Fabrication and biological evaluation of hydroxyapatite ceramics including bone minerals

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Biological apatite present in the bones and teeth of mammals contains various minerals, which create numerous nanoscale defects in their crystal structures. Substitution of the ions of these minerals into hydroxyapatite [Ca10(PO4)6(OH)2; HAp] induces considerable strain and various defects in the crystal structure of HAp. Although autogenous bone and synthetic HAp ceramics have been used clinically as bone grafts, autografting generally has better clinical results than artificial-bone grafting. In the present study, we fabricated HAp ceramics including bone minerals (bone HAp ceramics) as model materials to clarify the relationship between the nanoscale defect structure and bioactivity of the biological apatite. We also implanted bone HAp ceramics in the tibiae of rabbits, along with standard HAp ceramics without bone minerals (pure HAp ceramics) as a control, and examined the biological response of the living hard tissue to the implants histologically. The single-phase HAp and carbonate ion content of the bone HAp ceramics could be maintained by sintering at 1000°C for 5 h under a flow of carbon dioxide gas. The inclusion of trace elements and changes in the lattice constants were confirmed, and Raman spectroscopy indicated the presence of defects. Biological evaluation showed significantly more newly formed bone around the bone HAp ceramics at 4 weeks than around the pure HAp ceramics. These results demonstrated that bone HAp ceramics that include trace minerals and nanoscale defect structures may promote early-stage bone formation.

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1. Introduction

One of the trends in biomedical materials research is to produce materials that mimic the structure of natural hard tissue. Hydroxyapatite [Ca10(PO4)6(OH)2; HAp] is a key inorganic component of biological hard tissue, that has the ability to bond directly with the hard tissue of a host mammal. Therefore, HAp ceramics are clinically applied as bone and tooth root materials. However, autografting generally exhibits better clinical results than artificial-bone grafting. Biological apatite present in the bones and teeth of mammals contains various minerals, such as Na+, K+, Mg2+, F−, Cl− and CO32−.1) Thus, biological apatite has considerable strain and many nanoscale defects in its crystal structure. It is well known that the HAp lattice can easily accommodate a variety of both cationic and anionic substituents, thereby inducing modifications in the crystallinity, morphology, lattice parameters and stability of apatite structures.2)–5)

As concerns cations, sodium (Na) has been detected as an abundant trace element in addition to calcium (Ca) and phosphorus (P) in natural bone and dental minerals. The Na in biological apatite plays potential roles in cell adhesion, bone metabolism and absorption processes.6)–7) Magnesium (Mg) activates osteoblasts and osteoclasts, and not only promotes bone remodeling but also affects the nucleation and crystal growth of HAp.8)–9) Several studies have demonstrated the varied properties of potassium (K) for the control of biochemical processes and the important role K plays in the apatite mineral nucleation process.10)–11) As concerns anions, it is well known that fluor-
carbonate substitution. Golden and Ming et al.15) successfully prepared calcium hydroxide, magnesium chloride hexahydrate, sodium chloride, potassium chloride, and carbonate ions (CO₃²⁻) dissolved in aqueous suspension, Ca(OH)₂, MgCl₂·6H₂O, NaCl, KCl, and CO₃²⁻ ions, respectively. For the preparation of bone HAp powders, calcium hydroxide, magnesium chloride hexahydrate, sodium chloride, potassium chloride, and (NH₄)₂CO₃ were mixed in pure water with vigorous stirring at a rate of 200 rpm. An aqueous solution containing H₃PO₄ and NH₄F was added dropwise to the suspension over a period of 3 h. The pH of the mixed solution was maintained at 8.7 by the addition of hydrochloric acid (HCl) solution, and the mixed suspension was stirred for 3 h. For maturation of the precipitates, the precipitated suspension was aged at 37°C for 3 d. The resulting precipitates were washed and filtered repeatedly with pure water, and dried in an oven at 110°C for 2 d. The dried cakes were ground to fine powders and calcined in an electric furnace at 800°C (heating rate: 10°C·min⁻¹) for 1 h in steam and a CO₂ gas atmosphere. The calcined powders were crushed by ball-milling for 5 min using a zirconia pod and beads with a diameter of 10 mm. The dry-milled powders were crushed by ball-milling for 25 min using a zirconia pod and beads with a diameter of 2 mm in water, and then dried in an oven at 110°C for 2 d. Pure HAp powders were also prepared according to the same process, except for the addition of the bone minerals.

