Difference in Bioactivity, Initial Cell Attachment and Cell Morphology Observed on the Surface of Hydroxyapatite Ceramics with Controlled Orientation

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Hydroxyapatite ceramics has been demonstrated to be an appropriate material for biomedical applications owing to its bioactivity and high biocompatibility. It has an anisotropic crystal structure that belongs to the hexagonal system, and two types of crystal planes mainly appear on its crystal, which are a-plane and c-plane. Since these two crystal planes are very different in atomic elements, numbers and arrangements, they exhibit different nature (anisotropy). For this reason, it is said that crystal orientation might be intensifying its bioactivity and biocompatibility. However, the differences in biological features on these two crystal planes are not fully clarified yet. In this study, we have conducted an assessment to reveal anisotropic biological features of hydroxyapatite by using hydroxyapatite ceramics with controlled orientation fabricated by slip casting under a magnetic field. Tanase et al. have recently reported the difference in bioactivity on the two crystal planes by immersing crystal oriented hydroxyapatite ceramics into the simulated body fluid and found that c-plane oriented hydroxyapatite ceramics formed a precipitate layer earlier and thicker on its surface compared to a-plane oriented one. We first carried out Welch’s t-test on the difference in the thickness of the precipitate layer, reported previously to reveal the difference in bioactivity. Secondly, we conducted an osteoblast culture experiment on hydroxyapatite ceramics with controlled orientation to reveal the difference in initial cell attachment and cell morphology on the two crystal planes of hydroxyapatite using optical microscope observations. In the former case, the results of the Welch’s t-test indicated that the thickness of the precipitate significantly differed on each crystal oriented hydroxyapatite ceramics (P < 0.05). In the latter case, initial cell attachment seemed to be better on the a-plane oriented hydroxyapatite ceramics and also the morphology of the osteoblasts seemed to be rounded on the a-plane oriented hydroxyapatite ceramics compared to the c-plane oriented one. [doi:10.2320/matertrans.MBW200836]

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1. Introduction

Hydroxyapatite (HAp; Ca₁₀(PO₄)₆(OH)₂) is the major inorganic component of human hard tissues.¹⁻⁶ and it is a bioactive and biocompatible material widely utilized in biomedical applications.¹⁻⁸ It has an anisotropic crystal structure that belongs to the hexagonal system, and two types of crystal planes mainly appear on its crystal, which are a-plane and c-plane. Since these two crystal planes are very different in atomic elements, numbers and arrangements, they exhibit different nature (anisotropy). For example, the c-plane of HAp is mainly occupied by negatively charged phosphoric acid groups that adsorbs the amino acids in proteins, while the a-plane of HAp is mainly occupied by positively charged calcium ions that adsorbs the carboxyl groups in proteins.⁹,¹⁰ For this reason, it is believed that the bioactivity and biocompatibility of polycrystalline HAp might be intensified by crystal orientation. In fact, the human hard tissues are composed of HAp nanocrystals that form a self-organized structure exhibiting crystal orientation in a particular direction.¹¹ In the human long bone, for instance, the c-plane of HAp is oriented in the direction perpendicular to the longitudinal axis of the collagen fibril and the direction of the critical load.¹¹ The crystal orientation enhances the anisotropic properties of HAp; thus, the natural hard tissues must be selectively utilizing the anisotropic properties that depend on the two typical crystal planes of HAp. However, the difference in the biological features of each crystal plane of HAp has not been fully clarified yet.

On the other hand, it has been reported that the crystal orientation in materials can be controlled by applying an external magnetic field.¹²⁻¹⁴ Owing to the recent developments in superconducting technology, controlling the crystal orientation using a magnetic field has been extended to non-magnetic substances including HAp.²⁻⁵,¹⁵,¹⁶ Tanase et al.¹⁵ have recently reported the difference in the bioactivity of the two crystal planes by immersing crystal oriented HAp ceramics into the simulated body fluid, and they found that the precipitate layer formed earlier and thicker on the surface of c-plane oriented HAp ceramics than on the surface of a-plane oriented one. First, we carried out Welch’s two-tailed t-test on the difference in the thickness of the precipitate layer; by further analyzing the precipitate layers formed on samples used by Tanase et al. and by analyzing those formed on similar samples used in a duplicate experiment, to clarify the difference in bioactivity. Second, we conducted an osteoblast culture experiment on the HAp ceramics with controlled orientation to reveal the difference in the initial cell attachment and cell morphology on the two crystal planes of HAp by means of optical microscope observations.

