Hierarchical Self-Assembly of Nano-Fibrils in Mineralized Collagen

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A designed hierarchical structure was made by self-assembly of nano-fibrils of mineralized collagen resembling extracellular matrix. The collagen fibrils were formed by self-assembly of collagen triple helices. Hydroxyapatite (HA) crystals grew on the surface of these fibrils in such a way that their c-axes were oriented along the longitudinal axes of the fibrils. The mineralized collagen fibrils aligned parallel to each other to form mineralized collagen fibers. For the first time, the new hierarchical self-assembly structure of collagen−hydroxyapatite composite was verified by conventional and high-resolution transmission electron microscopy.

1. Introduction

Hierarchical assembly of nano-fibrils is ubiquitous in nature, such as in bone,[1] muscle,[2] intestine[3], etc. The hierarchical structure enables these tissues to play special roles in achieving different functions. For example, natural bone is of a complex nano-fibril system with an intricate hierarchical structure of mineralized collagen.[1] The assembly includes an orderly deposition of hydroxyapatite (HA) minerals within type I collagen matrix. The crystallographic *c*-axis of the hydroxyapatite is oriented parallel to the longitudinal axis of the collagen fibril.[4−6] Investigation and simulation of the hierarchical nano-fibril structure in nature can offer some new ideas in the design and fabrication of new functional materials,[7] such as tissue-engineering scaffold materials and biomimetic engineering materials. We selected the nano-fibrils of mineralized collagen as a self-assembly model system to search the possibility of synthesizing materials with such hierarchical structure.

The HA−collagen composite[8−10] synthesized by biomimetic strategy shows great promise in clinical application because of its compositional (and some structural) analogy to natural bone. A key step in the composite synthesis is control of nucleation and growth of calcium phosphate minerals by collagen matrix in aqueous media.[11,12] However, preparing any material with hierarchical structure of nano-fibril that resembles bone is challenging in that it involves dissimilar organic and inorganic nanophases, which have specific spatial relations with respect to one another.

Many groups have investigated the controlled nucleation and growth of crystals from organic templates in vitro such as poly(lactic acid),[13] reconstituted collagen,[14,15] peptide-amphiphile,[16] and many others. These studies suggest that the anionic groups on the surface of organic templates are nucleation sites of crystals. The anionic groups exposed in solution tend to concentrate the inorganic cations resulting in local supersaturation followed by nucleation of crystals. And some studies show a similar correlation between the crystallographic orientation of hydroxyapatite and the organic scaffold. However, to the best of our knowledge, hierarchical assembly of nano-fibrils of mineralized collagen that resembles the composition and structure of natural bone has never been available.

Here we report fabrication of a composite with hierarchical structure by self-assembly of nano-fibrils of mineralized collagen and show the ultrastructural association between collagen fibrils and mineral crystals by high-resolution transmission electron microscopy (HRTEM) images. The HRTEM images reveal more details on the mineral interaction with the surface of collagen fibrils and crystallographic *c*-axis preferential alignment with the collagen fibrils longitudinal axes in

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the system. Such results are crucial to understanding the possible mechanisms for collagen-mediated mineralization in calcified tissues in general.

2. Experimental Section

Chemical Agent. Acid-soluble collagen type I was obtained from rat-tail tendon using a protocol identical to that described by Pins et al.[17] The rat-tail tendon was dissolved in 10 mM HCl at room temperature for 7 h. The solution was centrifuged at 15 000 rpm at 4 °C for 45 min and then filtered. NaCl (0.7 M) was added to induce precipitation, and then the precipitate was collected by centrifugation (15 000 rpm, 4 °C, 30 min) and redissolved in 10 mM HCl. The acid-soluble mixture was gathered by dialysis against an aqueous phosphate buffer (20 mM disodium hydrogen phosphate, pH 7.4) at 4 °C for 12 h. The precipitated collagen was collected by centrifugation (15 000 rpm, 4 °C, 45 min), and then dissolved into a concentrated solution by dialyzing the pellets against a large volume of 10 mM HCl at room temperature for 12 h. The collagen solution was stored at 4 °C. $CaCl_2$, NaH_2PO_4, NaCl, and NaOH were analysis grade. As a solvent, deionized water was used.

