Preparation of spherical porous hydroxyapatite granules as support materials for microorganisms

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Suitable support materials for microorganisms are useful for bioreactors to achieve high efficiencies. Spherical porous hydroxyapatite (HA) granules about 5 mm in diameter composed of rod-like particles were prepared through hydrothermal treatment. The granules with and without pores tens of micrometers in size were prepared. These granules were immersed in a suspension containing microorganisms obtained from the soil, and the adhesion of microorganisms on the granules was examined. The adhesion of microorganisms on the surfaces of the HA granules was observed. The total metabolic activity of the microorganisms adhering onto the granule with pores tens of micrometers in size was higher than that adhering onto the granule without such pores. These results suggested that the HA granules had an affinity for microorganisms, and that granules with pores tens of micrometers in size are expected to be support materials for microorganisms.

Bioreactors involving microorganisms are widely utilized in various fields. In bioreactors, suitable support materials are used that provide the preferred properties such as high efficiency and easy handling.¹,² As support materials for microorganisms, polymer materials are widely used, but ceramic materials are also promising because of their chemical durability and stiffness.³ In the present study, hydroxyapatite [HA; Ca₁₀(PO₄)₆(OH)₂] was focused as support materials for microorganisms. HA is a main component of human bones and teeth, and shows great biocompatibility⁴,⁵ and adsorption properties.⁶ This biocompatibility may interact well with the attachment of microorganisms and the adsorption properties may increase the efficiency of bioreactors. There are several reports on the use of HA and HA-coated materials as support materials for microorganisms.⁷-⁹ We have also previously reported that HA shows superior properties for the adhesion of E. coli.¹⁰

For the application of HA as support materials for microorganisms, the design of structure is important. It is expected that spherical porous HA granules composed of rod-like particles that expose the a face of HA crystals are suitable. The spherical shape and porous structure permit easy handling and provide large surface areas for microorganisms, respectively. The exposure of the a face might be advantageous for the adhesion of microorganisms, because this face has positive sites¹¹ and the surfaces of microorganisms are typically negatively charged.¹² Moreover, the entanglement of the rod-like particles imparts non-brittle fracture behavior to the ceramics.¹³ In the present study, spherical porous HA granules composed of rod-like particles were prepared and their potential for supporting materials for microorganisms was examined.

Two types of spherical porous HA granules with and without pores tens of micrometers in size were prepared. A hydrothermal process was used to obtain the spherical porous HA granules composed of rod-like particles.¹⁴ Three grams of α-tricalcium phosphate [α-TCP, Ca₃(PO₄)₂] powder (Taihei Chemical Industrial, Japan) and 2.4 g of polymethylmethacrylate (PMMA) beads (Sekisui Plastics, Japan) as porogens, whose diameter was about 50 μm, were mixed with 1.9 g of a 15 mass % polyvinyl alcohol (PVA; Wako Pure Chemical Industries, Japan) solution. The resultant mixture was kneaded by hand, resulting in the formation of spherical granules about 5 mm in diameter. As a reference material, the granules without the PMMA beads were also prepared by mixing 10 g of α-TCP powder with 4.0 g of the 15 mass % PVA solution. The resultant mixture was again kneaded by hand and spherical granules about 5 mm in diameter were formed. The granules prepared with and without the PMMA beads were named Granule-H (high porosity) and Granule-L (low porosity), respectively. The granules were freeze-dried and sintered at 1200°C for 10 min for burning out the organic matters and sintering to obtain porous α-TCP granules. The twelve porous granules were placed in

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A Teflon®-lined autoclave (90 cm³) with 30 cm³ of distilled water and hydrothermally treated in vapor conditions at 105°C for 48 h, as shown in Fig. 1. After the hydrothermal treatment, the granules were washed and then immersed in distilled water at 90°C for 3 h to complete the conversion of the crystal phase to HA. The crystal phase of the products was examined by X-ray diffractometer (XRD; RINT-2200VL, Rigaku, Japan) with Cu Kα radiation. The morphology of the granules was observed using a scanning electron microscope (SEM; SU8000, Hitachi, Japan). The bulk density was calculated from the size and weight of the granules, and the porosity was determined from the obtained bulk density and the theoretical HA density (3.16 g·cm⁻³). Ten granules were considered to calculate the porosity. The specific surface areas of the granules were measured by the Brunauer–Emmett–Teller (BET) method using N₂ gas with a gas sorption analyzer (Autosorb-iQ, Quantachrome Instruments, USA).

Microorganisms were obtained from the soil in the Aobayama Campus of Tohoku University. Assuming the application of waste water treatment, a consortium containing various microorganisms was used. Ten grams of the soil was added to 50 cm³ of distilled water and stirred. The suspension was filtered with a filter paper which can keep particles of sizes larger than 20–25 μm. The filtered solution containing the microorganisms was used for the evaluation; 5 cm³ of the filtered solution containing microorganisms was mixed with 45 cm³ of Luria Broth and maintained at 30°C for 24 h for incubation. After incubation, the number of microorganisms was counted and a suspension containing 1 × 10⁸ cells·cm⁻³ of microorganisms was prepared by diluting the Tris-HCl buffer solution of pH 7.5. The granule was immersed in 1.2 cm³ of the as-obtained suspension and maintained at 30°C for 45 min for adhesion of the microorganisms. After 45 min, the granules were taken out and washed with phosphate buffer saline and immersed in a mixed solution of 1.14 cm³ Tris-HCl buffer and 0.06 cm³ Microbial Viability Assay Kit-WST (Dojindo Laboratories, Japan). This assay kit enabled the colorimetric detection of microbial metabolism by the reduction of 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium, monosodium salt (WST-8) to formazan in the presence of an electron mediator. After incubation at 30°C for 2 h, the absorbance at 450 nm was measured by UV–VIS spectroscopy (IVI-1240, AS ONE Co., Japan) and the metabolic activity of the microorganisms adhered on the granules was evaluated. Three granules were used for each sample, and the statistical difference was evaluated using the t-test. The microorganisms adhered on the granules were also observed by SEM.

