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Development of bioceramics with life functions by harnessing crystallographic anisotropy and their biological evaluations

Mamoru AIZAWA^{1,2,†}

¹School of Science and Technology, Meiji University, 1-1-1 Higashimita, Tama-ku, Kawasaki 214-8571, Japan

²International Institute for Materials with Life Functions, Meiji University, 1-1-1 Higashimita, Tama-ku, Kawasaki 214-8571, Japan

Hydroxyapatite (HAp) is a mineral component of vertebrate hard tissues, thus it assumes a pivot role as essential biomaterials for bone repair. The HAp crystal belongs to a hexagonal system, and has two types of crystal planes with different atomic arrangements: positively-charged calcium ions are packed mainly in the *a(b)*-plane, while negatively-charged phosphate ions and hydroxyl groups are packed mainly in the *c*-plane. In vertebrate long bone surfaces, HAp crystals have a *c*-axis orientation, which leads to the development of the *a(b)*-plane. On the other hand, in tooth enamel surface, HAp crystals have an *a(b)*-axis orientation, which results in the *c*-plane. However, the rationale behind the difference orientations between long bone and tooth enamel in different crystal planes remains poorly understood. Therefore, we would like to elucidate the effect of crystallographic anisotropy of HAp on its osteogenic functions. In particular, single-crystal HAp particles with preferred orientation to *a(b)*- and *c*-axes were successfully synthesized as the models to mimic bone and tooth enamel and by investigating the specific adsorption of acidic and basic proteins onto these particles, key understanding of bone regeneration process could be made toward developing bioceramics with life function. In addition, porous HAp ceramics with osteoinductivity and enhanced osteoconductivity properties was also developed using *a(b)*-plane-exposed HAp fibers to further explore the relationship. In this review, we will describe the development of bioceramics with life functions by harnessing the crystallographic anisotropy of HAp to provide greater insight on the biological properties of the resulting bioceramics, such as cell behavior *in vitro* and bone formation *in vivo*.

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1. Introduction

Hydroxyapatite [$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$; HAp] is an inorganic compound with a composition that is similar to that of human bone and teeth.^{1)–3)} HAp is a well-known osteoconductive material that can be used as a biomaterial for artificial bone, cement, and tooth roots.^{4)–9)} In addition, HAp also has been used as an adsorbent for bio-related substances such as proteins and amino acids as HAp does not cause any degeneration to bio-related substances during the adsorption process.^{10)–14)}

HAp typically has a hexagonal crystal structure with a $\text{P6}_3/\text{m}$ space group, as shown in Fig. 1, and its crystal unit cell is characterized as $a = b = 0.942 \text{ nm}$, $c = 0.688 \text{ nm}$, $\alpha = \beta = 90^\circ$, and $\gamma = 120^\circ$.^{15),16)} There are two major crystal planes, which are the *a(b)*-plane (*ac* and *bc* crystal faces) and the *c*-plane (*ab* crystal face). The *a(b)*-plane is rich in calcium ions and positively charged, while the

c-plane is rich in phosphate and hydroxide ions and negatively charged.^{2),10),11)}

Figure 1 also illustrates that the HAp crystals in the hard tissues, exhibiting in different orientations. For example, in living bone, the *c*-axes of HAp crystallites ($50 \text{ nm} \times 25 \text{ nm} \times 4 \text{ nm}$ in size) are parallel to the extending collagen fibers, resulting in the exposure of their *a(b)*-planes on the bone surface.^{17)–22)} On the other hand, in the dental enamel, HAp crystallites ($100 \mu\text{m} \times 50 \text{ nm} \times 50 \text{ nm}$ in size) form enamel prisms extending from the dentino-enamel junction, resulting in *c*-planes that are preferentially parallel to the enamel surface.^{22)–26)} Moreover, the *a(b)*- and *c*-planes exhibit anisotropic characteristics, such as resolvability, biocompatibility, and adsorptive activity.^{2),10),11),27)–29)}

However, the rationale behind the difference orientations between living bone and dental enamel in different crystal planes remains unclear. Therefore, we are now focusing on the crystallographic anisotropy of the HAp crystal to investigate and establish a new scientific principle related to “crystallographic anisotropy and life functions”, which will be discussed in this review.

