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Self-assembly phenomenon of hydroxyapatite nanocrystals on chondroitin sulfate

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Self-assembly phenomenon of hydroxyapatite (HAp) nanocrystals on chondroitin sulfate (ChS) templates was investigated. A HAp/ChS hybrid was synthesized through a precipitation method with a calcium hydroxide suspension and phosphoric acid solution containing the ChS. The preferential alignment of the crystallographic *c*-axis of the HAp crystals parallel to the long axis direction of the ChS templates was observed to occur with the chemical interactions between the HAp crystals and the functional groups of the ChS. This phenomenon was interpreted by the crystallochemical specific nucleation and growth of the HAp crystals regulated by the pre-organized functional groups of the ChS template. Practical implication may involve a biomimetic artificial bone or cartilage can be made through a precipitation method with suitable bio-organics.

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1. Introduction

Most biological materials, including the bone of human beings, are in the form of inorganic/organic nanocomposite [1–4]. The peculiar morphology, size, and crystallographic orientation of biominerals occur through electrostatical, structural, and stereochemical matchings between inorganic and organic materials. The functional groups which exist on the surface of bioorganic materials and pre-organization of them are believed to give the regiospecific nucleation sites and result in the self-assembly phenomena of the biominerals [5–8]. The bone of human beings is one of the good examples. It is composed of inorganic hydroxyapatite (HAp) and organic collagen and the crystallographic *c*-axis of HAp crystals are preferentially aligned parallel to the long axis of the collagen fibrils [7, 9, 10]. The crystallochemical specificity in the nucleation of hydroxyapatites on the collagen is believed to occur through the chemical interactions between hydroxyapatites and the nucleator molecules, like as carboxylate, glyco-, and phosphoproteins which exist in the architecture framework (in the form of holes and grooves) provided by collagen [11].

Chondroitin sulfate (ChS) is one of glycosamioglycans, which compose the extracellular matrix of cartilage and bone. Large amount of it in the cartilage permit diffusion of substances between blood vessels in the surrounding connective tissue. It is composed of repeating disaccharide units; one of two monosaccharides is N-acetyl-galactosamine sulfate (GalNAc–OSO_3^-) which contains a sulfate group and the other is glucuronic acid (GlcUA) which contains a carboxylate group. The combination of these two functional groups gives the ChS a very high density of negative charge and may let them to be nucleation sites for the HAp through binding the inorganic cation such as plus charged Ca ion [12–15].

In the present experiment, the self-assembly phenomenon of HAp crystals on ChS was investigated with the main focus on the crystallochemical specific nucleation and growth of the HAp crystals induced by the chemical interactions between the HAp crystals and pre-organized functional groups of the ChS template. The HAp/ChS hybrid was prepared by a precipitation method with calcium hydroxide and phosphoric acid solution containing the ChS. The electron diffraction pattern of the HAp crystals and the chemical shifts of the functional groups of the ChS were used to assess the self-assembly phenomenon of HAp crystals on the ChS template.

2. Experimental procedure

The 60HAp/40ChS hybrid (in wt %) was prepared by a precipitation method with a $Ca(OH)_2$ suspension and H_3PO_4 solution containing ChS. The $Ca(OH)_2$ powder was made by slaking of CaO which was made via the calcination of $CaCO_3$ (Wako Pure Chemicals Ind., Osaka, Japan) at 1050 °C for 3 h. The H_3PO_4 solution containing ChS was prepared by adding 4 g of ChS powder to the 39 mL of 0.7 M H_3PO_4 (Wako Pure Chemicals Ind., Osaka, Japan) solution with vigorous stirring until all the ChS power was dissolved completely. The ChS (Seikagaku Kogyo Co., Tokyo, Japan) used in this study was made from the bovine trachea

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TABLE I Composition of ChS

Composition (%)					
ΔDi-0S	ΔDi-6S	ΔDi-4S	ΔDi-disD	ΔDi-disE	ΔDi-tris
6.3	43.0	48.0	1.8	0.7	0.3

cartilage and the molecular weight was 13 000. The composition of it is as shown in Table I. The preparation of the hybrid was conducted by adding the H_3PO_4 solution with the ChS slowly into the 100 mL of 0.45 M $Ca(OH)_2$ suspension with vigorous stirring until pH became to around 9 at 36.5 °C. The precipitate was filtered and then dried at room temperature.