Procedure. The experimental method follows a processing route described by Bradt et al.,[15] but using a different buffer solution. Collagen was diluted at a concentration of 0.6 mg mL^{-1} by 10 mM hydrochloric acid at 4 °C. $CaCl_2$ solution (1.4 mL 0.1 M) was added into 10 mL of collagen solution and maintained for 10 min after mixture. NaH_2PO_4 solution (0.84 mL 0.1 M) was added and the pH was adjusted to 7.0 by 0.1 M NaOH solution. When the pH exceeded about 6.0, the solution became supersaturated and calcium phosphate started to precipitate with collagen. The solution was maintained at pH 7.0 for 1 h, after which the composite was harvested by centrifugation at 5000 rpm and suspended in deionized water to remove the salts. The centrifugation and suspension cycle was repeated 3×. After the last suspension the sample was freeze-dried. The precipitate was ground into fine powder for later examination.

3. Characterization

X-ray Diffraction. To investigate the structure and crystallinity of the samples, the powder was analyzed with an X-ray powder diffractometer, XRD (D/max-rA Rigaku diffractometer, Cu Kα radiation (λ = 0.15418 nm), Japan). The sample was scanned from 10° to 60° with a scan speed of 4°/min.

Infrared Spectroscopy. The sample was mixed with KBr in the mass ratio of 1 to 20. Infrared spectra were taken using a Perkin-Elmer system 2000 Fourier transform IR (FTIR) spectrometer in the range of 4000−400 cm^{-1}.

Scanning Electron Microscopy. Sample morphology and microstructure were examined by scanning electron microscopy (SEM). The samples were sputter-coated with a layer of gold about 10 nm thick for SEM (JSM-6301F) observations at 5 kV.

Transmission Electron Microscopy. To prepare a speciman for TEM, the powder was embedded in Spurr resin (SPI Supplies Division of Structure Probe, Inc.) for 12 h at 60 °C. Ultrathin sections no more than 50 nm thick were prepared using the ultracut (Leika ultracut UCT) technique and then transferred onto Formvar-coated (4% (w/v)) copper grids. The ultrathin sections were investigated using the JEOL 200CX instrument operated at 200 kV for conventional TEM examinations. The calcium phosphate crystals formed on the collagen fibers were assessed using selected area electron diffraction. The camera length of TEM analyses was 700 mm. HRTEM observation was carried out with a JEOL 2010F instrument operated in a transmission mode at 200 kV.

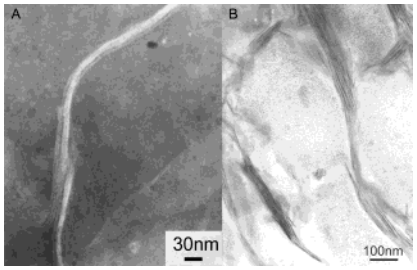

Figure 1. (A) Negative-stain (phosphotungstic acid) TEM of unmineralized self-assembled collagen fibrils reveals the diameter of the fibrils to be about 4.0 nm. (B) TEM micrographs of the unstained mineralized collagen fibrils reveals the diameter of the mineralized collagen fibrils to be 5.5−6.9 nm.

4. Results

Transmission Electron Microscopy. There are two possible ways to induce the formation of fibrils from monomeric collagen solutions.[18] One is to change the pH of a solution of collagen molecules at a certain temperature and ion strength, and a rigid, three-dimensional gel network will be formed. The other method involves changing the temperature of the neutral collagen solution from 4 °C to 25−33 °C. Figure 1A shows a TEM image in which typical collagen fibrils were formed under conditions of maximal lengthwise growth and minimal side-by-side aggregation. This mode of aggregation creates networks of exceedingly thin filaments that have an average diameter around 4.0 nm. The thin section was negatively stained using phosphotungstic acid.

In the collagen solution containing calcium and phosphate ions, collagen and calcium phosphate can self-assemble into a new composite when the pH of the solution is changed. The method mimics the physiology circumstance in biomineralization process in vivo.

Figure 1B is the TEM image of the composite prepared at the condition mentioned above. The composite consisted of an intertwined assembly of collagen fibrils bundles more than 1 μm long (Figure 1B). Each collagen fibril was surrounded by a layer of HA nanocrystals grown on the surface of the collagen fibrils. The sections for TEM examination were not fixed, and were not positively or negatively stained. The higher electron density in the TEM images corresponds to the HA crystal and the lower density corresponds to the collagen fibrils. As can be seen in the TEM image under high magnification (Figure 2), each mineralized bundle of collagen fibrils is 5.5−6.9 nm in diameter, which is thicker than that of the self-assembled collagen fibrils. On the basis of the above data, the self-assembled collagen nanofibrils act as the template of HA precipitation, and the thickness of HA crystal layer on the surface of the collagen fibrils was calculated to be 0.75−1.45 nm.

