Detailed Consideration of Physicochemical Properties of CO₃apatites as Biomaterials in Relation to Carbonate Content Using ICP, X-ray Diffraction, FT-IR, SEM, and HR-TEM

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CO₃apatites with different carbonate contents were synthesized at 69±1°C and pH 7.4±0.2 under different carbonate concentrations (0-0.3 mol/L) in the supplied solutions. Their physicochemical properties were analyzed using various methods. Inductively coupled plasma gave accurate chemical analysis data for calcium and phosphate contents. X-ray diffraction analysis showed a clear chemical shift at high carbonate content. A CO₃₂⁻ absorption peak area approximately proportional to carbonate content was observed through Fourier transmission infrared spectroscopy. Scanning electron microscopy and high-resolution transmission electron microscopy revealed a dramatic change of the crystal shape. Osteoblast proliferation at the surface of each CO₃apatite-collagen sponge indicated that osteoblasts deformed to expand and cover the surface of the sponge, and appeared to adhere well to the sponge.

Key words: CO₃apatites, Carbonate contents, Detailed analyses

INTRODUCTION

An inorganic substance of human hard tissue is CO₃apatite, which contains many truce elements such as Mg²⁺, Fe²⁺, Zn²⁺, Na⁺, CO₃₂⁻, HPO₄²⁻, F⁻, Cl⁻, etc., in addition to Ca²⁺, PO₄³⁻, and OH⁻ as the main components. In particular, enamel apatites contain approximately 1-3 wt% of carbonate, whereas dentin and bone contain approximately 3-5 wt% of carbonate. In the same vein, the crystallinity of teeth and bone are quite different. Enamel apatite is highly crystallized in contrast to dentin and bone, which are poorly crystallized. At this juncture, it should be mentioned that carbonate content and crystallinity seem to be related to the caries susceptibility of enamel and the resorbability of bone during daily metabolism.

Carbonate interferes with the crystallization of apatites and exerts a weakening effect on the bonds in the apatite structure. Due to these influences, the solubility of dental apatites containing carbonate increases and hence the susceptibility to caries. For synthesized CO₃apatites, their crystal features are dramatically changed. The apparent solubility of CO₃apatites over pH 4-8 at 37°C is related to their crystallinity. In particular, the degree of increase in the solubility of CO₃apatites having high crystallinity changes greatly in the region of 0-3 wt% of carbonate content—which approximates to that of enamel apatites. On the other hand, bone apatites have a higher carbonate content than enamel apatites and make a composite with 30-40 wt% of collagen.

To date, many researchers have reported on various kinds of material to be used as biomaterials, and all studies were conducted with duly sufficient attention to biocompatibility. However, the concept of delving into the compositions of biological hard tissues was not necessarily reflected during the development of these biomaterials. On this note about biomaterial composition, we have synthesized a CO₃apatite (CO₃Ap) with a chemical composition and crystallinity similar to bone at pH 7.4 and 60°C. CO₃Ap powder was mixed with a collagen solution, of which the antigenicity was removed by enzymatic treatment, and formed into CO₃Ap-collagen pellets. After insolubilization by UV irradiation, the composites showed remarkably reduced disintegration and maintained their shape. Further, they showed good biocompatibility when implanted beneath the periosteum cranii of rats.

With recent advances and current developments in tissue engineering, porous hard tissue biomaterials are expected. To invade the inner core, these materials need a much larger pore size than osteoblasts—where an average diameter of approximately 10 µm and without taking into account the deformation and projection length of osteoblasts. This is because osteoblasts sometimes deform with their expanded projections and become less likely to invade the deeper core by adhering to the walls of pores. Due to this mandatory requirement of large pore size, CO₃Ap was mixed with a neutralized
collagen gel whereby CO$_3$Ap-collagen mixtures with different CO$_3$Ap contents and porosity were lyophilized into sponges$^{1,2,9}$. SEM observation of CO$_3$Ap-collagen sponges showed favorable pores for cell invasion, with pore size ranging from 50 to 300 $\mu$m. Mouse osteoblast MC3T3-E1 cells were cultured in α MEM with 10% FCS for two weeks. Hematoxylin-eosin staining confirmed that osteoblast cells invaded well into the CO$_3$Ap-collagen sponge. However, to date, there is still no detailed consideration pertaining to the carbonate content of CO$_3$Apapatites as biomaterials. While there are reports on the crystallinity and solubility of carbonate apatites with different carbonate contents, the detailed role of carbonates for bone metabolism has not been fully clarified. Therefore, there is an urgent and pressing need to clarify the physicochemical properties of carbonate apatites. Fortuitously, with the development of advanced chemical analytical tools, detailed analyses can now be performed more accurately and then compared with previous reports.