For checking the crystal phases of the HAp/ChS hybrid, X-ray diffractometer (XRD) measurement was performed on the as-prepared precipitate. The microstructure was observed by transmitted electron microscopy (JEM-2000EX II, JEOL, Tokyo, Japan) with an accelerating voltage of 200 kV. The self-assembly phenomenon of the HAp crystals was assessed by the electron diffraction measurement on a HAp/ChS aggregate with a beam size of 280 nm. The chemical interactions between the HAp crystals and ChS templates were evaluated by the chemical shifts of the two functional groups of the ChS (carboxylate and sulfate groups) with diffuse reflectance Fourier transformed infrared (FT-IR) spectroscopy (Spectrum 2000, Perkin Elmer Co., Norwalk) before and after the precipitation. For IR spectroscopy measurements, the pulverized specimens were diluted with KBr powder by one tenth and the background noise was corrected with pure KBr data. The scanning resolution was 0.5 cm^{-1}.

3. Results and discussion

Fig. 1 shows the result of XRD measurement on the as-prepared HAp/ChS hybrid. Several HAp peaks denoted by ''H'' were observed. As the HAp peaks were comparatively broader than a normal HAp, it was considered that the HAp crystals grown had low crystallinity or small crystallite size.

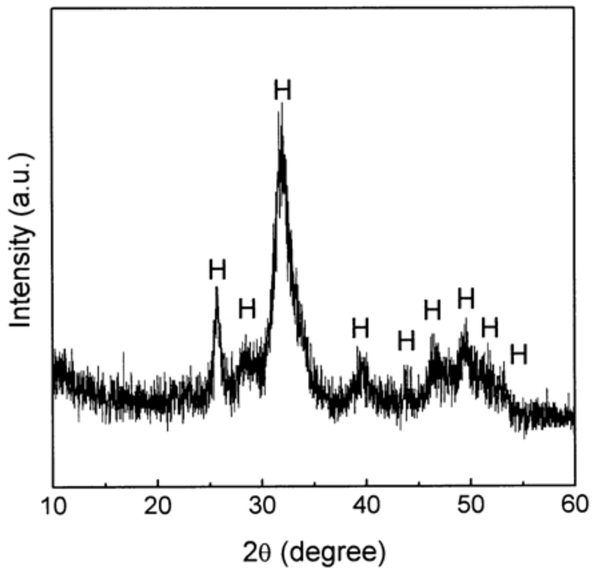

Figure 1 X-ray diffraction profile of the HAp/ChS precipitate.

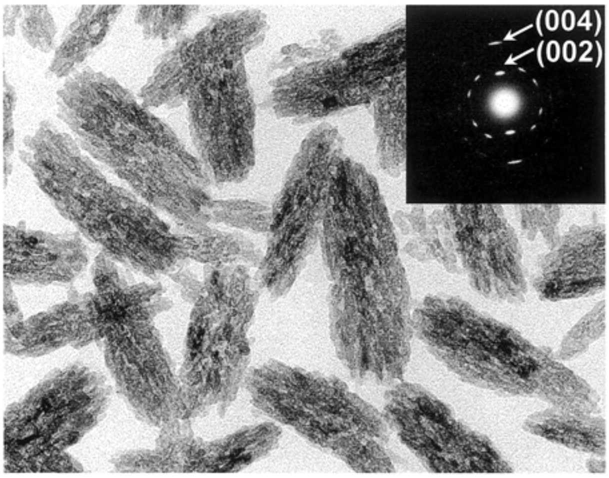

Figure 2 Microstructure and electron diffraction pattern of the HAp/ChS precipitate.

