Preparation of Nanotextured and Nanofibrous Hydroxyapatite through Dicalcium Phosphate with Gelatin

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Organized nanostructures of hydroxyapatite (HAp) were produced using a novel preparation route through the topotactic transition of dicalcium phosphate dihydrate (DCPD) containing gelatin molecules. A nanoscale texture of dicalcium phosphate (DCP) was formed by the dehydration of DCPD prepared in gelatin gel containing phosphate ions. A three-dimensionally oriented framework of HAp consisting of ca. 20 nm grains was prepared by a rapid hydrolysis of the nanotextured DCP with a sodium hydroxide solution. Nanoscale HAp fibers elongated along the *c* axis with a diameter of ca. 50 nm were also obtained by a moderate hydrolysis with ammonia water. The hierarchical architectures of the nanostructured HAp were successfully achieved by the phase transition associated with the specific interaction of the organic molecules.

Introduction

Hydroxyapatite [HAp; $Ca_{10}(PO_4)_6(OH)_2$] is the main inorganic component of hard tissues in vertebrates, such as bone and teeth.[1−3] For practical purposes, HAp has been utilized for artificial bones,[4,5] scaffold for tissue engineering, and liquid chromatographic packing[6] because of its high bioactivity and absorbability for various ions and molecules. Therefore, the structural control of HAp is important for the improvement of its performance. Many synthesis methods, including precipitation,[7] hydrothermal processes,[8] hydrolysis of salts,[9−13] and sol−gel routes,[14,15] have already been reported for the preparation of HAp crystals. Recently, shape-controlled HAp nanocrystals were prepared by a coprecipitation reaction with soluble collagen.[16−18] These efforts have met with partial success in the preparation of a nanocomposite of HAp−collagen which is similar to the nanostructures of real bone. The effects of gelatin,[19,20] silk fibroin,[21,22] chitosan,[23,24] and chondroitin sulfate[25,26] were also studied on the controlled production of HAp crystals. The presence of poly(acrylic) acid,[27] amino acid,[28−30] and inorganic ions[31,32] has been shown to influence the crystal morphologies with adsorption on specific surfaces. The preparation of whiskerlike and fibrous HAp crystals has been achieved by various techniques, such as hydrothermal synthesis,[33,34] homogeneous precipitation,[35−37] and the hydrolysis of α-tricalcium phosphate (α-TCP).[38,39] Despite these previous efforts, the control of macroscopic forms consisting of HAp

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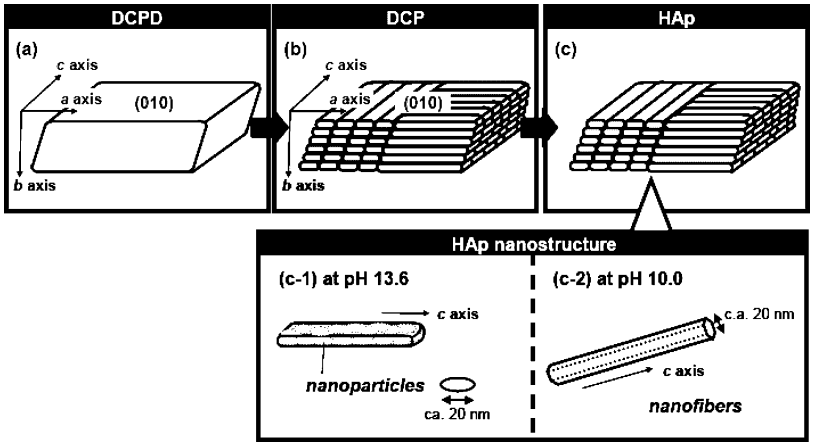

Figure 1. Schematic illustration of the overall process from precursor DCPD (a) to nanostructured HAp (c) through nanotextured DCP (b). Precursor DCPD (a) was prepared in gelatin gel containing phosphate ions. Nanotextured DCP (b) was obtained by the dehydration of precursor DCPD. Nanoscale structures of HAp consisting of textured particles (c-1) and fibers (c-2) were formed by hydrolysis at pH 13.6 and 10.0, respectively.

nanocrystals has been insufficient for mimicking real bone. A three-dimensionally oriented assembly of nanoscale units with a hierarchical architecture would be essential for improving the particular ability of artificial bone. Moreover, HAp crystals having a controlled macroscopic structure and a high specific surface area are also utilized for protein adsorbents, liquid chromatographic packing, and scaffold for tissue engineering.

