Different properties exhibited on the two typical crystal faces of hydroxyapatite in a simulated body environment

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Abstract. Hydroxyapatite is a main mineral constituent of hard tissues and is extensively used as a biomaterial in the medical field. Hydroxyapatite exhibits anisotropic chemical properties on its two typical crystal faces, the a-face and c-face, due to its hexagonal crystal structure. In polycrystalline bodies, such anisotropy can be enhanced by controlling the crystal orientation. Hydroxyapatite nanocrystals that constitute long bones also form a crystal oriented structure. Therefore, clarification of the difference between the properties of a-face and c-face in hydroxyapatite using in vitro experiments is useful to understand the structure and function of actual hard tissues. Hydroxyapatite ceramics with controlled crystal orientation were prepared by a slip casting method under application of a magnetic field. The fabricated hydroxyapatite ceramics had surfaces consisting mainly of a-face, or otherwise c-face hydroxyapatite. These hydroxyapatite ceramics were immersed into simulated body fluids to investigate the difference in bioactivity. The precipitation behavior observed on the surface of each hydroxyapatite ceramic was different. The thickness of the precipitate was increased and formed earlier on the HAp c-face compared to that on the a-face.

1. Introduction
In line with the aging population of Japan, there has been much emphasis on the development of biomedical materials for tissue engineering. One of the most typical biomedical materials extensively applied in the biomedical field is hydroxyapatite (HAp: Ca₁₀(PO₄)₆(OH)₂) [1-3], a type of calcium phosphate that comprises a substantial portion of the mineral constituent found in human hard tissues [4-9]. Therefore, HAp has received much attention from many researchers [6-8] and is considered to be a promising biomaterial with high biological affinity [8-9].
HAp is also known for its anisotropic properties; as a result of its hexagonal crystal structure, HAp exhibits anisotropic chemical properties depending on its two typical crystal faces, the a-face and c-face. One such example is the anisotropy in adsorption, and much research has been reported on the crystal face dependent adsorption properties of HAp [8, 10-15]. This adsorption dependency is due to the difference in the dominant atoms composing each crystal face of HAp; positively charged calcium on the a-face and negatively charged phosphate groups on the c-face [14-15]. For example, Kawachi et al. [8] reported that porous polycrystalline apatite with a larger a-face surface area than c-face had a higher selectivity for adsorption of bovine serum albumin (acidic protein) than lysozyme chloride (basic protein).

In polycrystalline bodies, such anisotropic properties can be enhanced by controlling the crystal orientation. Magnetic field is a useful tool to control the crystal orientation of magnetically anisotropic materials [16-25]. Due to the recent developments in superconducting technology, magnetic orientation has been extended to diamagnetic materials such as polymers [18] and ceramics [19-25]. For example, Kimura et al. [18] reported the magnetic orientation of various polymers, and Tanaka et al. [20] reported that the c-axis of ZnO can be oriented using a rotating magnetic field with subsequent sintering. The magnetic orientation of HAp was first reported by Inoue et al. [21]. Sakka et al. [22,23] disclosed the effect of agglomeration on the texture of HAp ceramics fabricated by slip casting under a high magnetic field, and concluded that highly textured HAp could be fabricated by slip casting with a well dispersed suspension of HAp single crystals in a high magnetic field followed by sintering. Furthermore, Akiyama et al. [24,25] reported that polycrystalline HAp with c-axis orientation could be formed by slip casting under a high magnetic field with mechanical sample rotation.

The hard tissues in humans are composed of HAp nanocrystals that form a self-organized structure exhibiting crystal orientation in a particular direction [26]. Taking the human long bone for example, the c-axis of HAp is oriented in the direction parallel to the ultimate load direction [26]. The crystal orientation enhances the anisotropic properties of HAp; therefore, the long bone selectively utilizes the anisotropic properties that depend on the two typical crystal faces of HAp. However, the anisotropy in bioactivity exhibited on these two crystal faces in a simulated body environment has not been investigated until now.