2. Materials and methods

2.1 Preparation of HAp powder including bone minerals

Bone HAp powders were prepared by wet synthesis. Table 1 shows the synthesis conditions of the bone and pure HAp powders. Ca(OH)₂, MgCl₂·6H₂O, NaCl, KCl, H₃PO₄, NH₄F and (NH₄)₂CO₃ were used as starting chemical precursors for Ca²⁺, PO₄³⁻, Na⁺, K⁺, Mg²⁺, Cl⁻, F⁻ and CO₃²⁻ ions, respectively. For the preparation of an aqueous suspension, Ca(OH)₂, MgCl₂·6H₂O, NaCl, KCl and (NH₄)₂CO₃ were mixed in pure water with vigorous stirring at a rate of 200 rpm. An aqueous solution containing H₃PO₄ and NH₄F was added dropwise to the suspension over a period of 3 h. The pH of the mixed solution was maintained at 8.7 by the addition of hydrochloric acid (HCl) solution, and the mixed suspension was stirred for 3 h. For maturation of the precipitates, the precipitated suspension was aged at 37°C for 3 d. The resulting precipitates were washed and filtered repeatedly with pure water, and dried in an oven at 110°C for 2 d. Pure HAp powders were also prepared according to the same process, except for the addition of the bone minerals.

### Table 1. Synthesis conditions of bone and pure HAp powders

|          | Pure HAp | Bone HAp | Volume/dm³ |
|----------|----------|----------|-------------|
| Ca(OH)₂  | 0.507    | 0.5350   |             |
| MgCl₂·6H₂O |         | 0.0107   |             |
| NaCl     | —        | 0.0226   | 1.0         |
| KCl      | —        | 0.0023   |             |
| (NH₄)₂CO₃ |         | 0.0795   |             |
| H₃PO₄    | 0.300    | 0.333    |             |
| NH₄F     | —        | 0.00263  | 1.0         |

2.2 Fabrication of HAp ceramics including bone minerals

The crushed powders (0.3 g) were uniaxially compressed at 100 MPa to form compacts 10 mm in diameter and 2–3 mm in thickness. The bone HAp ceramics were fabricated by firing the compacts at 1000, 1200 and 1300°C for 5 h in steam and a CO₂ gas atmosphere (flow rate: 100 cm³·min⁻¹). Pure HAp ceramics were also fabricated according to the above process.

2.3 Characterization of sample powders and the resulting ceramics

The crystalline phases of the resulting powders and ceramics were determined by powder XRD (MiniFlex, Rigaku Co., Japan) using Cu-Kα radiation at 30 kV and 15 mA. XRD data were collected under the following conditions: 2θ range of 10–50°; scan rate of 2° per minute; and sampling width of 0.02°. The lattice constants of the crushed ceramics were determined by refinement of XRD data collected under the following conditions: 2θ range of 10–50°; scan rate of 0.1° per minute and sampling width of 0.02° (40 kV, 40 mA, Ultima IV, Rigaku Co.). The PDXL refinement software was used according to the manufacturer’s instructions (Rigaku Co.). The infrared spectrum of the specimens was obtained using a Fourier
transform infrared spectrophotometer (FTIR; IR Prestige-21, Shimadzu Co.,) in the region of 400–4000 cm⁻¹ using potassium bromide pellets with a spectral resolution of 4 cm⁻¹. The Raman spectra of the ceramics were measured using a spectrometer (NRS-3000 series, JASCO, Japan). The spectra were measured in the frequency range of 100–4000 cm⁻¹.

