Creation of a composite of collagen and OCP crystals with large morphological anisotropy

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Collagen and octacalcium phosphate (OCP) crystals of micrometer size with anisotropic morphology were combined under physiological temperature and pH. OCP crystals elongated in the c-axis direction were grown in a reaction chamber where diffusion was controlled by a cation selective membrane. The size of crystal used was 40-50 μm in length, about 2 μm in width and aspect ratio of around 20–25. Atelocollagen was reconstituted and cross-linked in the presence of the crystals at 37°C for 20–48 h under stirring. Despite stirring and mixing during fabrication process, the long crystals assembled with reconstituted collagen fibers and arranged more or less parallel to each other along the collagen fibers. This suggested that OCP crystals and collagen fibers assembled through some interaction, by which crystals stayed on/between collagen fibers during fabrication process. Collagen content of resultant composites was arbitrarily changeable in the range of 20–90% by weight. Thus, the composite of long OCP crystals and collagen was newly designed for future application as a bone substitute material.

Key-words: Composite, OCP, Collagen

1. Introduction
Carbonate-containing hydroxyapatite (HAp), prototype of bone apatite, is currently widely accepted as an osteoconductive and biocompatible material. As other potent calcium phosphate for biomaterial, octacalcium phosphate (OCP: Ca$_8$H$_2$(PO$_4$)$_6$·5H$_2$O) has been nominated. This is based on the following reasons: Firstly, OCP is a metastable phase of HAp, therefore in an aqueous system, OCP transforms into HAp spontaneously. The crystal structure of OCP is an alternated structure of an apatitic layer and a hydrated layer where water molecules locate. Secondly, recent intensive studies for clinical application of OCP as a bone substitute material have shown that OCP, by itself, and the conversion process from OCP into apatite have the potential to enhance a new bone formation. In these studies, OCP had been used in forms of granules or coating on metal implants.

Aiming at developing a bone substitute material with flexibility and strength, we used OCP crystals elongated in the c-axis direction to construct a composite with collagen. A long OCP crystal was a key material from the following point of view: OCP grew in the c-axis direction preferentially under certain specific conditions at physiological pH and temperature and OCP transforms into apatite spontaneously. Since the transition is topotactic one, the original morphology and the c-axis direction of OCP crystal are preserved after transition. Regarding the mechanical property of a composite of calcium phosphate crystals and collagen, the crystalline component is responsible for strength and stiffness while it reduces the flexibility and elasticity. In this sense, fibrous crystals or whiskers are expected to increase flexibility of a composite. Fiberglass reinforced plastic (FRP) is a typical example of strengthening of a plastic. Type-I atelocollagen was used as source of collagen, since type-I collagen is a main organic component of bone and atelocollagen has a great deal of clinical usage due to lack of antigenicity. This study explored a method to unify collagen fiber and as-grown OCP crystal of micrometer in size with anisotropic morphology.

2. Materials and method
2.1 Synthesis of OCP crystals
Crystal growth was carried out in a diffusion controlled reaction chamber equipped with a cation selective membrane (Selemion, Asahi Glass Co.). The membrane system was developed in our previous studies to grow tooth enamel like apatite crystals under physiological condition. Briefly, the membrane separates Ca-solution from the phosphate solution in a reaction chamber: Ca$^{2+}$ ions diffuse into phosphate solution through the membrane and crystal formation of OCP (equation (1)) takes place on the phosphate side of the membrane.

$$8\text{Ca}^{2+} + 2\text{HPO}_4^{2-} + 4\text{PO}_4^{3-} + 5\text{H}_2\text{O} \rightarrow \text{Ca}_8\text{H}_2\left(\text{PO}_4\right)_6\cdot 5\text{H}_2\text{O} \quad (1)$$

Reaction condition to synthesize OCP crystals elongated in the c-axis direction was explored by a preliminary experiment performed at pH 6.0-7.4 and 37°C, using 5–100 mM Ca(CH$_3$COO)$_2$·H$_2$O and 5–30 mM NH$_4$H$_2$PO$_4$·(NH$_4$)$_2$HPO$_4$ (1: 1 molar ratio) solutions as ionic souces. In this study, therefore, crystal growth was carried out using 100 mM Ca solution and 10 mM phosphate solution at pH 6.5 and at 37°C for 3 days. After the reaction was terminated, crystals on the membrane were rinsed with double-distilled water and dried in air.

2.2 Fabrication of composite
Weighed out crystals were mixed with 200 mM triethanolamine hydrochloride buffer solution (37°C, pH 8.6) for 10 min and then an equivalent volume of atelocolagen (Cellmatrix Type-I ATM, Nitta Geratin) was added. Ateocolagen was reconstructed for 5–24 h and subsequently cross-linked in 0.01–0.1% dimethylsuberimidate dihydrochloride (Nacalai Tesque) solutions for 15–24 h. The theo-
retical amount of the reagent required to cross-link ε-amino groups of collagen molecules was 0.006%. Fabrication was carried out at 37°C with stirring. The mixing ratio of crystals and atelocollagen was varied. After cross-linking, the sample was thoroughly washed with double-distilled water using a tube mixer and centrifuged at 3000 rpm for 10 min. The washing process was repeated three times to remove the cross-link reagent and then the sediment was lyophilized for about 20 h.