Simulated body fluid (SBF) is a valid medium for evaluation of the bioactivity of such a material, and much research using SBF has been reported to date[27-34]. Kokubo et al. [31] reviewed the in vitro ability of various materials to form apatite layers on their surfaces in SBF and their corresponding in vivo bioactivities, and concluded that examination of apatite formation on a material in SBF is useful for predicting the in vivo bioactivity of a material. They also reported that a material that is able to form apatite on its surface in SBF can bond to living bone in a shorter period. Yamashita et al. [32] reported that the bone-like crystal growth on polarized HAp ceramics immersed in supersaturated SBF (1.5SBF) was accelerated on a negatively charged surface and decelerated on a positively charged surface. Tanahashi et al.[33] have investigated the surface functional group dependence on apatite formation on self-assembled monolayers (SAMs) in SBF and reported that SAMs of alkanethiols with negatively charged groups, such as phosphoric (-PO4H2) and carboxyl groups (-COOH), strongly induced apatite formation, while the positively charged amino group (-NH2) did not. Kim et al.[34] reported that the initial bone-like apatite formation took place earlier on the surface of HAp sintered at a lower temperature, which had a stronger negative charge, by attracting calcium ions to form calcium-rich amorphous or nano-crystalline calcium phosphate on its surface in SBF.

In this study, HAp ceramics with controlled orientation have been prepared by a slip casting method under the imposition of a magnetic field. The fabricated HAp ceramics had a surface mainly consisting of a-face or otherwise c-face of HAp, and in vitro experiment were carried out to examine the difference in bioactivity between the two typical crystal faces of HAp in a simulated body environment provided by SBF.

2. Experimental Procedure
2.1. Preparation of Crystal Oriented HAp Ceramics

HAp particles (HAp-200, Taihei Chemical Co.) were dispersed and suspended in ion-exchanged water using a dispersing agent (CELUNA D-305; Chukyo Yushi Co.). The mixture was mechanically ground in an agate mortar using a milling machine (Type ANM1000, Nitto Kagaku Co. Ltd.) to break down aggregated particles. After milling for over 4 hours, the slurry was poured into gypsum molds and slip cast, with or without the application of a 10 T magnetic field, to fabricate HAp green compacts with controlled orientation or with random orientation, respectively. In the former case, a green compact with a-face orientation was fabricated using a vertical magnetic field [22,23], while that with c-face orientation was fabricated using a horizontal magnetic field with mechanical mold rotation [24,25]. The green compacts were dried in air at room temperature for over 24 hours in the absence of a magnetic field, and were sintered at 1573 K in an electric furnace under atmospheric pressure. The sintered HAp ceramics were discoid in shape with diameters and thicknesses of approximately 12 mm and 2 mm, respectively. The crystal orientation and grain size distribution of the three HAp ceramics were then evaluated.

2.2. Evaluation of Crystal Orientation and Grain Size Distribution

The crystal orientation of the HAp ceramics was evaluated using X-ray diffraction (XRD; cat. no. 2035G403, Rigaku Corp.) with Cu-Kα radiation, after the ceramics surfaces were polished with emery paper of grade #2000. Scanning electron microscopy (SEM; VE-7800, Keyence Corp.) was used to evaluate the grain size distribution by examination of 82 to 104 grains in SEM images of each sample etched with HNO3.

2.3. Evaluation of Bioactivity using SBF

Conventional SBF (c-SBF) was prepared by dissolving NaCl, NaHCO3, KCl, K2HPO4, MgCl2·6H2O, CaCl2, Na2SO4, (CH2OH)3(CNH)2 (buffer) and HCl (pH adjuster) in pure water, as proposed by Kokubo et al.[27-31]. The ion concentration of c-SBF is shown in Table 1. The HAp ceramics were polished with 0.05 μm alumina buff to obtain a mirror-finish surface. The HAp ceramics were then immersed into c-SBF at a constant temperature of 310 K. The SBF was not renewed during this study. After 12, 24, 48 and 72 hours of immersion, the top surface and cross-section of the HAp ceramics were observed using SEM (VE-7800, Keyence Corp.) to evaluate the precipitation behavior on the surface.

3. Results and Discussion

3.1. Evaluation of Crystal Orientation and Grain Size Distribution

Figure 1 shows XRD patterns of the three HAp ceramics prepared. For the HAp ceramic fabricated in the absence of a magnetic field (Fig. 1(a)), the XRD pattern had reflections of various crystal faces and the high intensity peaks were very similar to that of HAp (JCPDS #73-0293), which indicates that randomly oriented HAp was successfully fabricated. However, the XRD patterns of the two HAp ceramics slip cast under magnetic fields were significantly different to that of the randomly oriented HAp ceramic. HAp ceramic fabricated under a vertical magnetic field (Fig. 1(b)) displayed an intense (300) reflection corresponding to the a-face, and c-face reflections were of very low intensity compared to that of the randomly oriented one. On the other hand, HAp ceramic fabricated under a horizontal magnetic field with mechanical mold rotation (Fig. 1(c)) displayed strong (002) and (004) reflections, which correspond to c-faces, and reflections of other faces were significantly suppressed.