Fig. 2 shows the microstructure of the HAp/ChS hybrid and electron diffraction pattern. Large numbers of the HAp nanocrystals (around 12 × 6 nm in size) were observed to occur on a ChS template (a HAp/ChS aggregate) which were around 150 × 50 nm in size. The crystallographic *c*-axis of the HAp crystals were observed to align preferentially parallel to the long axis direction of a ChS template and it was confirmed by the electron diffraction pattern; the (002) and (004) diffractions measured on a HAp/ChS aggregate showed small arching behavior (around 10 degrees).

Fig. 3 shows the FT-IR spectra measurements for the ChS and HAp/ChS hybrid. For the HAp/ChS hybrid, the asymmetrical stretching (ν_3) and bending (ν_4) modes of a $(PO_4)^{3-}$ ion were detected at around 1089, 1038, and 602, 565 cm^{-1}, respectively. The symmetric stretching modes (ν_1 and ν_2) of it were also found at around 963 and 472 cm^{-1}, respectively [16, 17]. Further, two stretching and an out-of-plane modes of a $(CO_3)^{2-}$ ion were also observed at around 1454, 1419, and 875 cm^{-1}, respect-

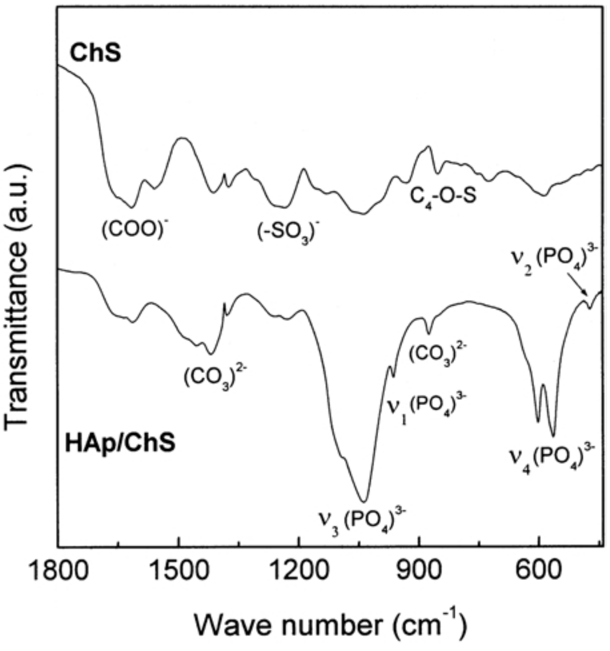

Figure 3 Diffuse reflectance FT-IR spectra of the ChS and HAp/ChS precipitate.

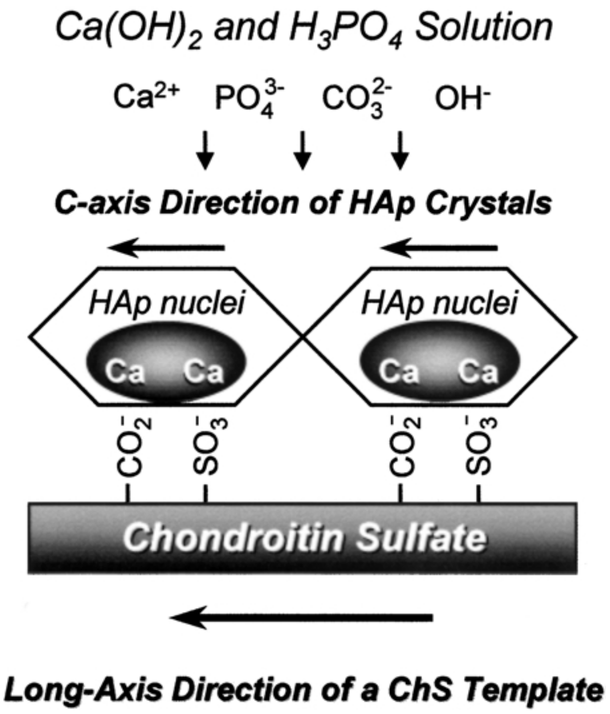

Figure 4 Schematic illustration for the crystallochemical specific nucleation and growth of the HAp crystals on the pre-organized ChS template.