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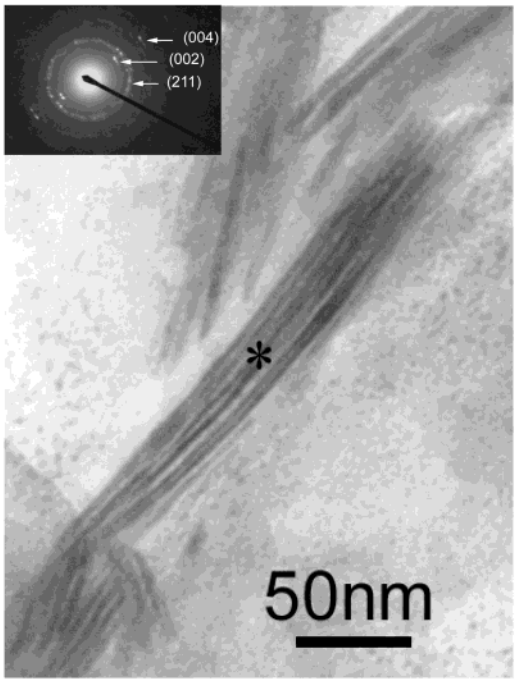

Figure 2. Higher magnification of the mineralized collagen fibrils. Insert is the selected area electron diffraction pattern of the mineralized collagen fibrils. The asterisk is the center of the area and the diameter of the area is about 200 nm.

To discern the relative orientation of the HA crystals with respect to collagen fibrils, the samples in which the isolated mineralized collagen fibrils could be observed were analyzed by electron diffraction. In all cases, the preferential alignment of the HA crystallographic *c*-axis with the collagen fibrils longitudinal axis was observed (Figure 2).

In the electron diffraction pattern, the ring-shaped diffraction was ascribed to 211 of HA. Because the two pairs of arc-shaped diffractions were respectively ascribed to 002 and 004 of HA, the *c*-axis of HA nanocrystals aligned from above-right to bottom-left, probably along collagen fibrils. The *c*-axes of nanocrystals were estimated from the width of the arc to be scattered with an angle of less than 30°. This *c*-axis alignment of HA nanocrystals is quite similar to the nanostructure observed for bone, indicating that the self-assembly of HA and collagen produced by osteoblasts in vivo could also be possible in vitro. The mineralization of collagen fibers in vivo is known to initiate at the hole zones between collagen molecules. It gradually proceeds to the overlap zone of collagen fibrils, and finally HA crystals are deposited along the periphery of the collagen fibrils with a preferred orientation of their *c*-axis parallel to the longitudinal direction of collagen fiber. Therefore, the 002 and 004 diffractions of the HA crystals show the arching behavior[19] found in bone because of the same preferential orientation of HA crystals.

High-Resolution Transmission Electron Microscopy. To study the relationship between the newly formed crystals and the collagen fibrils directly, HRTEM analyses at the lattice plane level were performed. The HA crystals associated with the surface of the collagen fibrils were also observed in Figure 3. The HRTEM