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2. Experimental Procedure

2.1 Fabrication of HAp ceramics with controlled orientation

Commercial powder of HAp (Taihei Chemical Industrial Co. LTD., HAp-200) was dispersed and suspended in ion-exchanged water using a polymeric dispersing agent. The particle size distribution of the initial HAp powder used in this study, measured using a laser scattering particle size distribution analyzer (HORIBA, LA-920), is shown in Fig. 1. The particles had a modal and median diameter of about 10 µm and more than 90% of the particles had a diameter between 4–30 µm. The prepared suspension was ground in an agate mortar using an automatic milling machine (Nitto Kagaku Co. Ltd., Type ANM1000) to disintegrate the aggregated particles.2,3) A well-dispersed slurry of the disaggregated HAp particles was obtained by milling the suspension for more than 14.4 ks (4 h). The slurry was then poured into plaster molds and slip cast inside the bore of a superconducting magnet generating a 10 T magnetic field in order to form HAp green compacts with controlled orientation. In addition, slip casting was carried out in the absence of a magnetic field to prepare a green compact without controlled orientation as a control. The slip casting conditions are summarized in Fig. 2.

The crystal orientation of HAp under a magnetic field is expressed in terms of the magnetization energy $U$ by the following equation.5)

$$U = -\frac{1}{2\mu_0} \chi B^2 V$$  (1)

Here, $\mu_0$, $\chi$, $B$, and $V$ are magnetic permeability in vacuum, magnetic susceptibility of HAp in the direction parallel to that of the external magnetic field, external magnetic flux density, and the volume of the HAp crystal, respectively.

![Fig. 1 Particle size distribution of HAp powder used in this study.](image)

![Fig. 2 Schematic view of slip casting conditions. (a) Slip casting under static magnetic field, (b) Slip casting under rotating magnetic field, (c) Control.](image)
density, and volume of the HAp crystal, respectively. Since the magnetic susceptibility perpendicular to the c-axis, \( \chi_\perp \), and that parallel to the c-axis, \( \chi_\parallel \), are considered to be different (\( \chi_\perp \neq \chi_\parallel \)) in hexagonal crystals, the HAp crystal rotates to minimize its energy. The magnetic susceptibility of HAp is anticipated to be \( \chi_\perp > \chi_\parallel (U_\perp < U_\parallel) \),3 so HAp with a-plane orientation can be fabricated under a static magnetic field2–4,16) and HAp with c-plane orientation can be fabricated under a rotating magnetic field.5,15–17)

The fabricated green compacts were completely dried in the absence of a magnetic field and were subsequently sintered in an electric furnace. Their crystal orientation was then observed by X-ray diffraction (XRD; Rigaku Corp., cat. no. 2035G403) using Cu-K\( \alpha \) radiation, after their surfaces were polished with an abrasive paper of grade #2000.

2.2 SBF immersion test using crystal oriented HAp ceramics

The fabricated HAp ceramics- the a- and c-plane oriented HAp ceramics and the control- were immersed in a conventional simulated body fluid (c-SBF),18) After 43.2, 86.4, 172.8, and 259.2 ks (12, 24, 48, and 72 h) of immersion, the cross-section of HAp ceramics were observed using the scanning electron microscope (SEM: Keyence Corp., VE-7800). The thickness of the precipitate layer formed on the surface of the HAp ceramics was then measured. The measured values were assessed by conducting a statistical analysis called Welch’s two-tailed t-test to clarify the difference in the bioactivity of the HAp ceramics. The thickness was measured at two randomly selected points from 8–12 SEM images collectively obtained from the further analyzed samples used by Tanase et al. and from the similarly characterized samples used in a duplicate experiment.

2.3 Osteoblast culture experiments using crystal oriented HAp ceramics

The osteoblastic cell line MC3T3-E1 (Riken BioResource Center, RCB1126) was used in this study. The cells were incubated in a petri dish with culture medium- alpha-minimum essential medium (\( \alpha \)-MEM; Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma) and a small amount of penicillin-streptomycin solution (Sigma). The petri dish was placed in an incubator providing fully humidified atmosphere kept at 310 K with 5% carbon dioxide (\( \text{CO}_2 \)). The culture medium was changed every 2–3 days and the cells were passaged just before they attained a confluent density.

For the culture experiments, the cells were collected in a fresh medium after being washed with phosphate buffered saline (PBS; Sigma) and detached with 0.25% trypsin-EDTA solution (Sigma). The cells were then seeded on to the fabricated HAp ceramics placed in a 48-well culture microplate at a cell density of \( 2 \times 10^4 \) cells/well. After incubating for a certain time period, the cells were washed twice with PBS and fixed using ethanol. The cells were then stained using Giemsa stain solution to enable observation.

