Behavior of hydroxyapatite crystals in a simulated body fluid: effects of crystal face

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Control of calcification behaviors in osteoconductive ceramics is important for development of novel materials. Calcification behaviors of osteoconductive materials in bony defects can be assessed using a simulated body fluid (SBF). Previous studies of hydroxyapatite (HAp) ceramics have not found a clear relationship between calcification behaviors and morphology, in particular the crystal face, of HAp. In this study we have investigated calcification behaviors of rod-shaped HAp crystals with controlled crystal face in a SBF. The aspect ratios of the rod-shaped HAp was taken to be the area ratio of (the a face)/(the c face) in HAp crystals. HAp with aspect ratios ranging between approximately 10 and 17 was synthesized under hydrothermal conditions. Scale-like precipitates, which should be nonstoichiometric HAp referred to as bone-like apatite, were formed on the HAp crystals soaked in the SBF. The formation rate of bone-like apatite was estimated by measuring decreases in calcium and phosphate ion concentrations of the SBF. The formation rate increased with increasing aspect ratios of the HAp crystals. The results show that bone-like apatite was preferentially formed on the a face of the HAp crystals and that the calcification behaviors of HAp crystals in a SBF can be controlled using the aspect ratio of the HAp crystal, that is, by controlling the crystal face of HAp.

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1. Introduction

Hydroxyapatite [HAp, Ca₁₀(PO₄)₆(OH)₂] ceramics are a typical osteoconductive material. HAp ceramics have been used as artificial bones. Neo et al. observed formation of nonstoichiometric HAp, also referred to as bone-like apatite, at the interface between natural bones and the HAp ceramic implanted in a bony defect of rat tibia.¹) The formation of a bone-like apatite layer has also been observed at the interface between bone tissue and other types of glass-ceramics after implantation.²) Hence, the formation of a bone-like apatite layer on the implanted materials is important for direct bonding of artificial materials to natural bone.

The ability of a material to form an apatite can be estimated using a type of simulated body fluid (SBF) proposed by Kokubo and colleagues.²⁻⁴) SBF mimics the inorganic ion concentrations of human blood plasma. Kim et al. reported that a bone-like apatite layer was formed on sintered HAp ceramics in a SBF.³) They suggested a formation mechanism of the bone-like apatite layer based on the zeta potential of HAp and compositional analysis of the bone-like apatite. According to their suggested mechanism, calcium-rich amorphous calcium phosphate is formed by interactions between negatively charged HAp and calcium ions. Subsequently, the amorphous calcium phosphate is transformed into bone-like apatite by incorporation of phosphate ions. Interactions between the charged material surface and inorganic ions in the surrounding fluid have been used to explain mechanisms for bone-like apatite formation on other osteoconductive materials in SBF.⁵⁻⁷) Therefore, surface charge of the osteoconductive materials is considered to be an important factor for bone-like apatite formation in a SBF.

HAp is a hexagonal crystal having a positively charged a face and a negatively charged c face. The charge difference is a result of calcium and phosphate ions on the surface of the a and c faces, respectively.⁸) Therefore, it is difficult to discuss the relationship between surface charge and bone-like apatite formation using HAp with a random crystal face. Effects of HAp surface charge on bone-like apatite formation in a SBF could be clearly observed if HAp with controlled crystal faces was used. In the present study, we investigate the behavior of HAp with controlled crystal faces in a SBF. To obtain HAp with different surface charges, we synthesized HAp crystals with various aspect ratios using a previously reported hydrothermal processing technique.⁹)

2. Experimental procedures

2.1 Synthesis of rod-shaped HAp through hydrothermal processing

Rod-shaped HAp crystals were synthesized based on a previously published technique⁹) with appropriate modifications to obtain HAp crystals with various aspect ratios. β-Tricalcium phosphate [β-TCP: Ca₃(PO₄)₂], Taihei Chemical Industrial Co. Ltd., Osaka, Japan) and dicalcium phosphate dihydrate (DCPD; CaHPO₄·2H₂O, Wako Pure Chemical Industries Ltd., Osaka, Japan) were mixed in a polyethylene vessel with zirconia balls and ethanol. The mass ratios of β-TCP:DCPD were 100:0, 75:25.