ively. It means that the PO_4 sites of the HAp structure, i.e. B-site, were partly substituted by carbonate ions [18]. Therefore, the HAp crystals formed on the ChS templates were identified as carbonate-containing hydroxyapatites. For the ChS, the asymmetrical stretching mode of $(COO)^-$, $-(SO_3)^-$ ions and C_4–O–S peak shoulder were detected at around 1632 and $1234\,cm^{-1}$, and $851\,cm^{-1}$, respectively. After formation of the HAp crystals on the ChS template, however, they were detected at around 1613 and $1228\,cm^{-1}$, respectively i.e. showed red chemical shifts. It can be known that, therefore, the preferential alignment of the HAp crystals on the ChS templates had occurred through the chemical interactions between the HAp crystals and the functional groups of the ChS.

From the results, it can be presumed that the self-assembly phenomenon of the HAp crystals is critically dependent on the chemical interactions between the HAp crystals and the functional groups of the ChS templates. For an explanation of preferred alignment of the HAp crystals observed in this work, the nucleation model based on the binding of calcium ion to the periodically arrayed functional groups of a ChS template [19, 20] may be appropriate, as Ca ion binding [21–26] and pre-organization of organic matrix are believed to be an initial step for crystallochemical specific formation of calcium phosphates on bioorganics [5–8]. In fact, the chondroitin 4-sulfate calcium salt was reported to have periodically arrayed functional groups. The repeat length per disaccharide is 0.913 nm and molecular chain has three-fold screw symmetry. The shape of unit cell is a trigonal prism with dimensions $a = b = 1.28$ nm, $c = 2.74$ nm, and $\gamma = 120°$ [20].

Negatively charged carboxylate and sulfate groups which are periodically arrayed on a ChS template will chelate the calcium ions and form a Ca–ChS complex. Then the cluster of a critical size can be formed by adsorbing the calcium, phosphate, carbonate, and hydroxyl ions on the Ca–ChS complex three-dimensionally and it may act as a nuclei for the crystal growth of HAp. After the nucleation, the HAp crystals will grow spontaneously with preferred orientation due to the chemical interactions with pre-organized functional groups of the ChS template as schematically shown in Fig. 4.

The results may suggest that the peculiar crystallochemical specific orientation of the HAp crystals occurs through the chemical interactions between the inorganic HAp crystals and the pre-organized functional groups of organic ChS template. However, the evidence of epitaxial growth of HAp crystals on the ChS template, i.e. an exact atom-for-atom match of the constituents of the HAp crystals with those of a ChS template constituent, could not be found in this work. The implication of the results is that the self-assembly phenomenon of the HAp crystals occurs *in-vivo* condition can be reproduced even *in-vitro* condition. Practical application may involve that a biomimetic artificial bone or cartilage can be made by a simple precipitation method with suitable bioorganics. The preparation of a biomimetic HAp/collagen/ChS nanocomposite is under investigation and will be published in the near future.

4. Conclusions

The self-assembly phenomenon of the HAp crystals on the ChS template was investigated. The (0 0 2) and (0 0 4) diffractions of the HAp crystals showed small arching angles and it means that the crystallographic *c*-axis of them are preferentially aligned parallel to the long axis direction of the ChS template. From the red chemical shifts of the carboxylate and sulfate groups of the ChS template after crystallochemical specific formation of HAp crystals, it can be presumed that the self-assembly phenomenon of the HAp crystals occurred through the chemical interactions between the HAp crystals and pre-organized functional groups of the ChS template.

Acknowledgments

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