The initial cell attachment efficiency was assessed after incubation for 10.8 ks (3 h) and the cell morphology was observed after incubation for 24 h by means of an optical microscope.

3. Results and Discussions

3.1 Characterization of the fabricated HAp ceramics

All of the fabricated HAp ceramics were discoid in shape with diameters of about 10 mm. Characteristics of the samples used for the SBF immersion test are described in Ref. 6).

The XRD patterns of the three HAp ceramics used for the osteoblast culture experiments are shown in Fig. 3. The XRD pattern of the control (Fig. 3(c)) showed reflections corresponding to various crystal planes and the major intensified peaks were similar to those in the reference data (HAp: JCPDS #73-0293), indicating that the control had a random orientation. The XRD pattern of HAp ceramics fabricated by slip casting under a static magnetic field showed intense reflections corresponding to (300); the a-plane, and planes perpendicular to the c-plane; planes relatively close to the a-plane (with inclination angle smaller than 20 degrees), while the reflections corresponding to the c-plane could be barely observed (Fig. 3(a)). In contrast, the XRD patterns of HAp ceramics fabricated by slip cast under a rotating magnetic field had intense reflections corresponding to (002) and (004); the c-planes, while the reflections corresponding to other planes were significantly suppressed (Fig. 3(b)). These results indicate that a- and c-plane oriented HAp ceramics were fabricated successfully. After the XRD analysis, the three HAp ceramics were buff-polished with alumina particles of 0.3 \( \mu \text{m} \) (Praxair Surface Technologies, Inc., alumina polishing compound) to reduce the effect of surface
The growth mechanism on the two crystal planes of HAp has been explained by Onuma et al. The growth rate of the precipitate layer is much larger on the c-plane oriented HAp ceramics compared to that on the a-plane oriented one. In addition, we can say that the thickness of the precipitate layer is distributed widely between the maximum and minimum value of 95% confidence interval of the a- and the c-plane oriented HAp ceramics. Thus, it may be inferred that the thickness of the precipitate layer formed on the two crystal oriented HAp ceramics after being immersed in the SBF for 12, 24, 48, and 72 h. The result of Welch’s two-tailed t-test is summarized in Table 1. The results revealed that the thickness of the precipitate layer formed on the crystal oriented HAp ceramics immersed in the SBF was significant at the significant level of \( \alpha = 0.05 \), i.e., \( P < 0.05 \), in every case assessed here.

Two questions arise from the obtained results. The first one is, why does the precipitate layer form earlier and is thicker on the c-plane oriented HAp ceramics than that formed on the a-plane oriented ones? The second question is, why is the 95% confidence interval wider in the case of the c-plane oriented HAp ceramics than that in the case of the a-plane oriented ones? The former question may be explained by the difference in the growth mechanism and/or that in the surface charge on the two crystal planes of HAp. The growth mechanism on the two crystal planes of HAp has been explained by Onuma et al.\(^{19-21}\) According to their study, the crystal growth mechanism on the a-plane is a combination of step flow mechanism and two-dimensional nucleation, while that on the c-plane is a multiple two-dimensional nucleation. Such difference in the growth mechanism may have caused the significant difference in the thickness of the precipitate layer. As for the surface charge, Tanahashi et al.\(^{22}\) have investigated the surface functional group dependence on apatite formation on self-assembled monolayers (SAMs) in the SBF and have reported that SAMs of alkanethiols having negatively charged groups such as phosphoric groups (-PO\(_2\)H\(_2\)) and carboxyl groups (-COOH) strongly induced apatite formation, while the positively amino groups (-NH\(_2\)) did not strongly induce apatite formation. Since the a-plane of HAp is positively charged and the c-plane is negatively charged,\(^{9,10}\) we may anticipate that this polarization difference is another reason why the thickness of the precipitate layer formed on the two crystal oriented HAp ceramics differed. In fact, the thickness of the precipitate layer formed on the control varied over different regions, showing polygonal shapes that well resembled the shapes of individual grains observed on the etched surface of the control (see Ref. 6)). Figure 5 shows the thickness of the precipitate layer formed on the control immersed in the SBF for 72 h along with the 95% confidence interval of the a- and the c-plane oriented HAp ceramics. It can be observed that the thickness of the precipitate layer is distributed widely between the maximum value of 95% confidence interval of the c-plane oriented HAp ceramics and the minimum value of 95% confidence interval of the a-plane oriented HAp ceramics. Thus, it may be inferred that the thickness of the precipitate layer formed is dependent on the nature of individual crystal planes of HAp. The latter question may also be explained by the different growth mechanism on the two crystal planes of HAp. Since the growth mechanism on the a-plane is a combination of step flow mechanism and two-dimensional nucleation, the precipitate layer must have formed gradually, meaning that perhaps the precipitate layer forms quite uniformly. Meanwhile, since the growth mechanism on the c-plane is a multiple two-dimensional nucleation, the nucleation must have taken place throughout the surface, meaning that the precipitate layer forms scabrous compared to that on the a-plane oriented one. In addition, we can say that the growth rate of the precipitate layer is much larger on the c-plane oriented HAp ceramics compared to that on the a-plane oriented one from Fig. 4. This large growth rate on the c-plane perhaps enhanced the asperity of the surface. Actually, the standard deviation of the thickness of the precipitate layer was approximately 2 times larger on the c-plane than that on the a-plane. We perhaps measured this asperity, and this may have appeared in the results as the wider confidence interval on the c-plane oriented HAp ceramics.
3.3 Initial cell attachment on the crystal oriented HAp ceramics