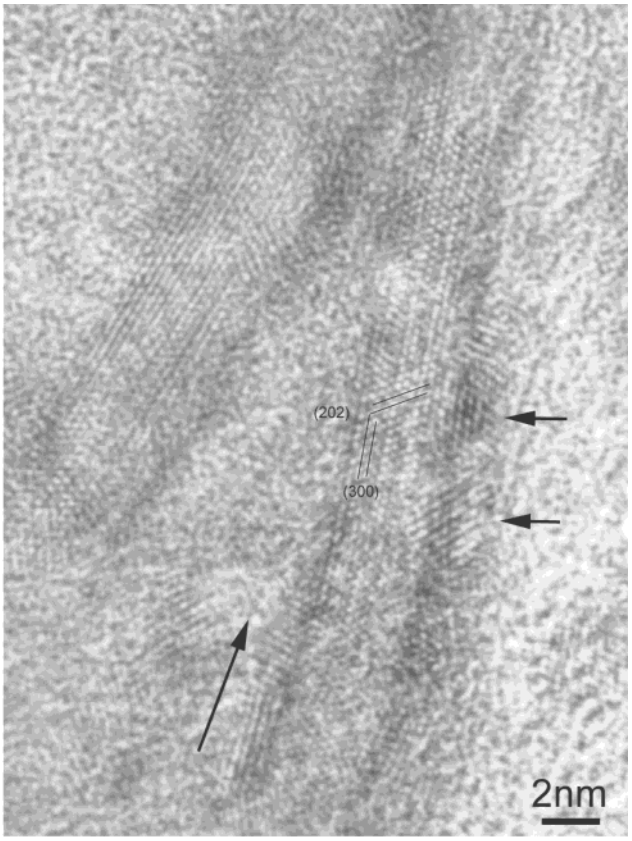

Figure 3. HRTEM image of mineralized collagen fibrils. Long arrow indicates the longitude direction of collagen fibril. Two short arrows indicate two HA nanocrystals.

analysis of the parallel-aligned mineralized collagen fibrils revealed that, not only on the side area of the collagen fibrils, but also in the middle area, crystal lattice is shown, and the electron density on the surface of the collagen fibrils is higher than that of the middle area. These findings indicate that the HA crystals grown on the surface of the collagen surround the fibrils.

The HRTEM image of the HA crystal located on the collagen surface reveals an apatite lattice plane of 0.27 nm, corresponding to the 300 apatite lattice plane, and a plane of 0.26 nm, corresponding to 202 (Figure 3), and a measured angle between them of 49.8° corresponding to the calculated angle of 51.8° in synthetic HA. The HA crystal is ascribed to 010 zone axis. Therefore, the *c*-axis of the HA crystal aligned parallel to the longitudinal direction of the collagen fibrils (which is also parallel to the long arrow direction in Figure 3). These results agree with the electron diffraction. On the other hand, the HA crystals layer on the collagen fibril surface is not uniform. Nanocrystals about 2−4 nm (short arrow in Figure 3), amorphous crystals, and crystal defects were observed with HRTEM. These observations may be explained by the surface charge property of type I collagen. In fact, there are several kinds of negatively charged, positively charged, and polar but uncharged, residue groups in collagen molecules. Among these functional groups, the carboxylate groups of collagen can critically affect the nucleation of the HA crystals on collagen membrane through chemical interaction.[20] The

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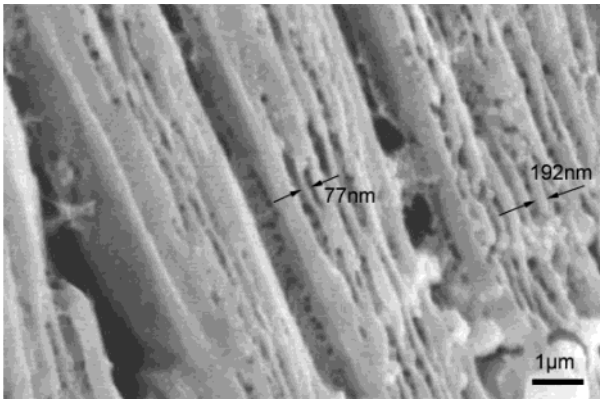

Figure 4. Scanning electron micrograph of mineralized collagen fibrils.

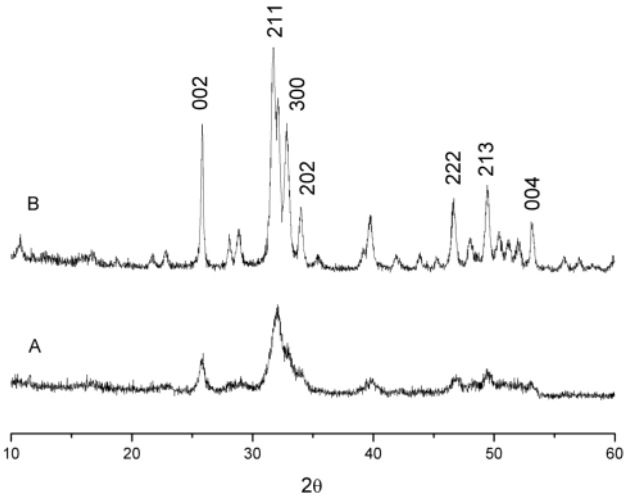

Figure 5. X-ray diffraction pattern of mineralized collagen fibrils (A) and commercially purchased HA (B).

possibility that the other charged or polar groups participate in the nucleation of HA crystals cannot be excluded. After nucleation, the crystal growth continues to cover the surface of the collagen fibrils.

