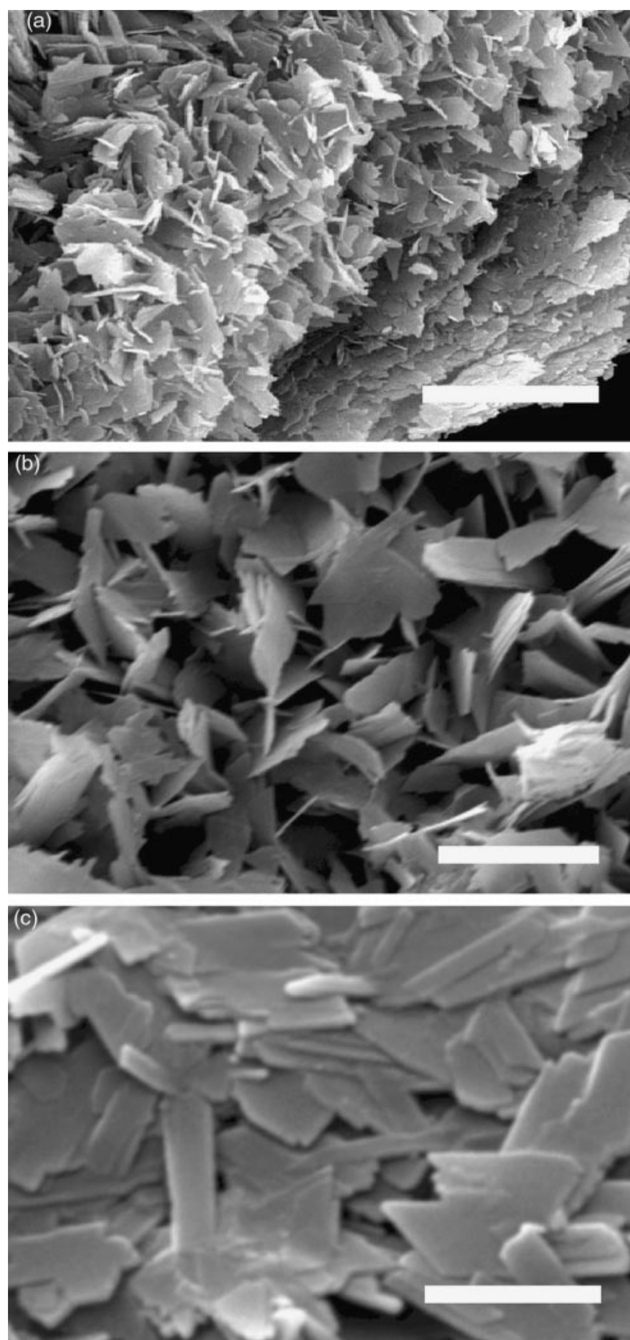

Fig. 3 SEM images of deproteinated samples of undeformed gelatin xerogels subjected to double diffusion for two days: (a) cross-section; (b) calcium side; (c) phosphate side. Scale bars: (a) and (c) 10 μm; (b) 5 μm.

Gelatin consists of ampholitic chain molecules positively charged below the isoelectric point (acid processed porcine skin, $pH_{iso} = 8.6$). Thus the phosphate ions may be immobilized by non-specific electrostatic interactions between the gelatin layers. These ions bonded to collagen and the crystal structure of OCP, which consists of hydrated layers alternating with HA layers parallel to the {100} faces,[10] suggest a means of constructing a model of the templated nucleation. It seems likely that in water solution the hydrated layer preferentially interacts with the phosphate ions immobilized on the ordered segments of triple helical molecules. In a tentative way it can be supposed that this phosphate layer acts as an intermediary in the local recognition of the organic matrix and OCP crystal surface. The flexibility, provided either by the molecular disorder and side group mobility in the matrix or by the structural nature of the hydrated layer of OCP, probably contributes to the interfacial collagen–phosphate–OCP(100) plane recognition. This recognition might also be the reason why the formation of OCP is induced in gelatin xerogel, in contrast with the formation of HA in gelatin,[5] polyglycolide[9] and chitin[11] gels or in water by simple mixing of the inorganic solutions used in this study at pH greater than 7, even if the variations of pH and calcium and phosphate concentrations caused by different diffusivities can not be ignored.[4,12]

The templated nucleation of OCP is substantiated by the reproducibility of the ordered crystallization and by the three-dimensional orientation of the OCP crystals grown inside uniaxially deformed gelatin films. The {100} faces of OCP crystals interact with the gelatin layers by setting the *c* axes parallel to the molecular chains. This crystal orientation indicates a real ordered nucleation induced by the organic matrix. As has been pointed out by Addadi *et al.*,[13] chains of calcium and phosphate ions, developed along the *c* axis