As a first step of our research into the carbonate content of CO$_3$Apapatites, we examined — in this study — the physicochemical properties of synthesized CO$_3$Apapatites in detail, using inductively coupled plasma (ICP), X-ray diffraction, FT-IR, SEM, and HR-TEM to obtain an index of carbonate apatites as hard tissue biomaterials.

**MATERIALS AND METHODS**

**Synthesis of CO$_3$Apapatites**

Hydroxyapatite (HAp) and CO$_3$Apapatites with five different carbonate contents (CO$_3$Ap$_{0.00}$, CO$_3$Ap$_{0.01}$, CO$_3$Ap$_{0.02}$, CO$_3$Ap$_{0.06}$, CO$_3$Ap$_{0.09}$) were synthesized at 60 $^\circ$C and pH 7.4±0.2. A 0.5 L solution of 0.2 mol/L Ca(CH$_3$COO)$_2$$\cdot$H$_2$O and a 0.5 L solution of 0.12 mol/L NH$_4$H$_2$PO$_4$ containing 0, 0.005, 0.01, 0.03, 0.06, and 0.3 mol/L (NH$_4$)$_2$CO$_3$ were added to a mechanically stirred solution of 1.3 mol/L acetate buffer. The suspensions were stirred for three hours and then kept at room temperature for a day. CO$_3$Apats were then separated by filtration, washed with distilled water, and dried at 60$^\circ$C. The samples were synthesized in duplicate.

**Identification by X-ray diffraction, FT-IR and chemical analysis**

X-ray diffraction was employed to identify precipitates and estimate the degree of crystallinity. Measurements were done with a Shimadzu X-ray diffractometer (DXI, Shimadzu Co. Ltd., Kyoto, Japan) with graphite-monochromatized CuKα radiation at 30 kV and 30 mA in a continuous scan mode (4° 2θ/min and 0.5° 2θ/min). FT-IR analysis was carried out with a Shimadzu spectrometer (FT-IR 8400S, Shimadzu Co. Ltd., Kyoto, Japan) by diffuse reflectance method using powder samples containing each apatite (concentration: 1 mg/100 mg KBr) with number of scans at 100.

Fifty milligrams of each CO$_3$Ap was dissolved completely in 0.1 N HCl solution. Calcium and phosphate concentrations (n=5) were determined using an inductively coupled plasma (ICP) analyzer (SPS7800ICP, SII NanoTechnology Inc., Tokyo, Japan). ICP multi-element standard solution (XSTC-22, SPEX CertiPrep Inc., Metuchen, NJ, USA) was used for calibration. Each 10 mg of CO$_3$Ap sample (n=5) was taken into a Conway dish, and carbonate concentrations were determined by the titration method as described by Conway$^{15}$. SEM and HR-TEM observations

Scanning electron micrographs (SEM) of crystals were obtained with a Hitachi instrument (S-4300, Hitachi Co. Ltd., Tokyo, Japan). HR-TEM observation was also carried out at high magnification. Each sample was embedded in epoxy resin, then sectioned very thinly (about 60 nm) with a microtome equipped with a diamond knife. These thin samples were put into the TEM holder and observed with a Topcon high-resolution transmission electron microscope (EM002B, Topcon Co. Ltd., Tokyo, Japan) at 200 kV.

**Preparation of CO$_3$Ap-collagen sponge**

0.5 wt% of calf skin collagen solution (Cellgen, Koken Co. Ltd., Tokyo, Japan), treated by the application of enzymes to minimize antigenicity, was neutralized with 0.1 N NaOH, then mixed immediately with 70 wt% of each CO$_3$Apapatite by dry weight. The mixture gels were put into 96-well culture plates. The plates were then frozen at −80°C for two hours and dried in a freeze dry machine (Eyela Co. Ltd., Tokyo, Japan) for 24 hours. The CO$_3$Ap-collagen sponges were subjected to UV irradiation — by placing them 10 cm from the UV lamp (10 W, 253.7 nm) — for four hours to become insoluble.

