Fabrication of solid and hollow carbonate apatite microspheres as bone substitutes using calcite microspheres as a precursor

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Spherical carbonate apatite (CO₃Ap) microspheres approximately 1 mm in diameter were fabricated by granulation of calcium hydroxide around a core followed by carbonation and phosphatization through dissolution-precipitation reaction. CO₃Ap microspheres with high uniformity could not be achieved without using a core. Solid CO₃Ap microspheres were obtained using a calcite core whereas hollow CO₃Ap microspheres were obtained using a NaCl core. The obtained microsphere was identified as B-type CO₃Ap by Fourier transform infrared analysis and the carbonate content was approximately 7–8 wt% regardless of the type of core used for sample preparation. The mechanical strength of both the solid and hollow CO₃Ap microspheres was sufficient for practical use as a bone substitute.

Keywords: Carbonate apatite, Microsphere, Hollow, Bone substitute

INTRODUCTION

As bone substitutes, hydroxyapatite (HAp: Ca₁₀(PO₄)₆(OH)₂) and β -tricalcium phosphate (β -TCP: β -Ca₃(PO₄)₂) are popular for clinical use and both show excellent osteoconductivity. However, HAp is not completely resorbed by the osteoclasts in the remodeling cycle of the osseous tissue because HAp is too stable and has extremely low solubility. On the other hand, the resorption process of β -TCP is a physicochemical dissolution, and it is reported that though β -TCP was successfully replaced by new bone at an active site of bone formation, it sometimes gave a poor prognosis at a less active site. The inorganic phase of bone composition has been idealized as HAp¹, but studies on both synthetic and biological apatites demonstrated that the bone mineral was a carbonate apatite (CO₃Ap) represented by a generalized chemical formula, Ca_{10-a}(PO₄)_{6-b}(CO₃)_b(OH)_{2-c}. CO₃²⁻ ion substitutes PO₄³⁻ or OH⁻ ion. The Ca²⁺ ion could also be substituted by various ions such as Mg²⁺, Na⁺ or Sr²⁺, and the OH⁻ ion by F⁻ or Cl⁻ ion^{2,3}. The substituted amounts of a, b and c varied to keep the electroneutrality of CO₃Ap. In the remodeling process of bone, osteoclasts produced a weak acidic environment of pH 3–5 in Howship's lacuna to dissolve the bone minerals. LeGeros and Ming reported the relationship between carbonate content in apatitic structure and solubility of apatite under weak acidic conditions⁴. The solubility of apatite increases with an increase in the carbonate content of apatite. This result indicates clearly that the carbonate content in apatitic structure plays a dominant role for in the dissolution of apatite. In other words, CO₃Ap is soluble in Howship's lacuna whereas HAp is not soluble. Therefore, CO₃Ap is a key factor if it is desired

that the artificial bone substitute will be replaced by new bone based on the bone remodeling process. Thus, CO₃Ap would be a better candidate as a bone substitute biomaterial. It is ideal for a bone substitute to have a similar structure as well as properties to bone⁵. Bone has a fully interconnected pore structure that allows the tissue ingrowth and nourishment of bone cells necessary for optimum bone growth or repair^{5,6}. Therefore, we have attempted to fabricate a calcium phosphate bone substitute with a fully-interconnected porous structure for a decade. Wakae *et al.* reported that a CO₃Ap foam with interconnected porous structure could be fabricated by hydrothermal treatment of α -tricalcium phosphate (α -TCP: α -Ca₃PO₄) foam in the presence of ammonium carbonate ((NH₄)₂CO₃) solution at 200°C for 24 h⁷. However, this CO₃Ap foam was very weak in mechanical strength and did not have the molding ability to obtain the desired shape. Later Karashima *et al.* reported that porous HAp blocks with molding ability could be obtained by the setting reaction of α -TCP foam granules⁸. In short, α -TCP foam was crushed into granules which had an interconnected porous structure, and the resulting granules were treated under hydrothermal condition with distilled water at 200°C for 24 h. However, the hydrothermal treatment resulted in HAp with high crystallinity and slower resorption rate. HAp with low crystallinity exhibited higher resorption rate and better bioactivity in bone graft space⁹. On the other hand, it was reported that closed-packing of microspheres of 1 mm in diameter gave 3-dimensionally interconnected pores of 200–500 μ m in size, ideal for ingrowth of blood vessels and new bone tissue^{10–13}. If we could prepare microspheres made of CO₃Ap, they are expected to be a good candidate for a bone substitute with excellent resorbability. Several researchers^{14,15} have reported on

preparation of spherical HAp or CO_3Ap with diameter of a few μm to 1 mm; however, there seems to be no report on spherical CO_3Ap of 1 mm diameter at present. Some preparation methods for ceramics granules with large size (more than 1 mm in diameter) were reported such as compaction/granulation¹⁶⁾, tumbling granulation¹⁷⁾, agitation fluidized-bed granulation¹⁸⁾ and other methods¹⁹⁾. Among them, tumbling granulation is a suitable for our purpose since large size ceramic microsphere can be prepared with high productivity and without valuable equipment.