2.3 Characterization of crystals and composite

OCP crystals and the lyophilized composites were characterized by means of an optic microscope, an X-ray diffractometer (XRD) (Rint2500, CuKα, 200 mA, 56 kV, Rigaku), a micro Laue camera (collimeter size: 100 μm in diameter, Ni filter, Rigaku) and a scanning electron microscope (SEM) (S4500, Hitachi). The content of the collagen in composite was estimated by heating the composite up to 1000°C by means of a thermo gravimetric (TG) analyzer (TG8120, Rigaku).

3. Results and discussion

Figure 1 shows OCP crystals grown on the membrane, using 100 mM Ca solution and 10 mM PO₄ solution of pH 6.5. Crystal length was 40–50 μm, width was about 2 μm and aspect ratio was around 20–25. Putting together the result of the preliminary experiment and that of our previous study,21,22 crystal length changed between about 1 μm and 150 μm in the pH range between 6.3 and 7.4. At pH lower than 6.5, length of OCP in the c-axis direction was extremely long, 100–150 μm, however, dicalcium phosphate dihydrate (DCPD) was formed as a byproduct. At pH higher than 6.5, crystal length of OCP decreased with an increase in solution pH. The width was in the range of 2–5 μm. Dependence of crystal width on reaction condition was much smaller than that of the length. Thus, it was possible to change the morphological anisotropy widely.

Collagen fiber and crystals assembled and unified after 15 h of cross-linking period, regardless of the concentration of the cross-linking reagent, crystal size and its morphology. However, when 0.01% of the cross-linking reagent was used, a 1-hour-reaction was not enough to unify them thoroughly and crystals separated from collagen fibers during the washing process. Figures 2a and 2b are SEM images of a composite, which was peeled from a lyophilized sample. Figure 2a (lower magnification) shows that reconstituted collagen fibers arranged almost parallel to each other in the composite and that crystals embedded into the collagenous matrix. Figure 2b (higher magnification) clearly shows parallel orientation of long crystals in the composite. Although stirring during reaction partially broke original long OCP crystals, morphological anisotropy was preserved after the fabrication. In our previous experiment of OCP crystal formation in bovine Achilles tendon,23 OCP crystals grew along the collagen fibers. In the present study, crystals unified with collagen fibers with orientation despite continuous stirring and mixing in the fabrication process. These results suggested that there was some interaction between OCP crystals and collagen fibrils, accordingly OCP assembled with collagen with orientation.

Figure 3a shows an X-ray micro-Laue photograph of a small piece of the composite, which was fixed at the apex of a needle (Fig. 3b). In Fig. 3b, the area irradiated by X-ray beam is indicated with an open circle. Diffraction spots in the Debye-rings indicate that there were crystallites with micrometer size in the composite. Figure 4 shows XRD patterns of OCP as-grown (the lower one) and a composite (the upper). These indicated that amount of OCP slightly decreased and apatite was formed (with index in Fig. 4) in the fabrication process. The broad background at about 6°–8° and 12°–24° in 2θ were due to collagen. Thus, OCP partial-
controlled arbitrarily. Suggested therefore that the collagen
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in a basic solution.11
8.6, because conversion of OCP into apatite was prompted
ascribed to the solution pH of the fabrication process, pH
ly converted to apatite during the fabrication reaction. Diffraction peaks of apatite that did not over-
the apex of a needle. The irradiated area is indicated by an open
ly converted to apatite after 3 weeks of implantation in a mouse.26 Granules of OCP implanted into rat calvaria
defects partially converted into apatite after 10–21 days.27 The size of OCP crystals used in this study was about 2 ×
40–50 µm (width × length). Even such large OCP crystals
partially converted into apatite (Fig. 4) during the fabrication
process at pH 8.6 and 37 °C for 20 h. Thus, OCP component
in the composite probably converts into apatite in vivo,
thereby providing a composite abundant in apatite. Since
both OCP and apatite have osteoinductive property and
the conversion process from OCP to apatite contributes
to enhance a new bone formation, the composite should work
as a useful bone substitute material. Study on the relationship
between crystal length, structure of composite and its
mechanical property is now on going.

4. Conclusion
Micron meter size OCP crystals with anisotropic morphology were combined with collagen fibers by reconstituting
and cross-linking atelocollagen in the presence of the crystals under physiological condition. Despite stirring and mixing
during the fabrication process, crystals assembled and unified with collagen fibers. Long crystals arranged more or
less parallel to each other along the collagen fibers. This suggested that OCP crystals and collagen fibers assembled
through some interaction, which would keep crystals staying on/between collagen fibers during further processing. Thus,
an innovative composite with a unique structure was constructed and the composite was expected as a bone substitute material for a future use.

References
2) H. Aoki, "Science and medical application of hydroxyapatite", Takayama Press System Center Co. Inc., Tokyo
3) E. B. Kenny, V. Lekovic, J. C. Sa Ferreira, T. Han, B. Dimitrijevic and F. A. Carranz, Jr., J. Periodontal., 57, 76–83
(1986).