The initial cell attachment efficiency was calculated after incubating for 3 h by dividing the number density of cells observed in an image taken using the optical microscope by the number density of cells seeded into the well. The results are shown in Fig. 6. It can be observed that the initial cell attachment efficiency on the a-plane oriented HAp ceramics was higher than that on the control, while that on the c-plane oriented one was lower than that on the control. This may be a result of the difference in the absorbability of proteins of the two crystal planes of HAp. Acidic proteins are believed to be adsorbed on the a-plane, while basic proteins are believed to be adsorbed the c-plane. In this study, perhaps, selective adsorption of bovine serum albumin (BSA; an acidic protein) in the FBS, which was added to the medium, occurred on the a-plane oriented HAp ceramics, and that might have promoted initial cell attachment. Kawachi et al. reported that porous HAp having a larger surface area of a-plane showed higher absorbability of albumin as compared to the isotropic HAp. Further, Bernard et al. reported that the biggest impact on MC3T3-E1 cell binding to HAp was found in the case of substrates with adsorbed BSA among bone sialoprotein (BSP), bone osteopontin (OPN) and BSA, and they concluded that BSA influences the adhesion and proliferation of osteoblasts to HAp. However, a further investigation on other effects such as those of cell adhesive proteins is required.

3.4 Cell morphology

Figures 7–9 show images of the Giemsa-stained cells on the three HAp ceramics after incubating for 24 h. The cells morphology was observed from the images. It appeared that the morphology of the cells on the a-plane oriented HAp ceramics was more rounded as compared to that of the cells on the c-plane oriented ones, as shown in Figs. 7 and 8. In contrast, the cells on the c-plane oriented HAp ceramics appeared to be elongating its nueraxons, as shown in Fig. 8. Both types of cells appeared to co-existed on the control, as shown in Fig. 9, without showing distinct partiality to either. The difference in the cell morphology observed here may have been caused by the difference in step of growth after the initial cell attachment or by the affinity between cell adhesion proteins and the atoms dominating the crystal planes.

However, further investigations are required to understand this phenomenon.

4. Conclusion

In this study, we have conducted two assessments to reveal the anisotropic biological features of HAp by using crystal oriented HAp ceramics fabricated by slip casting under a magnetic field. One was Welch’s t-test conducted on the difference in the thickness of the precipitate layer formed on crystal oriented HAp ceramics immersed in SBF, as recently
reported by Tanase et al., to clarify the difference in the bioactivities on the two crystal planes of HAp. The other was the cell culture experiment of MC3T3-E1 on crystal oriented HAp ceramics to reveal the difference in initial cell attachment and cell morphology on the two crystal planes of HAp by means of optical microscope observations. By conducting such assessments, the following results were obtained.

(1) There was significant difference in the thickness of the precipitate layer between the a-plane oriented HAp ceramics and the c-plane oriented ones ($P < 0.05$).

(2) The thickness of the precipitate layer appeared to be dependent on the nature of individual crystal planes of HAp.

(3) The initial cell attachment efficiency of MC3T3-E1 was higher on the a-plane oriented HAp ceramics as compared to that on the c-plane oriented ones.

(4) The crystal orientation of HAp appeared to affect the cell morphology of MC3T3-E1.

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REFERENCES