Though solid CO_3Ap microspheres can build up a 3-dimensional porous structure, if there was a hollow space inside the microsphere, the hollow space would allow effective permeation of blood and cells, and enable easier CO_3Ap resorption. Moreover, hollow-type HAp microspheres have received much attention as a controlled drug-delivery device of drugs or growth factors^{20–22)}. Thus a CO_3Ap microsphere with a hollow space is also expected to be a good candidate for a bone substitute with rapid bone formation.

In this study, the authors established a new fabrication method of both CO_3Ap solid and hollow microspheres of about 1 mm in diameter, and their physicochemical properties were investigated.

MATERIALS AND METHODS

Preparation of core

Two kinds of cores, that is, calcium hydroxide ($\text{Ca}(\text{OH})_2$), and calcium carbonate (CaCO_3 : calcite) were used for fabrication of a solid-type CO_3Ap microsphere, while sodium chloride (NaCl) was used for a hollow-type CO_3Ap microsphere. 0.2 g of $\text{Ca}(\text{OH})_2$ powder (Wako Chemical

Co. Ltd., Osaka, Japan) was placed in a stainless steel mold with inner diameter of 6 mm and pressed uniaxially under 100 MPa pressure. The $\text{Ca}(\text{OH})_2$ compact green block was crushed with a hammer and sieved to collect particles between 300 and 500 μm in size, these were used as the $\text{Ca}(\text{OH})_2$ core. The $\text{Ca}(\text{OH})_2$ compact was also treated in a stream of CO_2 gas saturated with water vapor at room temperature for 2 weeks to convert it into calcite^{23,24)}. The obtained calcite blocks were crushed with a hammer and sieved to collect particles between 300 and 500 μm in size, these were used as the calcite core. For fabrication of hollow-type CO_3Ap , water-soluble NaCl was used as the core material. A commercially available NaCl (Wako Chemical Co. Ltd.) was sieved to collect particles between 300 and 500 μm in size and these were used as the NaCl core.

Preparation of CO_3Ap microsphere

Each core was put into a rotary granulator (angle of inclination 40°), turned at a rotating speed of 40 rpm and misted with a small amount of water by atomist spray to wet the core surface. Then an appropriate amount $\text{Ca}(\text{OH})_2$ powder was added and the core was granulated. This procedure was repeated several times until the granule size attained approximately 1 mm in diameter. The $\text{Ca}(\text{OH})_2$ precursor microsphere with a different core, $\text{Ca}(\text{OH})_2$, calcite or NaCl was treated again in a stream of 100% CO_2 gas saturated with water vapor at room temperature for two weeks to convert it into calcite^{23,24)} and was used as a precursor. The calcite microsphere with the core was transformed into CO_3Ap in 1 mol/L Na_2HPO_4 solution at 60°C for 2 weeks²⁵⁾. Figure 1 shows a schematic representation of the preparation method of solid and hollow CO_3Ap microspheres.

Fig. 1 A schematic representation of the preparation method of solid and hollow CO_3Ap microspheres.

Characterization of the precursor and CO₃Ap microsphere
Sphericity of the precursor and CO₃Ap microsphere was evaluated according to the following equation (1).

$$\text{Sphericity} = L_{\max}/L_{\min} \quad (1)$$

where $L_{\max}$ means the maximum diameter of the microsphere and $L_{\min}$ means the minimum diameter.