It is widely known that crystal morphologies depend on the correlation between the driving force of crystallization and the diffusion of atoms, ions, molecules, or heat. Thus, a gel matrix has been used for the control of nucleation and morphology on aqueous solution-based crystal growth.[40−44] The formation and morphology of fluorapatite in gelatin were investigated.[45−47] In our previous study, hierarchically laminated calcium phosphate was produced through Liesegang periodic precipitation in a gel matrix of poly(acrylic acid) containing phosphate anions by diffusion of calcium cations.[48] In this work, we successfully produced nanostructured calcium phosphate from a precursor crystal of dicalcium phosphate dihydrate (DCPD, brushite, $CaHPO_4 \cdot H_2O$) prepared in gelatin gel. A hierarchical architecture consisting of HAp nanocrystals was achieved by a specific route from the precursor DCPD through dicalcium phosphate anhydrate (DCP, monetite, $CaHPO_4$). Figure 1 shows a schematic illustration of the procedure for the preparation of nanotextured and nanofibrous HAp crystals from precursor DCPD grown in a gelatin matrix through nanotextured DCP. The structural modification of octacalcium phosphate and TCP was achieved by the addition of gelatin,[49] proteins,[50] and poly(acrylic) acid.[51,52] However, nanostructured HAp has not been obtained by the phase transition of the precursor crystals. Because DCPD and HAp are similar in their ionic configuration of the calcium cation on the (010) face, the structural control of DCPD and DCP crystals with organic molecules would be important as a precursor of nanostructured HAp. Here, we synthesized DCP crystals having a three-dimensionally organized nanostructure from DCPD containing gelatin molecules. Subsequently, nanotextured and nanofibrous HAp crystals were prepared by the transition of nanostructured DCP crystals without deformation of the macroscopic shapes. A new family of HAp structures described here would be applied to a wide variety of biomedical applications because of their hierarchical architecture with a high specific surface area. Furthermore, this new preparation route through the topotactic transition of the precursor crystals is informative for the science and technology of materials processing.

Experimental Section

A stock solution containing 1.1 mol/dm^3 of ammonium dihydrogen phosphate ($NH_4H_2PO_4$, Kanto Chemical, 99.0%) was prepared by using purified water at room temperature. We dissolved 5.0 g of gelatin powder (Kanto Chemical) in 50 cm^3 of the stock solution at 70 °C. Then, 20 cm^3 of the gelatin solid containing phosphate ions was poured into a 50-cm^3 polypropylene vessel and cooled at 4 °C for 6 h to promote gelation. The same volume of a 2.7 mol/dm^3 calcium nitrate tetrahydrate [$Ca(NO_3)_2 \cdot 4H_2O$, Junsei Chemical, 98.0%] aqueous solution adjusted to the physiological pH of 7.5 with α,α,α-tris(hydroxymethyl) methylamine-HCl was loaded onto the gel in the vessel at 25 °C. A white powder of DCPD was formed in the gelatin matrix with the diffusion of calcium ions. The particles were precipitated on the bottom of the reaction vessel after the gelatin gel changed into a solid. Opaque crystals of DCP were obtained from the precipitates of DCPD by drying in air at 60 °C. HAp was prepared by the hydrolysis of the resultant DCP

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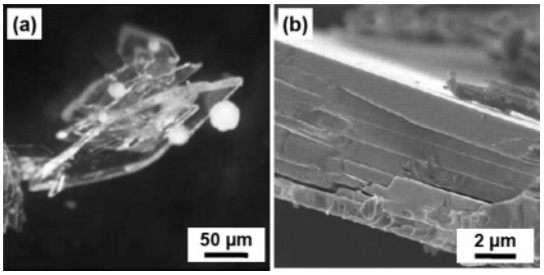

Figure 2. Appearance of precursor crystals of DCPD prepared in agar gel; an optical micrograph (a) and a SEM image (b).

crystals using a 10 mass% NaOH aqueous solution (pH 13.6) under stirring at 95 °C for 5 min or an NH_4OH solution (pH 10.0) at 95 °C for 2 h. The final products were washed with purified water and dried under ambient conditions.