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Table 1. Regents for preparing 1000 cm³ of the simulated body fluid

| Order | Reagent               | Amount   |
|-------|-----------------------|----------|
| 1     | NaCl                  | 7.996 g  |
| 2     | NaHCO₃                | 0.350 g  |
| 3     | KCl                   | 0.224 g  |
| 4     | K₂HPO₄·3H₂O           | 0.228 g  |
| 5     | MgCl₂·6H₂O            | 0.305 g  |
| 6     | 1.0 mol dm⁻³ HCl      | 40 cm³   |
| 7     | CaCl₂                 | 0.278 g  |
| 8     | Na₂SO₄                | 0.071 g  |
| 9     | (CH₂OH)₂CNH₂          | 0.0432 g |

and 10:90. The samples were ball milled for 48 h. After the ball milling process, powder samples were obtained by drying the milled samples at 100°C and sieving the dried powders.

Three different amounts (0.1, 0.2 and 0.4 g) of each of the three sample mixtures (i.e. nine samples in total) were put into a 90 cm³ Teflon® vessel with 20 cm³ of 2.0 mol·dm⁻³ (M) NH₄Cl (Wako Pure Chemical Industries Ltd.) solution. The pH of the NH₄Cl solution was adjusted to 7.3 using an ammonia solution (Wako Pure Chemical Industries Ltd.). The sample was encapsulated in an autoclave and then hydrothermally treated at 160°C for 24 h. The hydrothermally treated sample was collected by centrifugation, rinsed in ultrapure water and dried at 100°C for more than 12 h.

2.2 Soaking in SBF

SBF was prepared as follows: 700 cm³ of ultrapure water was put into a 1000 cm³ glass beaker, stirred with a magnetic stirrer and the reagent-grade chemicals listed in Table 1 were dissolved in the water in the order given in Table 1. Each reagent was allowed to completely dissolve before adding the next reagent. All reagents shown in Table 1 with the exception of the 1.0 M HCl solution were purchased from Nacalai Tesque, Inc., Kyoto, Japan. The 1.0 M HCl solution was prepared by diluting 35 mass % HCl solution (Wako Pure Chemical Industries Ltd.). The solution was kept at 36.5°C and the pH was adjusted to 7.25 by titrating with the 1.0 M HCl solution. After the pH adjustment, the solution was transferred to a volumetric flask and ultrapure water was added to adjust the total volume of the solution to be 1000 cm³.

Appropriate amounts of sample powder were soaked in 20 cm³ of SBF at 36.5°C for up to 7 days (d). The amount of powder was selected to maintain a surface area of 0.48 m² for each of the soaked powder samples. The samples were shaken at 150 rpm using a rotary shaker. The soaked powder was taken out after 6 h, 12 h, 1 d, 2 d, 3 d, 5 d and 7 d by centrifugation and the extracted powders were rinsed with ultrapure water and dried at 40°C for more than 12 h.

2.3 Characterization

The extracted powder samples were characterized using powder X-ray diffraction (XRD; RINT PC2100, Rigaku Co., Tokyo, Japan) using Cu Kα radiation (λ = 0.154056 nm). The crystalline phases of the samples were examined by powder XRD in the range 2θ = 20–40° with a scanning rate of 2.0°min⁻¹.

The chemical structures of the samples were characterized using Fourier transform infrared (FT-IR) spectroscopy (FT/IR-6100; JASCO Co., Tokyo, Japan), using the KBr tablet method under a N₂ gas atmosphere. The mass ratio of the KBr to the sample was 100:1. Each sample was scanned between 4000 and 400 cm⁻¹ at a resolution of 2 cm⁻¹.

The specific surface areas of the samples were measured using the BET method with N₂ gas as the adsorbate (NOVA 1000e, Yuasa Ionics Co. Ltd., Osaka, Japan). The samples were heat treated at 200°C for 2 h under vacuum conditions as a pretreatment before specific surface area measurement.

The morphology of the samples was observed using a scanning electron microscopy (SEM; JSM5600, JEOL Ltd., Tokyo, Japan), after application of a thin gold coating.

Zeta potentials of the samples in the SBF (pH = 7.25, 36.5°C) were investigated using a zeta potential measuring system (ELSZ-1, Otsuka Electronics Co. Ltd., Osaka, Japan). Zeta potentials were calculated based on the laser Doppler method. A slurry made by mixing 20 mg of sample powder with 10 cm³ of SBF was used for zeta potential measurement. For each sample the zeta potential measurements were repeated five times and the mean value and standard deviation were calculated.

The composition of the samples, including the Ca/P molar ratio, was measured using inductively coupled plasma atomic emission spectroscopy (ICP–AES: Optima 2000DV, PerkinElmer Japan, Kanagawa, Japan) following dissolution of 1 mg of sample powder in a 20 cm³ of 5 vol % nitric acid solution.