[†] Corresponding author: M. Aizawa; E-mail: mamoru@meiji.ac.jp

Fig. 1. Apatite structure and model of anisotropic crystal growth: single-crystal apatite particles with preferred orientation to $a(b)$ - and c -axes serve as models for hard tissues (bone and tooth enamel, respectively).

2. What are “bioceramics with life functions”?

In this review, we have defined “bioceramics with life functions” as ceramics that bring out “life functions” by interacting actively with cells and living organisms.³⁰⁾ In their research, the author and his collaborators have focused on the following three life functions: i) three-dimensional tissue regeneration,^{31)–33)} ii) osteogenic activity comparable to autogenous bone,^{34)–36)} and iii) biological defense (e.g., anti-tumor effect,^{37)–39)} enhancement of immune system,^{40)–42)} and infection resistance^{43)–45)}), and are conducting research projects addressing to these topics. This work can be classified into 1) basic research, 2) establishment of novel paradigms through medical-engineering collaborations, and 3) application of these paradigms through industry-academia collaborations. Due to the length constraints the current review, we shall describe the “development of a bioceramics with life functions having a bone-forming ability comparable to autogenous bone by harnessing the crystallographic anisotropy of HAp”.

3. Syntheses of single-crystal HAp particles with preferred orientation in $a(b)$ - or c -axis

In vertebrate long bone surfaces, HAp crystals have a c -axis orientation, which leads to the development of the $a(b)$ -plane; in the tooth enamel surface, such crystals have an $a(b)$ -axis orientation, which leads to the development of the c -plane. We hypothesize that the anisotropy of biological tissues is one of the important factors that contributes to the expression of life functions; based on this model, we have successfully synthesized “single-crystal HAp particles with two types of anisotropy” as the models to mimic bone and tooth enamel. The crystal preparation processes are described briefly below.

Firstly, we shall describe the process of synthesis of single-crystal HAp particles with preferentially exposed $a(b)$ -plane [hereafter, “apatite fibers (AF)”] as the model

for bone. AF can be easily synthesized by a homogeneous precipitation method using an aqueous starting solution containing $0.167 \text{ mol} \cdot \text{dm}^{-3}$ calcium nitrate tetrahydrate $[\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}]$, $0.100 \text{ mol} \cdot \text{dm}^{-3}$ ammonium hydrogen phosphate $[(\text{NH}_4)_2\text{HPO}_4]$, $0.50 \text{ mol} \cdot \text{dm}^{-3}$ urea $[(\text{NH}_2)_2\text{CO}]$, and $0.1 \text{ mol} \cdot \text{dm}^{-3}$ nitric acid (HNO_3).^{46),47)} The starting solution (750 cm^3) is refluxed at 80°C for 24 h to form octacalcium phosphate $[\text{Ca}_8\text{H}_2(\text{PO}_4)_6 \cdot 5\text{H}_2\text{O}]$; OCP, and the OCP is converted into HAp by further heating at 90°C for 72 h.

Next, we shall describe the process of synthesis of the single-crystal HAp particles with preferentially exposed c -plane (hereafter, “plate-shaped apatite”) as the model for tooth enamel. The plate-shaped apatite can be synthesized from the air-liquid interface of a starting solution via the degradation of urea with the enzyme urease.⁴⁸⁾ Briefly, a starting solution with a Ca/P molar ratio of 1.67 is prepared firstly by mixing $5.0 \text{ mmol} \cdot \text{dm}^{-3}$ calcium carbonate (CaCO_3), $3.0 \text{ mmol} \cdot \text{dm}^{-3}$ phosphoric acid (H_3PO_4), and $1.0 \text{ mol} \cdot \text{dm}^{-3}$ $(\text{NH}_2)_2\text{CO}$, and the pH is adjusted to 3.0 by the addition of aqueous HNO_3 . The starting solution is dispensed into glass petri dishes immediately after adding 2.73 cm^3 of urease (activity: $127 \text{ unit} \cdot \text{mg}^{-1}$) solution (0.1 mass %), and these dishes then are heated in an incubator at 50°C for 96 h. Following reaction, the interface product is collected and processed by heat treatment at 600°C for 2 h in an electric furnace at a heating rate of $10^\circ\text{C} \cdot \text{min}^{-1}$.