The ultrastructure of the powder particles was examined by high-resolution transmission electron microscopy (HR-TEM; JEM-2100F, JEOL Ltd., Japan) at 200 kV. The HR-TEM samples were prepared by dispersing the particles in ethanol and then collecting them on carbon-coated copper grids. The microstructure of the HAp ceramics was observed by SEM (JSM6390LA, JEOL Ltd.) at 10 kV. The SEM samples were prepared by fixing the ceramics on pieces of double-sided carbon tape, which were then deposited with platinum particles in a vacuum.

The relative density of the resulting ceramics was calculated by dividing the bulk density by the theoretical density of HAp (3.16 g·cm⁻³) according to Eq. (1):

\[
\text{Relative density} \, (\%) = \frac{\text{bulk density} \, (\text{g} \cdot \text{cm}^{-3})}{\text{theoretical density} \, (\text{g} \cdot \text{cm}^{-3})} \times 100
\]

The Ca, P, Na, K and Mg contents in the powders and ceramics were determined by inductively coupled plasma atomic emission spectrometry (ICP-AES: SPS7800, SII Nano Technology, Japan). The Cl⁻ and F⁻ ion contents were determined by ion-selective potentiometry (pH meter: F-55, Cl⁻-selective electrode: 6560-10C, F⁻—selective electrode: 6561-10C, HORIBA). The CO₂ content was determined by total organic carbon (TOC-5000A/SSM5000A, Shimadzu, Japan) analysis. To test the solubility of Ca²⁺ and PO₄³⁻ ions in the ceramics, bone and pure HAp ceramics fired at 1000°C for 5 h were soaked in 0.5 mol·dm⁻³ of tris(hydroxymethyl)aminomethane-hydrochloric acid (Tris-HCl) buffer (pH 7.3) at 37°C for 1, 3, 5, 7, 14, 21 and 28 days. The concentrations of Ca²⁺ and PO₄³⁻ ions in the Tris-HCl buffer after soaking were determined by ICP-AES.

2.4 In vivo evaluation

Cylindrical pure and bone HAp ceramics (diameter: ~4 mm, height: ~8 mm) fired at 1000°C for 5 h were used for the in vivo evaluation. The biological response of living hard tissue to the implanted pure and bone HAp dense ceramic samples was examined histologically using Japanese white rabbits (weight: ~3 kg, 16 weeks old) according to the guidelines of the laboratory animal center at Keio University. The specimens were implanted on the left and right sides of the tibia epiphysis of three rabbits for 4 and 24 weeks. After implantation for 4 and 24 weeks, undecalciﬁed abrasive sections were prepared using the samples and surrounding tissues, and Villanueva bone staining was performed. The bone contact and bone formation rates were measured using image analysis software (WinROOF, Mitani Co., Japan).

![Fig. 1. XRD patterns of HAp powders: (a) as-prepared bone HAp powder; (b) as-prepared pure HAp powder; (c) calcined bone HAp powder; and (d) calcined pure HAp powder (calcination conditions: 800°C, 1 h).](image)

### Table 2. Lattice constants of HAp powders

| Sample                        | Lattice parameters/nm | Lattice volume /nm³ |
|-------------------------------|-----------------------|---------------------|
| As-prepared bone HAp powder   | 0.936(3) 0.6889(17)    | 1.56805             |
| As-prepared pure HAp powder   | 0.9408(9) 0.6888(14)   | 1.58943             |
| Calcined bone HAp powder      | 0.9448(4) 0.6886(4)    | 1.59698             |
| Calcined pure HAp powder      | 0.9418(3) 0.6883(6)    | 1.58616             |
| Hydroxyapatite (ICDD #09-432) | 0.9418 0.6884         | 1.58084             |

Bone contact rate (%) = \( \frac{\text{Length in contact with bone (µm)}}{\text{Circumference of ceramics (µm)}} \times 100 \) (2)

Bone formation rate (%) = \( \frac{\text{Area of newly formed bone (µm}^2\)}{\text{Area around the ceramics (µm}^2\)} \times 100 \) (3)

3. Results and discussion

3.1 Characterization of bone and pure HAp powders

Figure 1 shows the XRD patterns of the as-prepared and calcined pure and bone HAp powders. As shown in Figs. 1(a) and 1(b), the XRD patterns of the as-prepared pure and bone HAp powders indicated that the crystalline phase was single-phase HAp with low crystallinity. When these powders were heated at 800°C for 1 h, the single-phase HAp was maintained and its crystallinity improved.