If the microsphere was a true sphere, sphericity would be 1. The composition of the precursors and CO₃Ap microspheres was evaluated by means of powder X-ray diffraction (XRD). The specimens were ground into fine powder for the XRD analysis. Diffraction patterns of the specimens were recorded with a vertically mounted diffractometer system (D8 ADVANCE, Bruker AXS Co. Ltd., Karlsruhe, Germany) using Ni filtered CuK α radiation generated at 40 kV and 40 mA. Specimens were scanned from 20° to 40° of 2 θ (where θ is the Bragg angle) in a continuous mode, and references for HAp and CaCO₃ and Ca(OH)₂ identifications were obtained from JCPDS cards. Fourier transform infrared (FT-IR) analysis of the CO₃Ap microsphere was also carried out using the KBr method with a FT-IR spectrometer (Spectrum 2000LX; Perkin-Elmer Co. Ltd., Massachusetts, USA). Quantitative analysis of the carbonate content in the CO₃Ap microsphere was performed using a CHN coder (CHN coder-MT-6, Yonako Analytical Instruments Corp., Kyoto, Japan). The apparent density of the CO₃Ap microsphere was obtained by dividing its weight by its volume and porosity was calculated by the following equation (2),

$$\text{Porosity of CO}_3\text{Ap microsphere} = 100 \times (d_{\text{CO}_3\text{Ap}} - d_{\text{CO}_3\text{Ap microsphere}}) / d_{\text{CO}_3\text{Ap}} \quad (2)$$

where $d_{\text{CO}_3\text{Ap microsphere}}$ and $d_{\text{CO}_3\text{Ap}}$ are the apparent density of a CO₃Ap microsphere and the theoretical density of CO₃Ap. The mechanical strength of the CO₃Ap microsphere was evaluated in uniaxial compression mode using a universal testing machine (AGS-J, Shimadzu Co., Kyoto, Japan) at a crosshead speed of 1 mm/min. The mechanical strength was obtained as a maximum load at fracture of the microsphere and the strength value was taken as the average of at least ten specimens. Surface and cross-sectional structures of the CO₃Ap microsphere were observed using scanning electron microscope (SEM) (S3400N, Hitachi High Technologies Co. Ltd., Tokyo, Japan) after sputter-coating with gold.

RESULTS

Figure 2 shows photographs of the precursors with various cores. When no core was used for granulation, the precursor showed an oval-like spherical shape and the particle were not uniform. However, the shape of the granule became more spherical when a core was used. Calcite and NaCl cores gave an almost spherical and homogeneous granule size. Table 1 shows the sphericity of the precursor microspheres when granulated with no core, Ca(OH)₂ core, calcite core and NaCl core. Though

Fig. 2 Photographs of the precursor microspheres with various cores.

Table 1 Sphericity with no core, Ca(OH)₂ core, CaCO₃ core and NaCl core

Sample name	Sphericity
No core	1.18±0.29
Ca(OH) ₂	1.12±0.22
Calcite	1.06±0.08
NaCl	1.03±0.14

sphericity was between 1.18 and 1.02 depending on the core materials, there are no significant differences between the values. However, the microspheres with a calcite core and NaCl core seemed to show high sphericity, so these were used for our experiment.

Figure 3 shows the XRD patterns of Ca(OH)₂ granules with calcite and NaCl cores before and after carbonation in a water-saturated CO₂ stream at room temperature for 1 day, 3 days, 7 days, and 14 days. Figure 4 shows the conversion ratio of Ca(OH)₂ to calcite with time for granules with calcite and NaCl cores. In both of the Ca(OH)₂ granules with calcite and NaCl cores, 70–80 mass% of calcite was formed after carbonation for 1 day. Carbonation occurred gradually from then and the rest of the Ca(OH)₂ was fully converted into calcite after carbonation for 14 days.

Figure 5 shows the XRD patterns of calcite microspheres with calcite and NaCl cores phosphatized in 1 mol/L Na₂HPO₄ solution at 60°C up to 14 days.

Fig. 3 XRD patterns of Ca(OH)_2 microsphere prepared using calcite core (left) and NaCl core (right) before and after carbonation for different periods. Ca(OH)_2 , NaCl and calcite are also included for comparison.

Fig. 4 Change in conversion ratio of Ca(OH)_2 microsphere to calcite microsphere with time during carbonation.

Figure 6 shows the conversion ratio of calcite into CO_3Ap with time. For the calcite granules with a calcite core, most of the calcite was converted into apatite phase (about 85 mass%) after phosphatization for 1 day. Phosphatization occurred gradually from then, as in carbonation, and only a small amount of calcite remained even after phosphatization for 14 days. It may be difficult for the calcite core to convert into apatite phase because it has a higher density than the outer shell. For the calcite granules with a NaCl core, though similar conversion of calcite into apatite phase occurred, the NaCl core was completely absent after 1 day. This means that the NaCl core completely dissolved out during phosphatization, and left a hollow space inside the microsphere. Formation of the hollow space was confirmed by SEM observation described later.