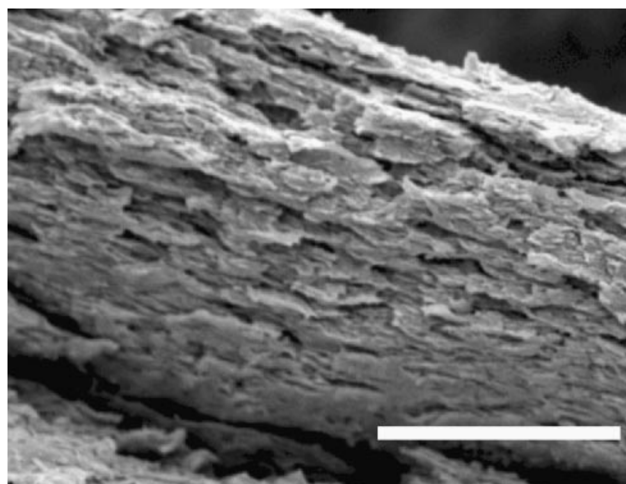

Fig. 4 SEM image of OCP deposition in a uniaxially deformed gelatin film. Scale bar 10 μm.

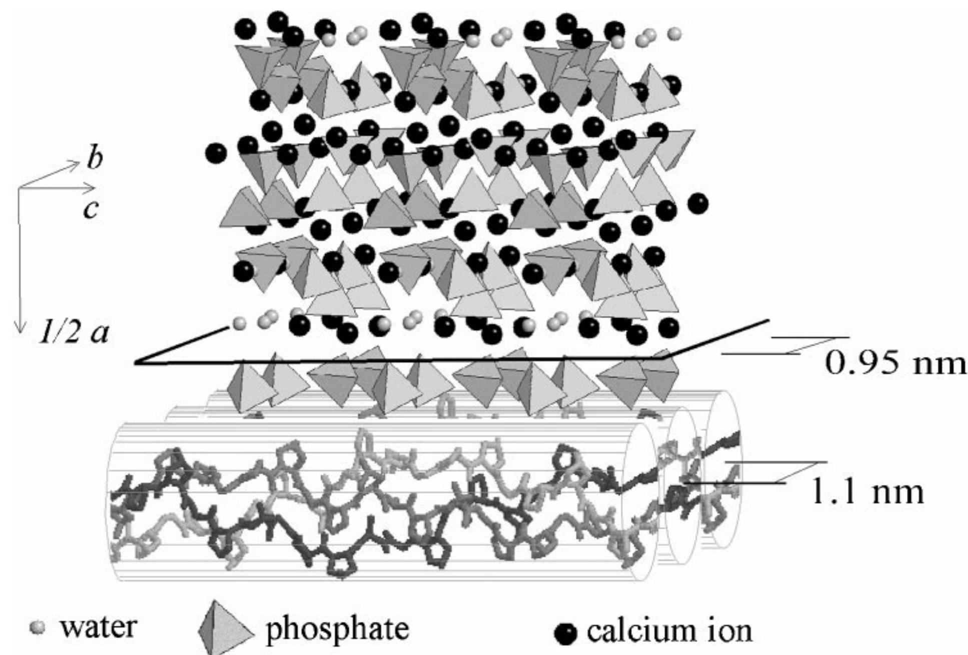

Fig. 5 Structural model for the ordered growth of OCP crystals on bundles of collagen molecules. The reported value of 1.1 nm corresponds to the spacing of the meridional reflection in air-dried samples. The highlighted hydrated OCP plane, on which the chains of calcium ions are separated by 0.953 nm, interacts with the chains of phosphate ions bonded to the collagen molecular chains in the oriented regions of the xerogel. This is probably the nucleation plane of the OCP crystals, which grow with the *c* axis parallel to the molecular axis of collagen and with the {100} faces parallel to the xerogel surface.

direction and separated by 0.953 nm, emerge at the surface of the hydrated OCP layer. This structural motif supports the model in Fig. 5 in which the oriented crystallization is supposed to be due to an ordered, even if not specific, interaction of the hydrated OCP layers with the phosphate ions bonded to the molecular chains separated by about 1.1 nm in the oriented regions of the xerogel. This should lead to an assembly of the plate-like crystals around the chain bundles with their *c* axes almost parallel to the direction of deformation, as shown by the SEM image in Fig. 4. The plate-like crystals grown on the phosphate side with the large {100} faces parallel to the sheets of gelatin films show no preferred orientation around the *a* axes.

When the sites of phosphate binding inside the xerogel are saturated, the active diffusion of anions from the phosphate to the calcium side gives rise on the calcium side (Fig. 3b) to the growth of plates with the characteristic OCP morphology[4,14] and almost perpendicular to the xerogel surface. So oriented crystals were not found in the uniaxially deformed xerogels probably because of the decreased ionic diffusion in the less swelled stretched samples.[8b]

It can be concluded that gelatin matrices represent an interesting medium to induce templated nucleation of OCP, which is considered an important precursor of biological HA deposition. Furthermore the role of the structure of the undeformed or uniaxially deformed xerogel in determining the architectural assembly of (100) plate crystals introduces a new route in the synthesis of organized crystalline materials for future application of organic–inorganic composites, as well as in the basic understanding of the physico-chemical processes which govern the oriented growth of mineral phases in structured substrates.

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