Table 2 shows the lattice constants of the HAp obtained by refining the XRD patterns of the pure and bone HAp powders. The lattice constant of the 𝑎-axes of the as-prepared pure HAp was slightly lower than that on the International Centre for Diﬀraction Data (ICDD) card (#09-432; HAp). This is due to the incorporation of a small amount of CO₂ in the atmosphere during wet synthesis. In wet synthesis, CO₂²⁻ ions are substituted mainly at the positions of the PO₄³⁻ ions of apatite, and the lattice constant of the 𝑎-axis of HAp decreases. The lattice constant of the 𝑎-axis of bone HAp was smaller than that of pure HAp. As the desired amount of CO₂²⁻ ions was
added in the synthesis of the bone HAp powders, more CO$_3^{2-}$ ions were incorporated in the apatite structure than in pure HAp, and the lattice constant of the $a$-axis decreased further as a result. The lattice constants of pure HAp after calcination were nearby in agreement with the decrease in the $a$-axis length of the bone HAp synthetic powder. The CO$_3^{2-}$ ions were eliminated during synthesis of pure HAp, the CO$_3^{2-}$ ions probably remained in the bone HAp. However, the lattice constant of the $a$-axis increased. This indicates the possibility that the added chloride (Cl$^-$) ions may have been incorporated into the apatite structure. Cl$^-$ ions, which have a large ionic radius, are scarcely incorporated into the apatite structure at the time of synthesis; it is considered, however, that they were incorporated into the structure when heated at 800°C. Based on their charge, the Cl$^-$ ions are expected to replace the OH$^-$ in the apatite. The hydroxyl group is present in HAp along the $c$-axis direction of the hexagonal structure. When Cl$^-$ ions are substituted in OH$^-$ sites, the $a$-axis changes greatly as compared with the $c$-axis, which has a spatial margin. For this reason, it is considered that the Cl$^-$ ions were substituted in the position of the hydroxyl group, and that the lattice constant of the $a$-axis increased remarkably as a result.

The FTIR spectra of the pure and bone HAp powders are shown in Fig. 2. These spectra indicate typical HAp absorptions. In the case of the pure HAp powders [Figs. 2(b) and 2(d)], absorption bands assignable to P–O stretching vibrations were observed at 960, 1030 and 1100 cm$^{-1}$, and bands assignable to O–P–O bending at 570 and 600 cm$^{-1}$. The absorption bands at 630, 1650 and 3570 cm$^{-1}$ indicate the existence of OH$^-$ groups, and absorption bands at 860 cm$^{-1}$ assignable to P–OH stretching vibrations of the HPO$_4^{2-}$ group partially substituting PO$_4^{3-}$ in HAp were detected in both samples. In addition, absorption attributed to CO$_3^{2-}$ groups was observed at 880, 1430 and 1460 cm$^{-1}$ in the bone HAp powders [Figs. 2(a) and 2(c)], indicating that the bone HAp was a carbonate-containing apatite. This is consistent with the large decrease in the $a$-axis length of the bone HAp synthetic powder. The CO$_3^{2-}$ groups in the HAp synthetic and calcined powder were derived predominantly through B-type carbonate substitution, moreover, in which CO$_3^{2-}$ is substituted for PO$_4^{3-}$. Absorptions attributable to the CO$_3^{2-}$ groups were also observed in pure HAp. Its absorption intensity was smaller than that of bone HAp, however, because a minute amount of CO$_2$ was absorbed from the atmosphere during the wet synthesis.

Table 3 shows the quantitative results for Ca and P elements of pure and bone HAp, together with those for Na, K and Mg elements in bone HAp. The Ca/P molar ratio of pure HAp was close to the theoretical value of apatite, 1.67. That of bone HAp was higher than 1.67, however, because the P content was reduced by replacing the PO$_4^{3-}$ group with the CO$_3^{2-}$ group. Furthermore, both as-prepared and calcined powders of bone HAp contained Na$^+$, K$^+$ and Mg$^{2+}$ ions. The K content changed slightly before and after calcination, but, the Na and Mg contents showed little change.