Figure 2 shows typical electron microscopic images of the two anisotropic apatite particles. Figure 2(a) shows the morphology of AF observed by scanning electron microscopy (SEM). These particles clearly exhibited a fibrous morphology. The ultrastructure of the AF was observed by higher-resolution transmission electron microscopy (HR-TEM). Figures 2(b) and 2(c) show bright-field images together with a selected area electron diffraction (SAED) pattern and a lattice image, respectively. We observed that these single-crystal AF grow along the c -axis direction to develop the $a(b)$ -planes.⁴⁶⁾ AF with the desired Ca/P

Fig. 2. SEM images (a, d) of single-crystal apatite particles with anisotropic structure and examination of their ultrastructure by HR-TEM (b, c, e, f). (a, b, c) AF. (d, e, f) Plate-shaped apatite. The inset in (b) shows the SAED pattern, which has clear spots arranged in rectangles; the spots that align along the long and short axes of the fiber were indexed as $(00l)$ and $(h10)$ spots, respectively, from the $[110]$ zone axis of the highly crystalline hexagonal structure. (c) Two types of orthogonal linear lattices are evident and the lattice spacings are determined to be 0.34 and 0.47 nm, which correspond to $d_{(002)}$ and $d_{(110)}$, respectively. The inset in (e) shows the SAED pattern, which exhibit clear hexagonally arranged spots. The lattice constants are determined to be $a = b = 0.94$ nm and $\gamma = 120^\circ$, which indicate that the observation direction is along the $[001]$ zone axis of the HAp crystal. (f) This lattice image shows a highly periodic arrangement of atoms. The lattice fringes have a spacing of 0.82 nm, which corresponds to $d_{(100)}$. This figure is redrawn based on a part of Figs. 2 and 3 in Ref. 50).

molar ratio was successfully synthesized by changing the Ca/P ratio of the starting solution.⁴⁷⁾

SEM images of the morphology of plate-shaped apatite are provided in Fig. 2(d); notably, the apatite's automorphism shows that it has a hexagonal plate shape. Figures 2(e) and 2(f) show bright-field images of the ultrastructure of the plate-shaped HAp together with the SAED pattern and the lattice image, respectively. We observed that this single-crystal plate-shaped HAp grow in the $a(b)$ -axis direction to develop the c -planes.⁴⁸⁾ The special environment employed for the synthesis, that is, using an air-liquid interface as the reaction field, may impart anisotropy to the growth direction of this HAp crystal. This plate-shaped HAp can serve as the model for tooth enamel. We have also reported on the changes in the crystalline phase and morphology of plate-shaped HAp undergo during heating.⁴⁹⁾

4. Protein adsorption on single-crystal HAp particles with preferred orientation to a - and c -axes

As mentioned in Section 3, the two single-crystal HAp particles, i.e., apatite fiber and plate-shaped HAp, can be synthesized as the models with preferentially exposed

$a(b)$ - and c -planes, respectively. In this section of the review, we examine the selective adsorption of negatively-charged bovine serum albumin (BSA) and positively-charged lysozyme (LSZ) on the different crystal planes of HAp.⁵⁰⁾ Protein adsorption experiments were performed in phosphate-buffered saline [PBS(-)] using synthetic HAp particles, as well as with isotropic HAp particles as a control. Here, commercially-available HAp powder (HAp-100, Taihei Kagaku, Japan) was used as the model of the "isotropic HAp particles". This HAp particle has an isotropic crystal structure as compared to the above-mentioned apatite fiber and plate-shaped HAp.