| Positive ion | Concentration [mol/m³] | Negative ion | Concentration [mol/m³] |
|--------------|-------------------------|--------------|------------------------|
| Na⁺         | 142.0                   | Cl⁻          | 147.8                  |
| K⁺          | 5.0                     | HCO₃⁻        | 4.2                    |
| Mg²⁺        | 1.5                     | HPO₄²⁻       | 1.0                    |
| Ca²⁺        | 2.5                     | SO₄²⁻        | 0.5                    |
These results indicate that the two HAp ceramics fabricated with a magnetic field had high crystal orientation to the a-face or c-face.

Figure 2 shows the grain size distribution of each HAp ceramic prepared in this study. More than 90% of the total grains had a diameter between 2 and 20 μm, with an average diameter around 11.5 μm for all of the three HAp ceramics, indicating that no significant differences were observed with respect to grain size and distribution.

3.2. Dependence of Precipitation Behavior on the Crystal Orientation of HAp

Figures 3 (a)-(c) show SEM images of the top surface of the three HAp ceramics immersed for 12 hours in SBF. No obvious precipitation was observed on the a-face oriented HAp ceramic; however, precipitation was evident on the randomly oriented and c-face oriented HAp ceramics. Moreover, the boundaries of the precipitated areas were composed of not curves, but lines on the top surface of the randomly oriented HAp. The precipitation behavior might depend on individual grains, because the precipitated areas were in polygonal shapes that resembled the shape and size of individual grains observed on the etched surface of the randomly oriented HAp ceramic, as shown in Fig. 4.

The variation in the thickness of the top surface precipitate that formed on the a-face and c-face oriented HAp ceramics immersed in SBF was evaluated over time for 12, 24, 48 and 72 hours. The average thickness of the precipitate was calculated from sixteen randomly selected positions measured from SEM cross sectional images. The calculated values are shown in Fig. 5. The precipitate became much thicker on the c-face oriented HAp ceramic compared to the a-face oriented one, which may indicate that the c-face has a greater ability to form a precipitate than the a-face. The thickness of the precipitate on the c-face oriented HAp ceramic seemed to become saturated at 48 hours of SBF immersion. Furthermore, precipitate formation occurred within a shorter time on the c-face oriented HAp than on the a-face oriented HAp. These results suggest that the c-face will bond to living bone in a shorter period than the a-face.
The difference in the precipitation time and thickness on a-face oriented and c-face oriented HAp may be due to the difference in the surface charge on the HAp faces [13-15]. Taking into account the results of Yamashita et al.[32], Tanahashi et al.[33] and Kim et al.[34], we may conclude that the faster and thicker precipitate formation on the c-face oriented HAp ceramic is due to the negatively charged surface, which strongly attracts calcium ions present in the SBF and accelerates precipitate growth. Slower and thinner precipitate formation on a-face oriented HAp is possibly due to the positively charged surface, which attracts chloride ions and restricts crystal growth, as mentioned in Reference 32. Considering this, the reason why the thickness of precipitate on the c-face oriented HAp exhibited a saturation plateau could be explained as follows. The precipitation rate on the c-face oriented HAp was initially fast, so that the concentration of calcium ions in the SBF must have decreased, leading to a reduction of the precipitation rate. Onuma et al. [35-37] reported that the growth mechanism was dependent on the a-face and c-face orientation of HAp. According to his study, the growth mechanism on the a-face was a combination of step flow and two-dimensional nucleation, while the growth on the c-face followed a multiple two-dimensional nucleation mechanism. These different growth mechanisms might also contribute to the significant difference in the thickness of the precipitate produced during SBF immersion.

4. Conclusion
HAp ceramics with controlled crystal orientation were prepared by a slip casting method under a magnetic field. According to the results of XRD and SEM observations, the fabricated HAp ceramics had surfaces mainly consisting of a-face or otherwise c-face HAp, with no significant difference in grain size and grain size distribution. An experimental investigation of the bioactivity dependency on the two typical crystal faces of HAp was conducted in a simulated body environment provided by SBF.
It was concluded that the thickness of the precipitate was increased and formed earlier on the HAp c-face compared to that on the a-face.

Acknowledgement
This work was partially supported by JSPS Asian Core Program “Construction of the World Center on Electromagnetic Processing of Materials”

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