The pH of the SBF after soaking sample powder was measured using a glass-electrode type pH meter (D–51, Horiba Ltd., Kyoto, Japan). Concentrations of calcium and phosphate ions in the SBF were measured using ICP–AES to determine the time dependent changes of these ions concentrations in the SBF. These measurements were conducted for three samples and the mean value and standard deviation were calculated.

3. Results

3.1 Characterization of hydroxyapatite with various aspect ratios

To determine the appropriate synthetic conditions of HAp, we characterized the nine samples described in section 2.1. Figure 1 shows powder XRD patterns of the powders before and after hydrothermal treatment. Powder diffraction files (PDF) #00-009-0432, #00-009-0169 and #00-009-0080 were used for identification of HAp, β-TCP and dicalcium phosphate anhydrous (DCPA, CaHPO₄). The samples were heat treated at 100°C before hydrothermal treatment, diffraction peaks assigned to β-TCP were detected. All of the β-TCP powder in the three compositions were completely transformed into HAp following the hydrothermal treatment. Diffraction peaks assigned to β-TCP and DCPA were detected in the samples of β-TCP:DCPD = 100:0 before hydrothermal treatment, 0.1, 0.2 and 0.4 g of the mixed powders (namely β-TCP:DCPD = 75:25) were transformed into HAp after hydrothermal treatment. Diffraction peaks assigned to β-TCP and DCPA were detected in the samples of β-TCP:DCPD = 10:90 before hydrothermal treatment. Crystalline phases of the hydrothermally treated samples containing 0.1 and 0.2 g of the mixed powders (namely β-TCP:DCPD = 10:90) were identified as HAp, while the hydrothermally treated 0.4 g of the mixed powder became a mixture of HAp and DCPA. In the samples with β-TCP:DCPD = 75:25 and 10:90 before hydrothermal treatment, DCPA was detected instead of DCPD. This is attributed to the dehydration of DCPD during the drying process at 100°C. Intensities of diffraction peaks assigned to DCPA increased with increasing DCPD fraction in the starting compositions.

The results shown in Fig. 1 demonstrate that we have found appropriate synthetic conditions for HAp formed as single phase for each composition. Therefore we selected a sub-set of three hydrothermally treated samples for subsequent analysis: the first...
The sample contained 0.4 g of the powder with $\beta$-TCP:DCPD = 100:0, the second sample contained 0.4 g of mixture of $\beta$-TCP and DCPD [$\beta$-TCP:DCPD = 75:25 (mass ratio)] and 0.2 g of mixture of $\beta$-TCP and DCPD [$\beta$-TCP:DCPD = 10:90 (mass ratio)], respectively.

The sample names are denoted as $T_xD_y$, where $x$ and $y$ indicate fraction of $\beta$-TCP and DCPD in the starting composition, respectively.

Figure 2 shows SEM images of HAp crystals of T100D0, T75D25 and T10D90. Rod-shaped crystals were observed in all of the samples. We defined the aspect ratio of the rod-shaped crystals as the ratio of long side to short side. The mean aspect ratios of the crystals were manually calculated using the sizes of one hundred crystals observed in the SEM images. The mean aspect ratios of the crystals in samples T100D0, T75D25 and T10D90 were 10.4 ± 6.8, 15.0 ± 8.6 and 16.7 ± 11.0, respectively. Significant differences in the aspect ratio values were confirmed between T100D0 and T75D25 as well as T100D0 and T10D90 by statistical computing.

Figure 3 presents the FT-IR spectra of HAp crystals of T100D0, T75D25 and T10D90. Absorption peaks of HAp were assigned based on previously reported data. Typical absorption peaks derived from phosphate ions ($PO_4^{3-}$) of HAp were detected at 1096, 1046, 1039, 959, 605, 574 and 561 cm$^{-1}$. The absorption peak detected at 3572 cm$^{-1}$ was derived from hydroxide ions (OH$^-$) of HAp in all of the samples. Weak absorption peaks derived from carbonate ions (CO$_3^{2-}$) incorporated into HAp were detected at 1401 cm$^{-1}$. The absorption peak detected at 873 cm$^{-1}$ was derived from hydrogen phosphate ions (HPO$_4^{2-}$) and/or CO$_3^{2-}$ incorporated into HAp.

Compositions, including the Ca/P molar ratio, of the HAp powders were measured by ICP-AES. The Ca/P molar ratios of T100D0, T75D25 and T10D90 were 1.58, 1.57 and 1.58, respectively. Specific surface areas of the HAp powders were measured using the BET method and found to be 4.8, 9.2 and 13.0 m$^2$·g$^{-1}$.
for T100D0, T75D25 and T10D90, respectively. Zeta potentials of the HAp powders in the SBF were measured as $-17.8 \pm 2.5$, $-7.7 \pm 1.3$ and $-4.2 \pm 3.1$ mV for T100D0, T75D25 and T10D90, respectively. The significant differences of zeta potential values in these samples were confirmed between T100D0 and T75D25 as well as T100D0 and T10D90 by statistical computing.