Figure 1 shows the XRD patterns of Granule-H and Granule-L before and after the hydrothermal and hot-water treatments. The crystal phases of both of the granules before and after the treatments were α-TCP and HA, respectively. All the organic substances were burned out through the sintering process, and α-TCP was completely changed into HA by the hydrothermal and hot-water treatments for both of Granule-H and Granule-L. Although the specimen was not in direct contact with the liquid water in the autoclave as shown in Fig. 1, the water vapor was speculated to condense on the specimen and react with α-TCP during the treatment, and the dissolution–precipitation reaction was speculated to occur. Since the HA was formed from α-TCP under a hydrothermal condition, the formed HA is speculated to have a calcium-deficient composition. Figure 2 shows the SEM images of the surfaces of Granule-H and Granule-L before and after the hydrothermal and hot-water treatments. Before the treatments, both Granule-H and Granule-L were porous and were composed of sintered particles. For Granule-H, pores...
about tens of micrometers in size were also observed. These pores were considered to be formed by the removal of PMMA through the sintering process. After the treatments, the granules were composed of rod-like particles with a high aspect ratio for both of Granule-H and Granule-L. The high aspect ratio was obtained by selecting a relatively low temperature and water vapor treatment.13) Table 1 shows the porosity, specific surface area, and surface area of one granule, which was obtained by multiplication of the specific surface by the average weight of the granules. The porosity of Granule-H was higher than that of Granule-L. The added PMMA beads were burned out, and the pores thus formed provided high porosity. The specific surface area of Granule-H was slightly higher than that of Granule-L. The difference in the surface area might be due to the difference in size of the particles constructing the granules, but it was difficult to find the difference from SEM images because the difference was small. On the other hand, the surface area of one granule of Granule-H was smaller than that of Granule-L, because the porosity of Granule-H is higher and the weight of one granule of Granule-H is less than that of Granule-L.

The SEM images of microorganisms on the surface of Granule-H and the absorbance obtained by the evaluation of metabolic activities of microorganisms on Granule-H and Granule-L are shown in Fig. 3. The microorganisms with a size of several micrometers were attached on the rod-like particles. The adhesion of microorganisms on the surface of the HA granules was observed, and this indicated that the granules showed an affinity for microorganisms. It has been reported that the rod-like shape of HA crystals formed from α-TCP under hydrothermal conditions are elongated along the c-axis and expose the a face.14) Therefore, the obtained granules composed of rod-like HA particles may be advantageous for the attachment of bacteria, because rod-like HA particles expose the a face, which has many positively charged cites due to Ca$^{2+}$.11) and the surfaces of most microorganisms are negatively charged.12) For both Granule-H and Granule-L, an increase in absorbance for the evaluation of metabolic activity was detected. This increase in absorbance is related to the metabolic activity of microorganisms on the granules. The detected metabolic activity for Granule-H was higher than that for Granule-L although the surface area of one granule of Granule-H was smaller than that of Granule-L. It is speculated that the pores made by the entanglement of rode-like particles are not sufficient for microorganisms to enter the granules, while the pores (about tens of micrometers in size) in Granule-H are sufficient for microorganisms to enter the granules. The HA granules, which are composed of rod-like particles and have pores about tens of micrometers in size, are expected to be useful as support materials for microorganisms. For the practical application, the comparison between the present HA granules and the currently used materials under the practical conditions is needed in the future study.

In conclusion, spherical porous HA granules, which were composed of rod-like particles and had pores tens of micrometers in size were prepared. The bacterial adhesion on the surface of the HA granules was observed, and it

| Table 1. Characteristics of granules after hydrothermal and hot-water treatments |
|-----------------|-----------------|-----------------|
|                | Granule-H       | Granule-L       |
| Porosity [%]   | 74 ± 1          | 42 ± 1          |
| Specific surface area [m$^2$/g] | 4.6          | 3.4          |
| Surface area of one granule [m$^2$/granule] | 0.25          | 0.39          |

Fig. 2. SEM images of granules. (a, b) Granule-H before hydrothermal and hot-water treatments, (c, d) Granule-L before the treatments, (e, f) Granule-H after the treatments, (g, h) Granule-L after the treatments. (a), (c), (e), (g): Low magnification, (b), (d), (f), (h): High magnification.
It was suggested that the granules had an affinity for microorganisms. These granules are expected to be useful for constructing bioreactors.

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Fig. 3. SEM images of microorganisms on the surface of Granule-H and the absorbance obtained by the evaluation of metabolic activities of microorganisms on Granule-H and Granule-L. The error bar indicates the standard deviation.