Figure 3 shows that the $a(b)$ -plane of HAp crystals is selective against the adsorption of acidic proteins BSA, as compared to c -plane of HAp crystals. In contrast, the c -plane of HAp crystals is selective against the adsorption of basic proteins LSZ. The underlying factor for this selective adsorption is hypothesized as follows: the $a(b)$ -planes of HAp crystals are rich in positively charged calcium ions; therefore, acidic negatively charged proteins are more easily adsorbed by the electrostatic force of attraction. On the other hand, the c -planes of HAp crystals are rich in negatively charged phosphate or hydroxide ions; therefore,

Fig. 3. Adsorption isotherms for BSA on apatite fiber (■, *a(b)*-plane), plate-shaped HAp (●, *c*-plane), and isotropic HAp (◆, control) ($n = 5$; error bars: standard deviation). This figure is redrawn based on a part of Fig. 5 in Ref. 50).

basic positively charged proteins are more easily adsorbed. These results indicate that the design and synthesis of HAp crystals with well-controlled orientations is an effective method to improve the crystals' specificity for the adsorption of bio-related substances. Understanding the selective absorption of proteins onto the apatite particles with different crystallographic anisotropy is of practical interest as this is the first interaction that occurs when the biomaterial is implanted *in vivo*.

5. Development of *a(b)*-plane-exposed HAp ceramics as a model of living bone and its cellular response

In vertebrate bones and tooth enamel surfaces, the respective *a(b)*-planes and *c*-planes of HAp crystals are preferentially exposed. However, the reason for HAp crystals exhibiting different orientations in different types of hard tissues is not yet understood. As such, appropriate two-dimensional ceramic models with preferentially exposed *a(b)*-plane are necessary to elucidate the rationale behind this natural phenomenon.

To address this issue, we have successfully fabricated dense HAp ceramic models that mimic the structure of living bones using composite powders of *a(b)*-plane-exposed single-crystal AF and wet-synthesized apatite gels (AG).⁵¹⁾ This process is a "templated grain growth (TGG)" method, in which template particles are AFs and matrix particles are AGs. The TGG method is described in detail in the review by Tani et al.⁵²⁾ The results of crystalline identification and ultrastructural observation showed that the resulting HAp ceramics maintain the *c*-axis orientation of the AF particles, and their higher *a(b)*-plane orientation degrees were maintained with small added amounts of AG. However, when the AG content exceeded 30 mass %, the orientation degree decreased.

Figure 4 shows SEM and TEM images at each stage of the fabrication process. Using HR-TEM, we demonstrated that the *c*-axis orientation of the resulting HAp ceramics was maintained at the nanoscale.⁵¹⁾ The influence of the degree of exposed *a(b)*-plane on the surface characteristics was examined by evaluating the surface zeta-potential and wettability. The results showed that increasing the degree of exposed *a(b)*-plane shifted the surface charge from negative to positive, and decreased the surface wettability.

Initial cell-attachment assays were performed on the above *a(b)*-plane-exposed HAp ceramics using MC3T3-E1 cells as the model for osteoblasts. The initial cell-attachment efficiency of the *a(b)*-plane-exposed HAp ceramics fabricated by AF or low-AG-content composite powders was lower than that of the isotropic HAp ceramics, which was used as the controls. The results showed that the attachment of cells decrease with increasing *a(b)*-plane degree. To our knowledge, this report was the first study that demonstrated the relationship between the surface potential of bioceramics and initial cell adhesion. However, the "*a(b)*-plane" was inferior to isotropic HAp in terms of initial cell adhesion. Nevertheless, we demonstrated that the *a(b)*-plane had a comparable cell proliferation with isotropic HAp, and could promote the differentiation of cells to osteoblasts, thereby leading to calcification.

Figure 5 shows the results of alizarin red staining of osteoblasts at 2 weeks after seeding on *a(b)*-exposed HAp ceramics. The red color indicates the calcified area. The two images showed that the cells that were seeded on the anisotropic ceramic advance further in the calcification stage than the cells that were seeded on the conventional isotropic apatite. The above results would bring one step closer to creating a whole new paradigm that extend the understanding and development of new knowledge in bioceramics toward the expression of a life function by harnessing "crystallographic anisotropy" of HAp. In light of these findings, anisotropic HAp would also offer as promising artificial bones with enhanced bone-formation ability.