3.2 Behavior of hydroxyapatite in SBF

Figure 4 presents the powder XRD patterns of the samples after soaking in the SBF. Diffraction peaks associated with HAp were detected in all the samples. No other crystalline phase was detected. Time dependent changes of the diffraction patterns were not detected in any of the samples, regardless of differences in aspect ratios of HAp crystals soaked in the SBF.

Figure 5 shows morphology of the as-prepared HAp crystals and HAp crystals soaked in the SBF for 7 days. After soaking in the SBF, the HAp crystals have several hundred nanometers of scale-like precipitates on the surface.

Figure 6 shows the time dependent changes of calcium and phosphate ions concentrations in the SBF. Initial values of calcium and phosphate ions concentrations are 2.5 and 1.0 mol m$^{-3}$, respectively. Both the calcium and the phosphate ions concentrations decreased with increasing soaking periods of HAp powders. The calcium and phosphate ions concentrations decreased most in T10D90 and least in T100D0.

Figure 7 shows time dependent changes to the pH of the SBF. The pH values of the SBF vary within the range 7.20–7.25. The aspect ratios of the HAp crystals in these samples did not have a significant effect on the pH of the SBF.

4. Discussion

According to the PDF for HAp, the ratio of reflection peak intensities of 211:112:300 is 5:3:3. The 211, 112 and 300 reflection peaks were detected at 31.8, 32.2 and 32.9° respectively. The 300 reflection peak intensities in all of the samples were nearly the same as or stronger than the 211 reflection peak intensities (Fig. 1). In addition, the 300 reflection peak intensities in all of the samples were stronger than 112 reflection peak intensities (Fig. 1). Several reports show that HAp crystals grow along the c axis direction and expose the a face.\textsuperscript{11–13} Hence the obtained HAp crystals with rod-shape also grew along the c axis direction and exposed the a face.

Aspect ratios of HAp crystals increased with increasing DCPD fractions in the starting compositions (Figs. 1 and 2). Increases in the aspect ratios of HAp indicate that crystal growth of HAp crystals in the direction of the a axis was inhibited. This can be explained by considering the Ca/P molar ratios of the initial samples. Before hydrothermal treatment, the samples showed crystalline phases of β-TCP or a mixture of β-TCP and DCPA (Fig. 1). The Ca/P molar ratios of DCPA and HAp are 1.0 and 1.67 (stoichiometric composition), respectively. Thus, increasing the DCPA fraction in the samples before hydrothermal treatment
led to a reaction condition that was rich in phosphate ions for HAp. As the a face of HAp is rich in calcium ions, the excessive phosphate ions were adsorbed on the a face. As a result, crystal growth in the a axis direction was inhibited.

The zeta potential of the HAp crystals in SBF was negative even though the HAp crystals exposed the a face of HAp is rich in calcium ions. This implies adsorption of anions, such as phosphate, hydroxide and chloride ions, from the SBF on the HAp crystals. We calculated the area ratio of (the a face)/(the c face) based on the aspect ratio of HAp crystals. In doing this, we regarded the HAp crystals as hexagonal prisms. Figure 8 shows the relationship between the area ratios and zeta potential values. The zeta potential values of the HAp increase linearly with increasing the area ratio. The a face of HAp has positively charge, and hence the negatively zeta potential values got smaller with increasing the fractions of the a face. These findings suggest that the zeta potential of HAp is controllable by the area ratio and thus by the aspect ratio.

Crystalline phase of samples were not changed following soaking in the SBF, though precipitates with a scale-like structure should be bone-like apatite. Based on previously reported methods, we calculated decreases in the ion activity product (IAP) for HAp to discuss the formation rate of bone-like apatite in the SBF.