Next, we discuss the anions present in the pure and bone HAp powders. Figure 3(a) shows the carbonate content of the pure and bone HAp powders. The as-prepared and calcined powders of bone HAp contained more CO$_3^{2-}$ ions than that of pure HAp. This is because large numbers of CO$_3^{2-}$ ions were absorbed into the apatite structure of the bone HAp through the addition of a carbonate source during synthesis. Because the calcination was carried out in an atmosphere of CO$_2$ gas, moreover, the carbonate content of the calcined powder of bone HAp did not decrease. Figure 3(b) shows the fluorine content of

![Fig. 2. FTIR spectra of HAp powders: (a) as-prepared bone HAp powder; (b) as-prepared pure HAp powder; (c) calcined bone HAp powder; and (d) calcined pure HAp powder (calcination conditions: 800°C, 1 h).](image-url)

| Sample                  | Ca Mass % | P Mass % | Na Mass % | K Mass % | Mg Mass % | Ca/P molar ratio |
|-------------------------|-----------|----------|-----------|----------|-----------|-----------------|
| As-prepared bone HAp    | 37.40     | 16.38    | 0.45      | 0.24     | 0.10      | 1.67            |
| As-prepared pure HAp    | 37.76     | 17.72    | —         | —        | —         | 1.65            |
| Calcined bone HAp       | 40.37     | 17.61    | 0.44      | 0.32     | 0.10      | 1.77            |
| Calcined pure HAp       | 39.12     | 18.40    | —         | —        | —         | 1.64            |
as-prepared and calcined bone HAp powder. Both the as-prepared and calcined powders contained approximately 0.004 mass% of fluorine. Although the fluorine content predicted from the nominal composition was 0.05 mass%, the actual content was only about one-tenth the amount added at the time of synthesis. Yao et al. reported wet synthesis at 100°C of HAp with a fluorine content of 2.5 mass%. Thus, in this study, the incorporation of F ions may have been suppressed by competition with other anions. Figure 3(c) shows the chlorine content of as-prepared and calcined bone HAp powders. Both the as-prepared and calcined powders contained approximately 0.4–0.5 mass% of chlorine. The chlorine content predicted from the preparation amount was 0.1 mass%, and unlike fluorine, more chlorine was thus incorporated than expected. Cl ions have a large ionic radius and are not incorporated in large amounts into the apatite structure. The excess Cl ions may have been adsorbed on the powder surface.

TEM micrographs of the pure and bone HAp powders are shown in Fig. 4. Needle-shaped particles approximately 0.1–0.5 μm in size were observed in both samples, and there were almost no differences in morphology. These products were taken immediately after synthesis before they had aged. Effects such as inhibition of grain growth due to added minerals were therefore not observed. In addition, as the selected area electron diffraction image showed concentric patterns, both products may be polycrystalline.

3.2 Fabrication of bone HAp ceramics and some properties

XRD patterns of bone HAp ceramics sintered at 1000, 1200 and 1300°C for 5 h are shown in Fig. 5. To be omitted the XRD pattern, all the pure HAp ceramics fired at 1000–1300°C were still single-phase HAp after firing. The bone HAp ceramics fired at 1000°C were single-phase HAp, but the bone HAp ceramics fired at 1200 and 1300°C contained trace amounts of CaO. To confirm the proportion of CaO, quantitative determination of the crystal phase by the reference intensity ratio was performed. The proportions of CaO in the ceramics fired at 1200 and 1300°C were 0.54 and 0.85 mass%, respectively.

The lattice constants of the HAp phases of the respec-
tive ceramics are shown in Table 4. The lattice constant of the pure HAp ceramics was close to the value on the ICDD card under all conditions, although the \(a\)-axis lattice constant of bone HAp increased. The \(\text{Cl}^-\) ions incorporated into bone HAp during calcination were maintained even after firing.