X-ray diffraction (XRD) patterns were recorded on a Rigaku RAD-C system with Cu Kα radiation. The morphology of the particles was observed with a field-emission scanning electron microscope (SEM; Hitachi S-4700) and a field-emission transmission electron microscope (TEM; Philips TECNAI F20). Fourier transform infrared (FTIR) spectra and thermogravimetry were performed with a BIO-RAD FTS-60A and a Seiko Instruments TG/DTA6200, respectively. The specific surface area was calculated by the Brunauer−Emmett−Teller methods using nitrogen adsorption isotherms obtained at 77 K with a Micromeritics TriStar 3000.

Results and Discussion

Precursor crystals were prepared in gelatin gel containing phosphate ions by the diffusion of calcium ions from a $Ca(NO_3)_2$ solution loaded on the gel. Clear and platy crystals 1−2 mm in width were slowly grown at the top of the gel matrix with the introduction of calcium ions (Figure 2). Since the gel network of gelatin molecules was weakened by the penetration of calcium ions, the grown crystals were then precipitated on the bottom of the reaction vessel. Because the diffraction peaks of the (020) and (040) planes of DCPD were mainly observed for as-grown crystals (Figure 3a), DCPD was produced by the reaction in the gel. The top face was assigned to be the (010) plane because platy crystals were easily arranged parallel to the surface of the holder for the XRD measurement. A weight loss estimated by thermogravimetry indicated that the DCPD crystals contained ca. 2 mass% of organic compounds. The absorption peak at 1380 cm^{-1} in the FTIR spectra was associated with the interaction of Ca^{2+} and COO^- of the gelatin (Figure 4a−c). A slight shift of the (020) peak to a lower angle was observed in the XRD pattern, although other peaks were not changed from the standard. These results indicate that the gelatin molecules in the DCPD crystals were incorporated in a hydrated water layer parallel to the (010) plane.

We found that the transition of DCPD prepared in the gelatin gel to DCP instantly proceeded at 60 °C in air, whereas dehydration of DCPD is usually observed at 115−220 °C. When the gelatin covering the platy crystals was removed by washing with purified water, heating at a relatively high temperature above 200 °C was required for the phase transition. The dehydration of DCPD was suggested to be precipitated through instabilities in the chemical environment

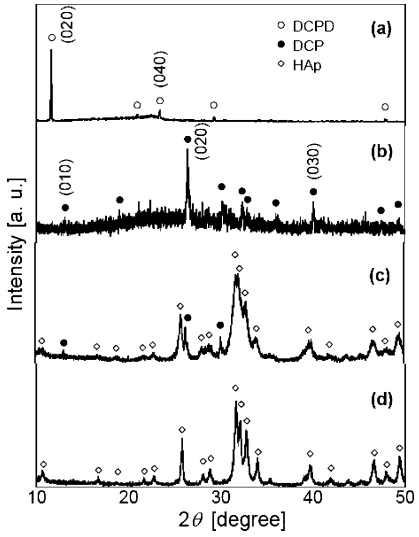

Figure 3. XRD patterns for crystals as-deposited (a), dried at 60 °C (b), dried and treated for 5 min in $NaOH_{aq}$ at 95 °C (c), and dried and treated for 2 h in NH_4OH_{aq} at 95 °C (d). Whereas a small amount of DCP remained after a rapid hydrolysis with NaOH, the signals of DCP disappeared with a prolonged treatment.

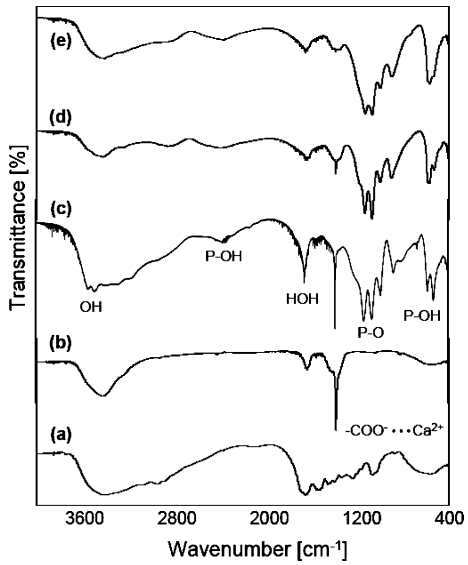

Figure 4. FTIR spectra for gelatin (a), a mixture of gelatin and calcium ions (b), as-deposited precursor crystals (c), dried crystals at 60 °C (d), and commercial DCPD (e). The absorption peak at 1380 cm^{-1} was not observed for pure gelatin and DCPD.