Scanning Electron Microscopy. The mineralized collagen is a three-dimensional network of collagen fibrils on which crystals of calcium phosphate have settled. A scanning electron micrograph of the sample is shown in Figure 4. The mineralized collagen fibrils are aligned parallel to their longitude axes. The diameters of the mineralized collagen fibrils ranged from 77 to 192 nm. The calcium phosphate nanocrsytals can be seen to aggregate on the surface of collagen fibrils. This observation is in agreement with the TEM results.

Crystallographic Structure. The crystallographic structure of the calcium phosphate was studied with IR spectroscopy and X-ray diffraction. Figure 5 shows the X-ray diffraction pattern of the mineralized collagen and commercially purchased HA powder with good crystallinity. The inorganic phase in the mineralized collagen fibrils was determined as apatite calcium phosphate and no peaks from other Ca−P materials were present in the XRD pattern. The results are consistent with the electron diffraction results. The intensities of the peaks also proportionately correspond to the values of pure HA. There is no texture because the mineral in the mineralized collagen has random orientation in the powder during XRD examination.

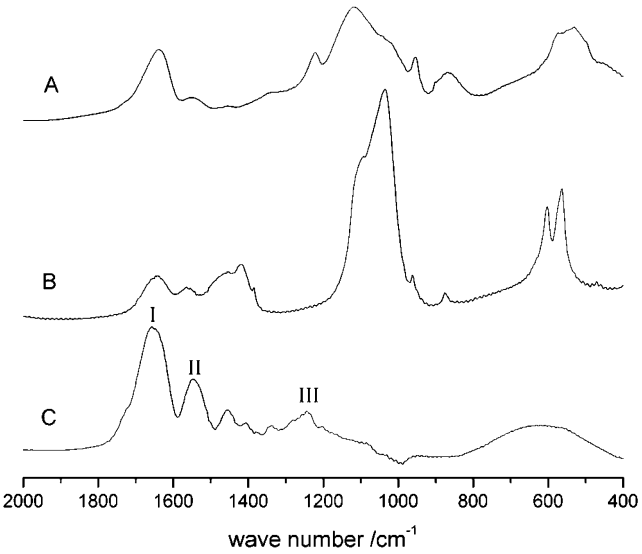

Figure 6. IR spectra of mineralized collagen fibrils (A), precipitated HA (B), and reconstituted collagen fibrils (C).

Table 1. Peak Positions of the Phosphate Bands in the IR Spectra of Precipitated Calcium Phosphate and Mineralized Fibrils Compared with Values from the Literature for Hydroxyapatite and Octacalcium Phosphate (OCP)[a]

calcium phosphate	mineralized fibrils	HAP[b]	assignment
562	568	571	ν_4
601		602	ν_4
		632	OH vibrational mode
961	950	962	ν_1
1031	1029	1050	ν_3
			ν_3
			ν_3
			ν_3
1089	1113	1089	ν_3

[a] The wave number in cm^{-1} is given. [b] HAP values are from ref 30.

The IR spectra of mineralized fibrils, calcium phosphate precipitated under the same conditions in the absence of collagen, and reconstituted collagen fibrils are shown in Figure 6. The IR spectrum of the mineralized fibrils appears as a superposition of the spectra of the collagen fibrils and the calcium phosphate. The mineralized collagen fibrils and the precipitated calcium phosphate show the typical peaks of the phosphate bands in hydroxyapatite. The peak positions of these bands are reported in Table 1. The positons of the peaks are nearly identical for the mineralized fibrils and the precipitated calcium phosphate but differ slightly from the values for pure hydroxyapatite.[21]

Weak bands of carbonate appear at 1417 and 870 cm^{-1} in the spectrum of precipitated calcium phosphate and the spectrum of mineralized collagen fibrils. It is reasonable that the crystals of the mineralized collagen fibrils contain carbonate,[22] as CO_3^{2-} is probably incorporated into the solution from the air during mineral precipitation.

The amide I peak, predominantly corresponding to the C═O stretch, observed at 1657 cm^{-1} of reconstituted

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collagen fibrils, shifted to 1651 cm^{-1} of mineralized collagen fibrils.The red shift of the amide I peak indicates that the C═O bonds in the peptide chain were weakened because of the formation of new chelate bonds between Ca ions and C═O bonds.[23] This indication implies that the carbonyl groups on the surface of collagen molecules are the nucleation sites of calcium phosphate. The intensity of the amide I, II, and III peaks of mineralized collagen fibrils decreased so severely that the amide II and III peaks almost disappeared. The calcium phosphate crystals grow on the surface of collagen fibers and wrap the nucleation site groups entirely or partly, which leads to a decrease of the intensity of amide I, II, and III.