The FTIR spectra of pure HAp ceramics showed absorption attributable to \(\text{PO}_4^{3-}\) and \(\text{OH}^-\) as in the case of pure HAp powder. In addition, bone HAp ceramics showed absorption attributable to \(\text{CO}_3^{2-}\) ions. Thus, the carbonate-containing apatite content in the bone HAp ceramics after sintering could be maintained by flowing carbonic acid and water vapor during firing. The position of the carbonate

ions in the bone HAp ceramics was predominantly type A, which is \(\text{CO}_3^{2-}\) substituting \(\text{OH}^-\).

The Raman spectra of the pure and bone HAp ceramics are shown in Fig. 6. The peak at around 963 cm\(^{-1}\) is attributed to \(\nu_1\text{PO}_4\), the peak at around 450 cm\(^{-1}\) to \(\nu_2\text{PO}_4\), the broad peak in the range of 1000–1200 cm\(^{-1}\) to \(\nu_3\text{PO}_4\), and the peak at around 550–600 cm\(^{-1}\) to \(\nu_4\text{PO}_4\).\(^{26}\) Focusing on the peak at 450 cm\(^{-1}\) attributable to the \(\nu_2\text{PO}_4\) bending mode, we see that pure HAp showed a single peak, whereas bone HAp showed an increase in the number of peaks. In addition, the number of peaks attributable to the \(\nu_4\text{PO}_4\) bending mode at around 600 cm\(^{-1}\) also increased. This was considered to be due to the change in the \(\text{PO}_4^{3-}\) position of bone HAp caused by \(\text{CO}_3^{2-}\) and \(\text{Cl}^-\) ions, thereby changing the surrounding environment of \(\text{PO}_4^{3-}\).

Type B carbonate substitution breaks the symmetry of planar \(\text{PO}_4^{3-}\),\(^{27}\) and type A substitution induces distortion in the atomic arrangement of carbonate-containing apatite, which causes voids and loss of the \(\delta_3\) helical axis.\(^{28}\) Thus, the number of peaks increased in bone HAp as compared with pure HAp, and it is considered that the symmetry was distorted due to the introduction of defects.

Figure 7 shows SEM micrographs of the surfaces of the pure and bone HAp ceramics, together with the values of their relative densities. Compared with pure HAp, the grain size of bone HAp was smaller. The difference in grain sizes was particularly remarkable in the specimens fired at 1200 and 1300°C: pure HAp was approximately several micrometers in size, whereas bone HAp was approximately one-tenth that size. This may be because the mineral com-

\[\text{Table 4. Lattice constants of HAp ceramics}\
\]

| Sample          | Firing temperature/°C | Lattice parameters/\text{nm} | Lattice volume/\text{nm}^3 |
|-----------------|------------------------|------------------------------|-----------------------------|
| Bone HAp ceramics | 1000                   | \(0.94380(10)\), \(0.68835(10)\) | 1.59302                     |
| Pure HAp ceramics | 0.9418(2)              | \(0.6886(4)\)               | 1.58685                     |
| Bone HAp ceramics | 1200                   | \(0.94345(12)\), \(0.68873(14)\) | 1.59272                     |
| Pure HAp ceramics | 0.94143(17)            | \(0.6887(3)\)               | 1.58584                     |
| Bone HAp ceramics | 1300                   | \(0.94267(9)\), \(0.68831(9)\) | 1.58912                     |
| Pure HAp ceramics | 0.94156(14)            | \(0.6892(3)\)               | 1.58743                     |

Fig. 5. XRD patterns of bone HAp ceramics sintered at (a) 1000°C, (b) 1200°C, and (c) 1300°C for 5 h.

(Fig. 6. Raman spectra of (A) wide and (B) narrow scans: (a) bone and (b) pure HAp ceramics sintered at 1000°C for 5 h.)
ponents not incorporated in the crystal structure segregated at the grain boundary to inhibit grain growth. The relative density of the pure HAp ceramics fired at any temperature exceeded 90%, whereas the relative density of the bone HAp ceramics fired at 1200 and 1300°C was lower than 90%. The relative density of bone HAp ceramics may be greatly affected by desorption of carbonate ions with baking at temperatures of 1200°C or higher.