Figure 7 shows the FT-IR spectra of the calcite microspheres with calcite (solid) and NaCl (hollow) cores phosphatized in 1 mol/L Na_2HPO_4 solution at 60°C for 14 days. The FT-IR spectrum of HAp is also shown as a reference. The spectra for both the solid- and hollow-type CO_3Ap microspheres show several absorption bands due to PO_4 group at $1200\text{--}900\text{ cm}^{-1}$ and $650\text{--}500\text{ cm}^{-1}$, and CO_3 group at $1550\text{--}1350\text{ cm}^{-1}$ and at about 870 cm^{-1} . The absorption bands for PO_4 group corresponded

Fig. 5 XRD patterns of calcite microsphere prepared using calcite core (left) and NaCl core (right) before and after phosphatization for different periods. Calcite, NaCl and HAp are also included for comparison.

to those for HAp. The absorption band for CO_3 clearly shows that carbonate ions, CO_3^{2-} are involved in the apatite lattice, because the XRD patterns only showed formation of the apatite phase.

Figure 8 shows the SEM photographs of the solid (a) and hollow CO_3Ap microsphere (b) at low and high magnification. As shown in these photographs, granular CO_3Ap crystals were seen on the surfaces of the microspheres, and the apparent crystal size seemed to be slightly smaller in the solid CO_3Ap microspheres. Figure 9 shows the SEM photographs of cross-sections of the solid (a) and hollow CO_3Ap microspheres (b) at low and high magnification. The bright section in the central part of solid microsphere showed the calcite core in intimate contact with the shell. However, the surrounding CO_3Ap crystals seemed to be loosely packed towards the outer surface. With the hollow CO_3Ap microsphere, a hollow space was clearly seen in the center part of the microsphere indicating the NaCl core had dissolved out. The hollow-type CO_3Ap microsphere showed a more closely packed structure than the solid-type one.

Table 2 summarizes the physicochemical properties, such as carbonate content, apparent porosity and compressive strength for the solid- and hollow-type

Fig. 6 Change in conversion ratio of calcite microsphere to with CO_3Ap microsphere with time during phosphatization.

Fig. 7 FT-IR spectra of CO₃Ap microspheres prepared using calcite core and NaCl core.

CO₃Ap microspheres. Carbonate content is 7.9 mass% in the solid microsphere, and 8.4 mass% in the hollow-type microsphere, which is very close to the average carbonate content of 7.4 mass% in human bone. The apparent density of the solid microsphere was 1.60 g/cm³ ($n=30$) and the resultant porosity was 48.1 vol%. On the other hand, the apparent density of the hollow microsphere was 1.07 g/cm³ ($n=30$) and the porosity was 65.2 vol%. The compressive strength of the microspheres was 1.4 ± 0.4 N for the solid-type and 1.9 ± 0.3 N for the hollow-type. Unexpectedly, the hollow-type showed higher strength than the solid-type.

DISCUSSION

The present study clearly demonstrated that solid and hollow CO₃Ap microspheres could be successfully prepared using a novel method to fabricate CO₃Ap based on dissolution-precipitation reaction. When microspheres of 1 mm in diameter were close-packed, for example, a 3-dimensional porous structure with pores of approximately 500 μ m in size would be constructed²⁵. It was reported that interconnected pores with 300–500 μ m in diameter are enough large for bone cells such

Fig. 8 Surface SEM images for CO₃Ap microsphere prepared using calcite core (a) and NaCl core (b).

as osteoblasts¹³⁻¹⁶⁾ to penetrate into the pores. A pore size of 500 μm seems to be ideal for cell penetration and cell revascularization. Therefore, fabrication of a microsphere with 1 mm diameter makes it easy to construct a 3-dimensional porous structure with pores of approximately 500 μm in size. For spherical granulation, selection of the core material is especially important to control the sphericity of the obtained microsphere. As shown in the present study, for the tumbling granulation method with no core, the granule size was not homogenous and its shape was not spherical, but oval-like. Moreover, its strength was too low and it was easily broken. When a core was used for the tumbling granulation, more spherical microspheres were obtained. As shown in Table 1, sphericity of the precursors was closer to 1. Although there was no statistically significant difference in sphericity between microspheres with different cores, decreasing order of each standard deviation becomes no core > $\text{Ca}(\text{OH})_2$ core > NaCl core > calcite core. This result indicates that the sphericity of microsphere with no core is uneven when compared with that of microsphere with NaCl core or calcite core. Apparent density of the core was measured at 1.80 g/cm^3 for calcite and 2.13 g/cm^3 for NaCl . Apparent density of $\text{Ca}(\text{OH})_2$ was lower than

calcite. The order in apparent density of the core agreed well with that in uniformity of the resultant precursor microsphere. This indicates that the heavier core was more efficient for spherical granulation with uniform size. If the core is light in weight, it would easily bounce in the granulator and effective granulation would be difficult. On the other hand, a heavy core would granulate more effectively and densely. Thus, the NaCl and calcite cores with higher apparent densities gave microspheres with higher uniformity.