Bone-like apatite contains Na⁺, Mg²⁺, HPO₄²⁻ and CO₃²⁻. Although its composition is different from the stoichiometric composition of HAp [Ca₁₀(PO₄)₆(OH)]₂, we assume that composition of bone-like apatite is the same as stoichiometric composition of HAp to simplify the calculation of the IAP. The IAP was calculated using the following equation.

\[
IAP = \left(\gamma_{Ca^{2+}}\right)^{10}\left(\gamma_{PO_4^{3-}}\right)^{6}\left(\gamma_{OH^-}\right)^{2} \times [Ca^{2+}]^{10}[PO_4^{3-}]^{6}[OH^-]^{2}
\]

where \(\gamma\) is the activity coefficient. The activity coefficients for Ca²⁺, PO₄³⁻ and OH⁻ under physiological conditions are 0.36, 0.06 and 0.72, respectively. Dissociation constants of phosphoric acid are given by:

\[
\begin{align*}
H_2PO_4^- & \rightleftharpoons H^+ + HPO_4^{2-} ; K_1 = 6.22 \times 10^{-3} \\
H_2PO_4^- & \rightleftharpoons H^+ + HPO_4^{2-} ; K_2 = 6.58 \times 10^{-8} \\
HPO_4^{2-} & \rightleftharpoons H^+ + PO_4^{3-} ; K_3 = 6.61 \times 10^{-13}
\end{align*}
\]

Activity coefficients of H⁺, H₂PO₄⁻ and HPO₄²⁻ are 0.81, 0.61 and 0.23, respectively. In addition, we assumed that the following equilibriums existed:

\[
\begin{align*}
Ca^{2+} + H_2PO_4^- & \rightleftharpoons CaH_2PO_4^{3+} ; K_4 = 31.9 \\
Ca^{2+} + HPO_4^{2-} & \rightleftharpoons CaHPO_4^{2-} ; K_5 = 6.81 \times 10^{2} \\
Ca^{2+} + PO_4^{3-} & \rightleftharpoons CaPO_4^{2-} ; K_6 = 3.46 \times 10^{6}
\end{align*}
\]

Values of the equilibrium constants are taken at 37°C. Activity coefficients of CaH₂PO₄⁺ and CaPO₄²⁻ are 0.72 and 0.72, respectively.
SBF after soaking the samples.

**Figure 9.** Time dependence of the decreases in log(IAP) values of the SBF after soaking the samples.

**Figure 10.** Time dependence of the surface area ratio of the \( a \) face to the \( c \) face of HAp and the decrement of log(IAP) of the SBF.

Figure 9 presents the change of the IAP values, namely decrement of log(IAP), from the initial value of the SBF. The IAP values decreased with increasing soaking periods for all of the samples. The decreases in the IAP values in T75D25 and T10D90 were greater than the decrease for T100D0 for nearly all of the soaking periods. After 7 days, the decrease of IAP values in T10D90 was significantly greater than the decrease in T75D25. **Figure 10** shows the relationship between the decrease of the IAP values of the SBF and the crystal face area ratio, namely (the \( a \) face)/(the \( c \) face). At all the soaking periods, the IAP value decreases the least for T100D0 and the most for T10D90. The aspect ratios of T100D0, T75D25 and T10D90 were 10.4, 15.0 and 16.7, respectively. The decrease of the IAP values is the result of bone-like apatite formation in the SBF. Therefore, these findings indicate that bone-like apatite formation preferentially occurred on the \( a \) face of HAp crystal and rate of calcification of HAp can be controlled by the crystal aspect ratio. Understanding the relationship between the zeta potential and bone-like apatite formation in the SBF is important for the design of novel osteoconductive materials. In this study, we found that the zeta potential values of HAp crystals are dependent on the crystal aspect ratio, namely surface area ratio of (the \( a \) face)/ (the \( c \) face) (Fig. 8). The results suggest that bone-like apatite preferentially forms on the \( a \) face. In other words, formation rate of bone-like apatite on the \( a \) face of HAp crystal is higher than that on the \( c \) face. This implies activation energy for bone-like apatite formation on the \( a \) face of HAp is smaller than that on the \( c \) face. It is difficult to provide a convincing explanation of the effects of the zeta potential of HAp on bone-like apatite formation. However, our results show that the formation of bone-like apatite on HAp, which has negatively zeta potential, was enhanced by decreasing the magnitude of zeta potential of HAp. In future studies we will investigate the effects of the zeta potential on bone-like apatite formation in the SBF.

5. Conclusions

We investigated behaviors of HAp crystals with various aspect ratios, namely HAp crystals with controlled crystal face, in a SBF. Rod-shaped HAp crystals with aspect ratios ranging from approximately 10 to 17 were synthesized under hydrothermal conditions. Scale-like precipitates, which were bone-like apatite crystals, were formed on the HAp crystals soaked in the SBF. The formation rate of bone-like apatite increased with increasing aspect ratios of the HAp crystals. These results imply that bone-like apatite was preferentially formed on the \( a \) face of the HAp crystals and that the calcification behavior of HAp crystals in a SBF can be controlled using the crystal aspect ratios. These findings are applicable for development of new types of ceramic biomaterials with highly functionality.

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