Table 5 shows the Ca, P, Na, K and Mg contents determined by ICP-AES. Pure HAp reached values around the Ca/P molar ratio of 1.67 under all conditions, but, the Ca/P molar ratio of the bone HAp ceramics was larger than the 1.67 stoichiometric ratio, as well as that of the as-prepared powder and calcined powder. It is thought that the bone HAp ceramics maintain their content of carbonate-containing apatite in which carbonate is substituted at the phosphate position even after firing at temperatures of 1000°C or higher.

Table 5. Chemical compositions of HAp ceramics determined by ICP-AES

| Sample            | Firing temperature | Ca Mass % | P Mass % | Na Mass % | K Mass % | Mg Mass % | Ca/P molar ratio |
|-------------------|--------------------|-----------|----------|-----------|----------|-----------|------------------|
| Bone HAp ceramics | 1000               | 39.86     | 17.46    | 0.47      | 0.34     | 0.10      | 1.76             |
| Pure HAp ceramics |                   | 39.11     | 18.06    | —         | —        | —         | 1.67             |
| Bone HAp ceramics | 1200               | 39.84     | 17.92    | 0.45      | 0.36     | 0.10      | 1.72             |
| Pure HAp ceramics |                   | 39.49     | 18.61    | —         | —        | —         | 1.64             |
| Bone HAp ceramics | 1300               | 40.99     | 18.16    | 0.43      | 0.33     | 0.10      | 1.74             |
| Pure HAp ceramics |                   | 39.67     | 18.41    | —         | —        | —         | 1.67             |

This is because almost all the CO$_3^{2-}$ ions present in the pure HAp structure were desorbed by firing at temperatures of 1000°C or higher. On the other hand, the bone HAp ceramics maintained their carbonate content even after firing. Most notably, the carbonate content of the ceramics fired at 1000°C was 2.5 mass %, close to the target value of 3.5 mass %. Flowing carbonic acid gas during firing made it possible to maintain a certain carbonate content, even when firing at temperatures of 1000°C or higher.

Figure 9 shows the elution amounts of (a) Ca$^{2+}$ and (b) PO$_4^{3-}$ ions per unit mass. The elution amounts of Ca in bone HAp ceramics were higher than those in pure HAp ceramics. Because bone HAp ceramics contain more carbonate ions than pure HAp ceramics, bone HAp ceramics have a higher solubility. In the initial period, there was no significant difference between the degrees of P elution by the pure and bone HAp samples, but, after 21 days, the P of the bone HAp had eluted slightly more than that of pure HAp. As given in Table 5, the Ca/P molar ratios of bone HAp and pure HAp ceramics are 1.76 and 1.67, respectively; and, bone HAp is Ca-rich and phosphorus-less compared to pure HAp, so that there was little difference in the elution of P from bone and pure HAp ceramics. Substitution of ions into the HAp crystal causes the formation of vacancies and changes the electron density distribu-
tion in the crystal structure, and the solubility changes accordingly. Among the ions contained in bone HAp, F⁻ and Cl⁻ ions are known to decrease solubility, whereas Mg²⁺ and CO₃²⁻ ions increase solubility. CO₃²⁻ ions were the most abundant in bone HAp; thus, since bone HAp containing six kinds of ions has more defects at the starting point of dissolution than pure HAp, the solubility of bone HAp ceramics may be improved.