Apparent porosity of solid microsphere was approximately 48% due to inter granular space of

Table 2 Parameters of carbonate content, apparent porosity and compressive strength for solid and hollow carbonate apatite microspheres

	Solid	Hollow
Carbonate content (mass%)	7.9 $\pm$ 1.5	8.4 $\pm$ 0.8
Apparent porosity (%)	48.1 $\pm$ 1.4	65.2 $\pm$ 1.1
Compressive strength (N)	1.4 $\pm$ 0.4	1.9 $\pm$ 0.3

Fig. 9 Cross-sectional SEM images for CO_3Ap microsphere prepared using calcite core (a) and NaCl core (b).

particles as shown in Figs. 8(a) and 9(a). Apparent porosity of the hollow microsphere (approx. 65%) was higher than that of the solid CO₃Ap microsphere because of the hollow space in its center as indicated in Fig 9(b). It is well known that a hexagonal closest packing structure is built by piling up microspheres, the space occupied by these microspheres is 74%²⁷⁾, that is, the theoretical porosity of a hexagonal closest packing structure is 26%. Therefore, total porosity of the solid CO₃Ap microsphere is $(1 \times 0.74 \times 0.481 + 0.26) \times 100 = 61.6\%$, and total porosity of the hollow microsphere is $(1 \times 0.74 \times 0.652 + 0.26) \times 100 = 74.3\%$.

The mechanical strength was 1.4 ± 0.4 N (solid) and 1.9 ± 0.3 (hollow), that is, the strength of the hollow microsphere was slightly higher than the solid microsphere. Huang *et al.* reported that a hollow HAp microsphere (500–800 μm in diameter) prepared by a glass conversion process showed a rupture of the sphere at compressive load of at least 350 mN²⁶⁾. However, it is impossible to compare the strength with the above value, because the particle size and thickness of the shell were different. The mechanical strength of 1.9 N in the present study is high enough for practical use as bone substitute. It was expected that the mechanical strength increased with wall thickness of hollow sphere, thus the solid microsphere should have a higher mechanical strength than the hollow one. However, the observed mechanical strength was unexpectedly higher in the hollow microsphere. This was probably due to a difference in microstructure of the CO₃Ap phase between the solid microsphere and hollow one. The SEM photographs of the cross section of the microspheres revealed that CO₃Ap crystals were more densely packed in the hollow microsphere as shown in Fig. 9. It is thought that NaCl used as the core of the hollow microsphere can be smoothed off more easily than the calcite core. Around core would make it possible to coagulate Ca(OH)₂ powder more densely on the surface during granulation. Densely packed CO₃Ap was formed by successive carbonation and phosphatization of Ca(OH)₂ and this led to the high mechanical strength of the hollow microsphere. In the present study, though the core size was fixed around 300–500 μm , a solid or hollow CO₃Ap microsphere with desired size and shell thickness could be fabricated by changing the core size and granulation conditions.

FT-IR spectra showed that CO₃²⁻ ions were included in the CO₃Ap microspheres. According to data from the literature, carbonate ions can be classified into A-type which gives peaks at 880, 1,465 and 1,545 cm⁻¹, and B-type which shows different peaks at 872, 1,412 and 1,460 cm⁻¹^{28,29)}. In this experiment, the carbon peaks observed for the specimens closely matched that of B-type carbonate. The absorption bands at 1,410 and 1,460 cm⁻¹ belong to the vibration modes of C-O of the CO₃²⁻ group in B-type CO₃Ap, in which PO₄³⁻ was partially substituted by CO₃²⁻. These results showed that a B-type CO₃Ap solid microsphere could be obtained through dissolution-precipitation reaction.

CONCLUSION

The present study demonstrated that solid and hollow CO₃Ap microsphere could be easily prepared based on a four-stage method: (1) preparation of the core (calcite (solid), NaCl (hollow)), (2) granulation with Ca(OH)₂ using the core, (3) conversion of the Ca(OH)₂ to calcite by carbonation, and (4) conversion of the calcite to CO₃Ap by phosphatization. It was expected that the fabricated solid and hollow CO₃Ap microspheres can be used for a constituent of resorbable bone substitute with a 3-dimensional porous structure.

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