of the water molecule.[53] Thus, the presence of gelatin, which is a highly water-absorbing molecule, promoted the dehydration of DCPD even at 60 °C. Concurrently with the crystal phase transition, the products became opaque and exhibited a porous structure, as shown in Figure 5a and b, although the macroscopic platy form was hardly changed. Since the XRD pattern (Figure 3b) indicated that the main face of the plates was also identified to be the (010) face of DCP, the topotactic transition, in which the crystal lattice of the product phase shows one or more crystallographically equivalent and orientational relationships to the crystal lattice of the parent phase, occurred with the dehydration of the precursor crystal of DCPD. However, the crystallographic orientation of the resultant DCP was partially different from that of the parent

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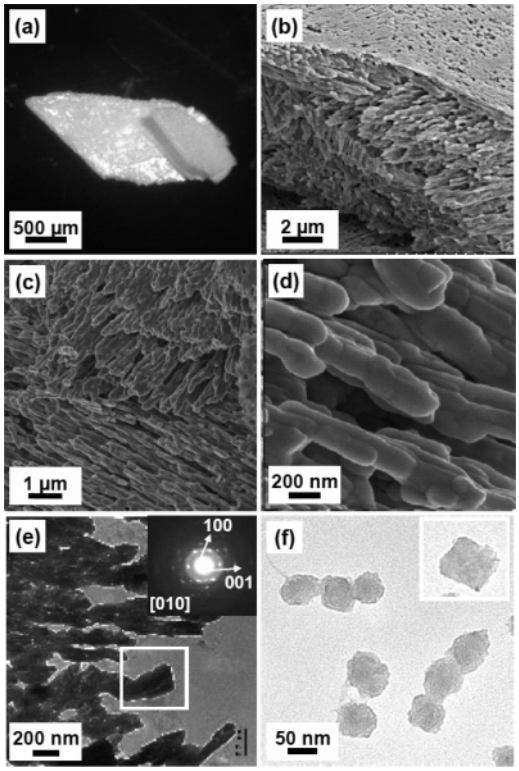

Figure 5. Appearance of crystals dried at 60 °C; an optical micrograph (a), SEM images (b, c, and d), and TEM images (e and f). The inset of e is an electron diffraction pattern for the rectangle area.

phase. Thus, the topotactic transition from DCPD into DCP was imperfect in this reaction system.

In contrast to the similarity of the macroscopic morphology, the microstructure of the products was drastically changed with the transition. A laminated lattice architecture was found to be an attachment of rectangular units with a 2−5 μm length and 100−200 nm width (Figure 5b−e). TEM images show that the nanoscale building blocks with a size of ca. 50 nm made up the rectangular units (Figure 5f). However, the spot pattern of electron diffraction assignable to a single-crystalline DCP shows that the lattice of the rectangular units was elongated to [100] and [001] directions (inset of Figure 5e). The angle of the lattice (ca. 104°) is associated with the unit structure of the DCP crystal. Broadening of the FTIR peak assigned to the carboxy groups (COO^-) of gelatin suggests the change of the status of gelatin molecules with the phase transition from DCPD to DCP (Figure 4c and d). Thus, the textured architecture is deduced to be composed of iso-oriented nanocrystals covered with gelatin. Commercially available crystals of DCPD (Junsei Chemical) were transformed into DCP by heating at 200 °C in air. However, a porous body or a lattice structure was not formed, whereas cracks parallel to the (010) face were observed as a result of the shrinkage by removal of the water layers of DCPD. No phase transition at 60 °C was observed when the gelatin was simply mixed with the commercial DCPD powder. Thus, the presence of gelatin inside the DCPD crystal was essential for the promotion of the transition into the hierarchically porous structure of DCP. Because the porous structure was formed by the volume change on dehydration, the porosity of the lattice structure of DCP was theoretically calculated to be 37.3% from the density of DCPD (2.328 g cm^{-3}) and DCP (2.929 g cm^{-3}). Recently, Cölfen et al. reported that polymer-mediated calcite consists of three-dimensionally, well-aligned nanocrystals that are scaffolded to a so-called mesocrystal.[14−16] The nanotextured DCP consisting of iso-oriented nanocrystals may be classified into a family of mesocrystals. In this case, however, the lattice morphology was produced through the topotactic transition of single-crystalline DCPD. Thus, the iso-oriented structure would not be ascribed to the oriented attachment of nanoscale units directed by the adsorbed polymers, as suggested for the formation of mesocrystals.