3.3 Biological evaluation of bone HAp ceramics

The response of living hard tissue to bone HAp ceramics was examined using Japanese white rabbits. Pure HAp ceramics were used as a control, and cylindrical ceramics fired at 1000°C were used as the implanted specimens. All the rabbits were in good health during the experimental period, and there were no signs of inflammation, carcinogenesis or infection around the implant site. Figure 10 shows the results of the histological evaluation of the samples after Villanueva bone staining. Histological observation of the samples implanted after 4 weeks showed that bone tissue was directly bonded with the surface of both the bone and pure HAp ceramics. In addition, the appearance of osteoblasts on osteoids was observed with the bone and pure HAp ceramics. Histological observation of the fluorescence also showed that each of the ceramics was bonded with calcified bone, which appeared green. Both the bone and pure HAp ceramics were almost completely covered with mature calcified bone after implantation for 24 weeks, demonstrating that they had biocompatibility and osteoconductivity. Since both samples were dense sintered ceramics, moreover, the materials remained without being resorbed after 24 weeks. The edges of the pure HAp ceramics were smooth, however, while the edges of the bone HAp ceramics were jagged. It seems, moreover, that dissolution progressed further on the surface of the bone HAp ceramics.

The (a) bone contact and (b) bone formation rates calculated by image analysis are shown in Fig. 11. The bone contact rate of the bone HAp ceramics after 4 weeks was higher than that of the pure HAp ceramics. We have previously examined the ability to promote osteoblast differentiation on bone HAp ceramics and reported that the alkaline phosphatase activity of bone HAp ceramics was higher than that of pure HAp ceramics in the early and/or middle stages of differentiation. It is considered that...
osteoblasts could be induced around the ceramics from the initial stage, resulting in an improved bone contact rate for bone HAp. The bone contact rates did not differ for the two samples after 24 weeks; since apatite has excellent osteoconductivity originally, however, HAp slowly contacted the surrounding newly formed bone, and the bone contact rate of the pure HAp ceramics approached that of the bone HAp ceramics. A similar tendency was also seen.

Fig. 10. Histological evaluations of bone and pure HAp ceramics. Visible light: osteoid: purplish red; calcified bone: light brown; hypocalciified bone: orange. Fluorescence: osteoid: red; calcified bone: green; hypocalciified bone: orange. I: HAp ceramics. Insets: highly magnified images of the dotted square areas.

Fig. 11. Determination of (a) bone contact and (b) bone formation rates in bone and pure HAp ceramics. Error bars represent the standard deviation. (n = 6, *p < 0.05)
in the bone formation rate. There was no significant difference between the bone formation rates of the bone and pure HAp ceramics implanted for 24 weeks; however, the bone formation rate of the bone HAp ceramics implanted for 4 weeks was approximately 1.5 times higher than that of the pure HAp ceramics. In addition, the bone surrounding the pure HAp ceramics after 4 weeks had a high proportion of hypocalcified bone, while the bone HAp ceramics were already surrounded by mature calcified bone. These results demonstrated that bone HAp including trace minerals and nanoscale defect structures may promote early-stage bone formation.

4. Conclusions

Bone HAp was synthesized by adding ions of six kinds of minerals contained in biological bone, and its material properties and biocompatibility were examined. The as-prepared and calcined bone HAp powders were single-phase HAp that contained carbonate ions. The chemical compositions of the bone HAp powders before and after calcination were almost identical, and the six kinds of ions added during synthesis were present. The lattice constants of the a-axis of the bone HAp powders changed from before to after calcination, suggesting that ions were incorporated into the crystal structure of HAp by calcination. When ceramics were fabricated using bone HAp powders, CaO was formed at temperatures of 1200°C or higher, but single-phase HAp was obtained at 1000°C. The CO$_3^{2-}$ ion content in bone HAp ceramics was maintained by flowing carbonic acid gas during firing, and the maximum content of CO$_3^{2-}$ ions was 2.5 mass%. The Raman spectra showed a division of peaks attributed to PO$_4^{3-}$ of the bone HAp ceramics, and indicated the presence of defects. In the biological evaluation by implantation of the ceramics into rabbits, histological observation showed that bone tissue bonded directly with the surface of both the bone and pure HAp ceramics. At 4 weeks after implantation, there was significantly more newly formed bone and a significantly larger mineralized zone around the bone HAp ceramics compared to the pure HAp ceramics. At 24 weeks, there were no significant differences between the bone HAp and pure HAp ceramics. These results demonstrate that bone HAp ceramics may promote early-stage bone formation and offer potential for use as high-performance biomaterials for artificial-bone grafting.

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