5. Discussion

Mineral nucleation and growth in association with collagen is an important aspect of vertebrate calcification. Nylen et al.[24] first made the suggestion for surface-mediated mineralization on collagen in a study of tendon calcification, and subsequently the concept has been mentioned in other work.[25−27] None of these previous studies, however, provide direct and unequivocal data for surface events. In this study, the TEM and HRTEM results demonstrate directly for the first time that crystals are associated with collagen fibril surfaces. The most compelling data in this regard are those obtained from HRTEM observation of the mineralized collagen fibrils. One dichotomous mineralized fiber was selected as a study subject (Figure 3). The angle between the two branches of fibrils is about 15°. In the selected area, HA crystal lattice was observed on both fibrils surface. The phenomenon that electron density on the surface of the collagen fibrils is higher than that of the middle area can be more clearly observed on both collagen fibrils. Here the crystals appear to be disposed along contours that are interpreted as the cylindrically shaped surfaces of collagen fibrils. In fact, if the fibrils were almost parallel to each other, HA crystal might be supposed to be plate-shaped under lower magnification.

It may be noted that the TEM and HRTEM results of the mineralized collagen fibrils have been obtained with unstained samples so that collagen fibrils themselves are not immediately apparent on microscopy. The stain treatment can increase the contrast of organic components in specimens, which can make a more precise correlation of putative surface mineral deposition and fibril structure. But at the same time it may introduce artifacts in the small, newly forming crystals. Other studies, such as using immunocytochemical approaches and atomic force microscopy to minimize potential artifacts in mineral crystals, are ongoing in this laboratory.

The microscopy on the final product alone cannot establish the sequence of the reactions. The experiments of turbidity measurement by using a photometer, as described by Bradt et al.,[15] were performed to monitor the kinetics of the mineralization process. The turbidity of the reaction solution changed during the process and remained constant after about 1 h, which indicates that the process was terminated. Here we presume that the formation of collagen fibrils and precipitation of calcium phosphate crystals initiate simultaneously based on the turbidity monitoring of the reaction solution (data not shown) and the result of microscopy on the final product.

Summarized results of this study are depicted in a schematic diagram of the self-assembled HA/collagen

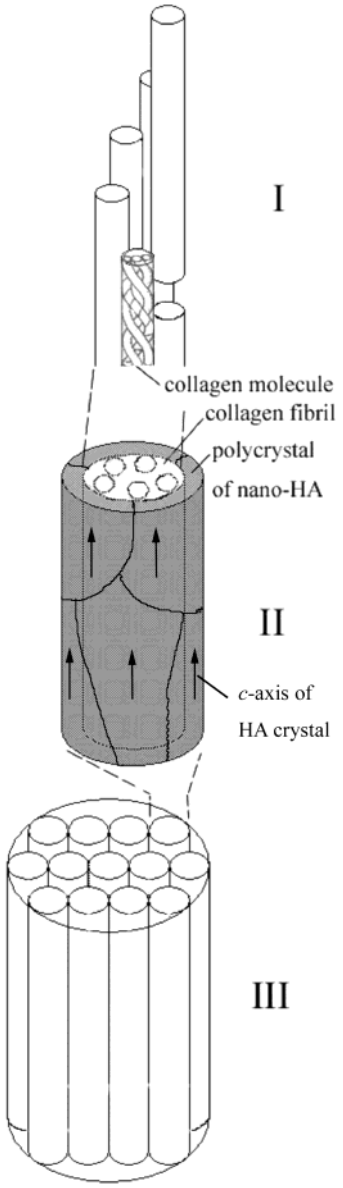

Figure 7. Scheme of the hierarchical structure of self-assembled HA−collagen composite. (I) The lowest level of the hierarchy is the organization of collagen molecules. The collagen fibrils are formed by self-assembly of collagen triple helices. Collagen molecules in fibrils arrange in so-called 5-stranded models. (II) The second level of the hierarchy is the organization of collagen fibrils with respect to HA crystals. The HA crystals grow on the surface of these fibrils in such a way that their *c*-axes are oriented along the longitudinal axes of the fibrils and they surround the fibrils. Arrow indicates the direction of *c*-axes of the HA crystals. (III) The third level of the hierarchy is the organization of the mineralized collagen fibrils. The mineralized collagen fibrils align parallel to each other to form mineralized collagen fibers.