Gelatin molecules played a dual role in the construction of the lattice architecture of DCP. Initially, gelatin gel provided a matrix for the formation of gelatin-incorporated precursor crystals of DCPD. Since the gel matrix suppressed the rapid mixing of Ca^{2+} and PO_4^{3-}, the crystal growth of DCPD gradually proceeded with the inclusion of gelatin molecules. The results of the thermogravimetry and FTIR spectra supported the specific interaction between DCPD and the gelatin molecules. The gelatin molecules incorporated with DCPD played another role in the generation of a hierarchical architecture. The phase transition from DCPD to DCP at 60 °C is assisted with the dehydration promoted by the gelatin molecules. At the same time, the specific interaction of the gelatin molecules with the (100) and (001) faces restricted the crystal growth during the phase transition and produced the lattice architectures with rectangular units. The negatively charged carboxy groups (COO^-) of the gelatin molecules would show a particular interaction with the specific faces. The oriented attachment of the nanoscale building blocks of DCP covered with gelatin resulted in a rectangular unit mainly exhibiting a (010) face. Therefore, the dual role of gelatin molecules effectively leads to the emergence of DCP hierarchical architecture. Calcium carbonate was prepared by the same procedure using a gel of poly(acrylic acid) having carboxy groups.[48] In this case, however, large platy crystals of precursor DCPD were not obtained because the gel network was firmly maintained after the diffusion of calcium ions. Consequently, the transformation of gelatin gel into a solid with the crystal growth of DCPD was also essential for the production of the lattice architecture of DCP.

Nanotextured DCP was transformed to HAp with hydrolysis in alkali solutions at 95 °C (Figure 3c and d). The structure of HAp strongly depended on the condition of hydrolysis. When HAp was synthesized at 95 °C for 5 min at high pH (pH 13.6) in a NaOH solution, the macroscopic morphology and the microscopic lattice structures consisting of small needlelike crystalites remained (Figure 6a−c). Furthermore, the needlelike crystals were composed of nanoparticles with an average size of 20 nm (Figure 6d). Because the *c* axis of HAp was parallel to the surface of the plate, the topotactic transition from DCP to HAp was achieved. On the other hand, hydrolysis at a low pH (pH 10.0) using an NH_4OH solution for 2 h produced aggregates of nanofibrous HAp without deformation of the macroscopic platy morphology (Figure 7). The average diameter of the hexagonal fibers was estimated to be ca. 50 nm. The hexagonal habit indicates that the HAp fibers were elongated

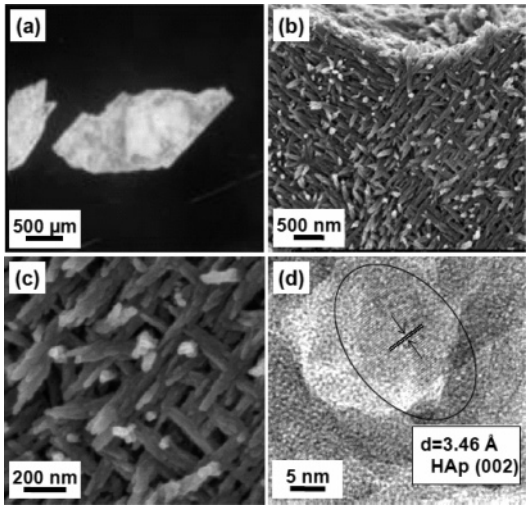

Figure 6. Appearance of crystals dried at 60 °C and treated in $NaOH_{aq}$ at 95 °C; an optical micrograph (a), SEM images (b and c), and a TEM image (d).

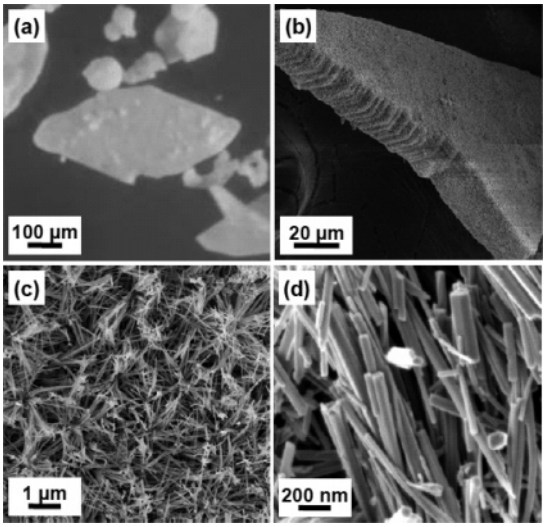

Figure 7. Appearance of crystals dried at 60 °C and treated in NH_4OH_{aq} at 95 °C; an optical micrograph (a) and SEM images (b, c, and d).