Finally, we consider that the reason for the difference in the orientation between living bone [*a(b)*-plane] and dental enamel (*c*-plane) is due to the different cellular responses induced by the distinct crystal planes. In future studies, we hope to determine the actual mechanism by using the *a(b)*-plane-exposed ceramics developed in the present work as the models for living bones. Although the mechanism in which the *a(b)*-plane promotes cell differentiation has not yet been elucidated, the specific adsorption of serum proteins on the ceramic surface (and subsequent effects on protein conformation) may affect cell differentiation. We are currently working on the research to test out this hypothesis. In addition to *a(b)*-plane-exposed HAp ceramics, we have also succeeded in creating *c*-plane-exposed HAp ceramics via a reactive template grain growth route.⁵³⁾ In near future, we hope to elucidate the mechanism, in which the *a(b)*-plane promotes cell differentiation

Fig. 4. SEM images at each stage, starting powder, compact, and dense ceramics of fabrication process for $a(b)$ -plane-oriented HAp ceramics as a model of living bone, together with the ultrastructure of the final products: (a) low-magnification TEM image, (b) SAED pattern of crystal grain #1, and (c) intermediate-magnification and (d) higher-magnification HR-TEM images of crystal grain #1. Scale bars: (a) 200 nm, (c) 5 nm, and (d) 1 nm. (a) Three crystal grains (#1, #2 and #3) are seen. Grain boundaries also can be observed. (b) These SAED patterns show clear spots, corresponding to the $[110]$ zone axis of the hexagonal structure with higher crystallinity. The lattice parameters and axial angle determined for these patterns were $a = b = 0.94$ nm, $c = 0.69$ nm, and $a = b = 90^\circ$, values that are in good agreement with those of the HAp crystal structure. (c) Two types of orthogonal linear lattices with different cycles can be observed. The lattice spacings were determined to be 0.47 and 0.34 nm. Those values are in good agreement with the 0.472 and 0.344 nm values for $d_{\text{spacing}(110)}$ and $d_{\text{spacing}(002)}$, respectively. (d) The HAp crystal unit cell observed from the $a(b)$ -plane direction has a rectangular shape that is 0.688 nm higher and 0.944 nm long. In this lattice image, the same rectangle with a highly periodic sequence can be observed. SAED patterns and lattice images similar to those of crystal grain #1 were obtained for the other two crystal grains (#2 and #3). Therefore, the resulting HAp ceramics maintained the preferred orientation in the $a(b)$ -plane. This figure is redrawn based on a part of Figs. 2, 3 and 7 in Ref. 51).

Fig. 5. Evaluation of calcification by alizarin red staining of human osteosarcoma-derived osteoblast cell line MG63 cultured on the two HAp ceramics for 2 weeks: (a) isotropic HAp ceramics, (b) $a(b)$ -plane oriented HAp ceramics.

by comparing proteins adsorbed onto isotropic and crystallographic anisotropy-controlled HAp ceramics based on the study of proteomic analysis and other techniques.

6. Development of novel bioceramics with osteoinductivity and enhanced osteoconductivity properties

In the previous section, we described the research showing that the $a(b)$ -plane of HAp promoted the differentiation of osteoblasts and calcification. We postulated that $a(b)$ -plane-exposed HAp ceramics with three-dimensional porous structure has better osteoconductivity property. The term “osteoconductivity” refers to the ability to facilitate bone formation in the presence of osteoblasts. In addition, porous HAp ceramics exposed on the $a(b)$ -plane may have “osteoinductivity” property, that is, osteogenesis in the absence of osteoblasts. Artificial bone with osteoinductivity property could potentially replace autogenous bone, which is currently the gold standard in the orthopedic field.

We have successfully fabricated HAp ceramics with well-controlled porous structures using the above-mentioned apatite fiber.^{54)–56)} These porous HAp ceramics were constructed by intertwining individual fibers, such that the pore structures were mostly opened pores. In addition, we succeeded in synthesizing silicon-containing AF (Si-AF), in which silicon (Si) was added to the above-mentioned single-crystal HAp particles with preferred orientation to the *c*-axis (i.e., AF).⁵⁷⁾ Silicon is known to promote bone formation.⁵⁸⁾ Porous Si-containing HAp ceramics (hereafter, Si-HAp ceramics) with porosities of 70 and 85 % can be fabricated easily by adding carbon beads with diameters of 20 and 150 μm as pore-forming agents into Si-AFs, follow by firing the green compacts at 1300 $^{\circ}\text{C}$ for 5 h in a steam atmosphere. **Figure 6** shows the typical microstructures of such porous HAp and Si-HAp ceramics. We can observe macro pores of about 150 μm and micro pores of about 20 μm in the four kinds of porous ceramics, depending on the diameters of the pore-forming agents.