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composite comprising multiple levels of hierarchical organization (Figure 7). The lowest level of this hierarchy is the organization of collagen molecules. The collagen fibrils are formed by self-assembly of collagen triple helices. The most widely accepted model for packing of collagen molecules is that collagen molecules in five-stranded fibrils form a 5_1 or 5_4 helix, which was originally proposed by Smith.[28] Considering the diameter of the collagen molecule is 1.5 nm,[29] the diameter of the collagen fibrils in the five-stranded packing model should be approximate 4.0 nm, which was verified by the TEM observation of self-assembled collagen fibrils. The second level of the hierarchy is the organization of collagen fibrils with respect to HA crystals. The HA crystals grow on the surface of these fibrils in such a way that their *c*-axes are oriented along the longitudinal axes of the fibrils and surround the fibrils. This fact implies that the nucleation and growth of HA crystals are not random but controlled by the collagen fibrils. The diameter of the mineralized collagen fibril was observed by TEM and HRTEM to be 5.5−6.9 nm. The third level of the hierarchy is the organization of the mineralized collagen fibrils. The mineralized collagen fibrils align parallel to each other to form mineralized collagen fibers.

Because of the potential importance of design of these materials, including those used for mineralized tissue repair, and due to its resembling the natural structure of mineralized extracellular matrix, a similar self-assembled structure was studied by early research. Recently, Stupp andco-workers[16] reported the pH-induced self-assembly of a peptide−amphiphile (PA) to make a nanostructured fibrous scaffold with fibers able to direct mineralization of hydroxyapatite throughout the surface of the fibers. The results of TEM and electron diffraction are similar to those of mineralization of collagen fibrils in this study. It can be argued that the observations of mineral on the fiber surfaces suggest plate-shaped HA crystals grow in parallel arrays. In this study, however, the HRTEM images provide direct evidence that the first-formed HA crystals are polycrystalline and form a crystalline layer to cover the surface of the collagen fibrils. The HA crystal lattice was observed not only on the side area of the collagen fibrils but also in the middle area. Here the crystals appear to be disposed along contours that are interpreted as the cylindrically shaped surfaces of collagen fibrils. HA crystals formed a hollow tube with collagen fibril as the core. On radial direction, the crystal thickness of the axial area is thinner than that of circumference area. Therefore, TEM photos of mineralized collagen fibrils show smaller contrast in the axial area than the circumference area (Figures 2(B) and 3). The schematic diagram of the HA crystal layer covering the surface of the collagen fibril in the self-assembled HA−collagen composite coincides with the experiment results, rather than showing plate-shaped HA crystals grown in parallel arrays. By inference, the model of a mineral layer covering the surface of fibrils may be applicable to the other systems in which mineralization occurs on fibrils' surfaces.

This experiment of collagen mineralization shows that the collagen fibrils can nucleate HA crystals on their surfaces. And more important is the direct observation that the crystals covering the surface of the collagen fibrils and the *c*-axes of the HA crystals are aligned with the longitudinal axes of collagen fibrils (Figure 3). Here the epitaxial growth mechanism may be a possible explanation of the assembly. Previous papers have proved that the negatively charged groups of the collagen molecules are the nucleation sites of HA crystals.[20] The positions of the oxygen in the hydroxyl groups of HA crystals have the epitaxial relationships with those in the carboxylate groups of collagen fibrils. The novel self-assembled structure could be interesting in the design of new functional materials, including those for bone tissue repair. The nanoscale organization resembles that of HA crystals in mineralized tissue in which the HA crystals also align their *c*-axes with the longitudinal axes of the collagen fibrils. The alignment is the most important characteristic of the bone minerals.

6. Conclusion

We have made the designed hierarchical self-assembly of mineralized collagen nano-fibrils. The TEM observations at several magnifications verify that the composite contains multiple levels of hierarchical organization. HA crystals grown on the surfaces of the collagen fibrils surrounded the surface of the collagen fibrils with their *c*-axes oriented along the longitudinal axes of the collagen fibrils. The mineralized collagen fibrils align parallel to each other to form mineralized collagen fibers.

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