along the *c* axis, whereas the direction of the fibers was random in the aggregate. Gelatin was completely removed with the hydrolysis process in the alkali solutions. The specific surface area was estimated to be ca. 70 and 30 m^2/g for nanotextured and nanofibrous HAp, respectively. The lower value for the fibrous HAp was consistent with the further crystal growth under a moderate reaction condition. Since the specific surface area of powdery HAp originating from natural bone was 80−100 m^2/g,[54] the granularity of the nanotextured HAp appears to be similar to that of the biomineral HAp. The overall process from precursor DCPD to nanostructured HAp was summarized in Figure 1.

The transition of DCPD and DCP to HAp with hydrolysis proceeds in an alkali solution as expressed in eqs 1 and 2

$$5CaHPO_4 \cdot 2H_2O \rightarrow Ca_5(PO_4)_3OH + 2H_3PO_4 + H_2O \quad (1)$$

$$5CaHPO_4 + H_2O \rightarrow Ca_5(PO_4)_3OH + 2H_3PO_4 \quad (2)$$

Recently, thick plates consisting of *c*-axis-oriented HAp nanoneedles were prepared from commercially available platy DCPD crystals under a high pH condition.[11] We confirmed that nanoscale HAp needles perpendicular to the surface were formed through the nucleation at the surface of commercial DCPD plates. We also found that hydrolysis with a moderate condition at a relatively low pH (pH 10.0) produced aggregates of thin plates of HAp in the platy mother crystal. However, a hierarchical architecture consisting of nanostructured HAp was not obtained from the commercially available DCPD. Consequently, the specific interaction between gelatin molecules and DCPD/DCP crystals is essential for the preparation of nanotextured and nanofibrous HAp. A rapid hydrolysis at a high pH induced the topotactic transition of DCP to HAp without a drastic variation of the nanostructure. The nanotextured architecture of the resultant HAp was inherited from the lattice structure of the parent crystal because the size of the unit crystals and the angle of the lattice (ca. 104°) were not changed with the phase transition. On the other hand, the lattice changed into a fibrous structure of HAp with a moderate hydrolysis under low pH conditions. The gradual transition promoted the crystal growth with elongation of the *c* axis of the hexagonal HAp structure. In this case, the width of the HAp fibers was influenced by the size of the rectangular units of the parent DCP crystals.

The HAp crystal has two types of crystal planes with different charges: positive on *a* planes and negative on *c* planes. Thus, novel properties could be produced by controlling the orientation of the crystal planes achieved by modifying the morphology of HAp crystals. For example, it is expected that HAp fibers elongated along the *c* axis exhibit a high specificity of adsorption to a negatively charged acidic protein due to the wide surface of the *a* plane. Fibrous HAp has already been prepared using the hydrothermal synthesis and homogeneous precipitation method.[33−39] However, the diameter of the fibers was larger than several hundred nanometers and the specific surface area was insufficient for the adsorbents. The nanotextured and nanofibrous HAp crystals prepared in this study exhibited a hierarchical architecture, providing a high specific surface area with a controlled crystal face and macroscopic pores. The fibrous structure is also effective for the interaction with organic compounds. Thus, the novel types of nanostructured HAp would be utilized as a functional material in a wide variety of applications, including protein adsorbents, artificial bones, liquid chromatographic packing, and scaffold for tissue engineering.

Conclusions

This study provided a new preparation route through the topotactic transition of precursor crystals for the formation of a hierarchical architecture. Novel types of nanotextured and nanofibrous HAp were successfully produced through the topotactic transition of DCPD containing gelatin molecules. Nanotextured HAp with an oriented framework of ca. 20 nm grains and nanofibrous HAp elongated along the *c* axis with a diameter of ca. 50 nm were formed from nanostructured DCP prepared by dehydration through the specific interaction with gelatin molecules. The hierarchical architectures of the nanostructured HAp providing a high

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specific surface and macroscopic pores would be applicable for various biomedical applications.

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