In order to showcase the osteoconductivity property (osteogenesis in the presence of osteoblasts) of the above porous HAp ceramics, we implanted these materials into porcine tibia to examine the formation of bone inside and around the specimens. Bone formation, in other words, osteoconductivity property, was confirmed inside and around all of the implanted specimens. Notably, the amounts of calcified bone in Si-HAp ceramics were higher than those observed with pure HAp ceramics. Moreover, a comparison of the Si-HAp ceramics with porosities of 70 and 85 % revealed that the proportion of calcified bone was higher in the former than in the latter. These results indicated that the inclusion of silicon and optimization of porosity could lead to promotion of bone formation (osteoconductivity).

In addition, minimal ectopic bone formation was observed in the specimens that were implanted in the porcine adipose tissue, but ectopic bone formation could be observed in pure HAp and Si-HAp ceramics with a porosity of 70 % when the specimens were implanted into muscle tissue, as shown in **Fig. 7**. Notably, ectopic ossification of the specimens with a porosity of 70 % occurred in all the 8 specimens. The control of porosity might be one of the important factors that contribute to the expression of

Fig. 6. Microstructure of apatite-fiber-derived porous ceramics with osteoinductivity and enhanced osteoconductivity properties: (a, b) pure HAp ceramics, (c, d) Si-containing HAp ceramics. Porosities: (a, c) approximately 70 %, (b, d) 85 %. This figure is redrawn based on a part of Fig. 2 in Ref. 52).

Fig. 7. Hematoxylin and eosin (HE) and Masson's trichrome (MT) staining of specimens removed from muscle tissue (decalcified sections). Higher magnification: highly magnified images of the dashed areas in the low-magnification images. HE staining: nucleus, blue; cell membrane, red. MT staining: muscle fiber, red; collagen fiber, violet. White asterisk: bone formation area. Black arrow: residual material. This figure is redrawn based on a part of Fig. 9 in Ref. 52).

osteointductivity (i.e., bone formation in the absence of osteoblasts) property.

At present, autogenous bone grafts are the gold standard in the field of orthopedic surgery. The porous HAp ceramics with porosity of 70 % described in this study showed comparable osteointductivity property as autogenous bone. Therefore, our findings will be a cornerstone in guiding functional design for the development of next-generation artificial bone-like substitutes for autogenous bone.

7. Conclusions

Our findings on harnessing the crystallographic anisotropy HAp demonstrated a progressive step forward to the expression of life functions by new approaches, and would also offer as promising artificial bones with enhanced bone-formation ability, including osteointductivity property. On a side note, we are also conducting research on the regeneration of other tissues (including bone,^{31),59)} liver,^{32),60)} and blood vessels³³⁾ and on biological defenses (including anti-tumor effects,^{37)–39)} enhancement of immune system function,^{40)–42)} and infection resistance^{43)–45)}). Further information can be obtained by visiting the website for our laboratory (http://www.isc.meiji.ac.jp/~a_lab/) or The Meiji University International Institute for Biomaterials with Life Functions (http://www.isc.meiji.ac.jp/~a_lab/institute/).

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Mamoru Aizawa is a Professor at Faculty of Science and Technology, Meiji University, and also a director at International Institute for Materials with Life Functions. He received his Ph.D. in Engineering from Sophia University in 1996. After working Kao Corporation, Sophia University, he moved as an associate professor to the Meiji University at 2003 and then promoted the professor at 2008. In addition to the above, he has worked as a visiting scholar and project leader at the University of Cambridge (2001.10–2002.9) and the Kanagawa Academy of Science and Technology (2009.10–2013.9), respectively. He is specialized in biomaterials and tissue engineering on the basis of ceramics.