**Culture of osteoblasts**

Mouse osteoblast-like MC3T3-E1 cells derived from untransformed mouse bone marrow were obtained from the RIKEN Cell Bank (Tsukuba, Japan) and maintained in a continuous culture at 37°C in a 5% CO$_2$ humidified atmosphere. Cells were grown in DMEM (Dulbecco's Modified Eagle's Medium) solution supplemented with 10% heat-inactivated FBS, Penicillin (100 U/ml) and streptomycin (100 μg/ml) were added to the media. The osteoblast-like cells (2.5×10$^5$ cells/well) were grown for one week in α MEM (minimum essential medium alpha modification) with 10% heat-inactivated FBS in 96-well plates containing CO$_3$Ap-collagen sponges at 37°C in a 5% humidified atmosphere. The culture medium was replaced every three days. The specimens were first fixed by replacing the culture medium with 2%...
glutaraldehyde, and then dehydrated in an ethanol-water series (60-100%). Finally, sponges with cells were observed with an SEM (S-4300, Hitachi Co. Ltd., Tokyo, Japan).

RESULTS

The chemical compositions of synthesized CO$_3$Aps analyzed using an inductively coupled plasma (ICP) chemical equipment are shown in Table 1. The calcium content of the samples was not significantly different from each other, although it was lower than that of the stoichiometrical 10 mmol/g of theoretical hydroxyapatite Ca$_{10}$(PO$_4$)$_6$(OH)$_2$. Total phosphate content was also lower than the stoichiometrical 6 mmol/g and decreased greatly at high CO$_3$ content of CO$_3$Ap$_{0.3}$. As for the CO$_3$ content, it increased gradually and then greatly at high CO$_3$ content of CO$_3$Ap$_{0.3}$. Total value of phosphate and carbonate contents approached the stoichiometrical phosphate content of 6 mmol/g. However, at higher content, it exceeded the stoichiometrical value. The CO$_3$ content of CO$_3$Ap$_{0.06}$ (<CO$_3$ content=0.80±0.07 mmol/g, i.e., 4.8 wt%) was similar to that of bone.

Table 1 Chemical compositions of precipitates

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ca (mmol/g)</th>
<th>P (mmol/g)</th>
<th>CO$_3$ (mmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HAp</td>
<td>9.01±0.09</td>
<td>5.74±0.09</td>
<td></td>
</tr>
<tr>
<td>CO$<em>3$Ap$</em>{0.06}$</td>
<td>8.45±0.09</td>
<td>5.48±0.07</td>
<td>0.12±0.05</td>
</tr>
<tr>
<td>CO$<em>3$Ap$</em>{0.03}$</td>
<td>8.49±0.19</td>
<td>5.62±0.12</td>
<td>0.21±0.09</td>
</tr>
<tr>
<td>CO$<em>3$Ap$</em>{0.02}$</td>
<td>8.72±0.09</td>
<td>5.50±0.11</td>
<td>0.58±0.06</td>
</tr>
<tr>
<td>CO$<em>3$Ap$</em>{0.06}$</td>
<td>8.78±0.16</td>
<td>5.46±0.15</td>
<td>0.80±0.07</td>
</tr>
<tr>
<td>CO$<em>3$Ap$</em>{0.3}$</td>
<td>8.56±0.12</td>
<td>4.69±0.11</td>
<td>1.57±0.03</td>
</tr>
</tbody>
</table>

Stoichiometrical compositions of hydroxyapatite
Ca$_{10}$(PO$_4$)$_6$(OH)$_2$: Ca=10 mmol/g, P=6 mmol/g

Fig.1 shows the X-ray diffraction pattern of each CO$_3$Ap. Crystallinity, which can be evaluated in terms of relative peak intensity and half-value width of the (300) reflection, decreased with increasing CO$_3$ content as given in previous reports. Even at the lower content of CO$_3$ ions, crystallinity decreased greatly. The (300) reflection peak shifted at high CO$_3$ content (Fig.2). This meant that most CO$_3$ ions were substituted into PO$_4$ positions.

FT-IR spectra expressed as percent transmittance became broader over the wave number range of 400-4000 cm$^{-1}$ with increasing CO$_3$ content (Fig.3). The FT-IR spectra of CO$_3$Aps showed clear CO$_3$ ion absorption peaks at 1410-1450 cm$^{-1}$. The expanded relative area mostly due to CO$_3$ ion absorption, when expressed as absorbance, increased with increasing CO$_3$ content (Fig.4).

Scanning electron microscopy (SEM) showed that hydroxyapatite had a needle-like crystal feature, and that the crystal feature of CO$_3$Aps changed dramatically to become flake-like and broccoli-like as shown in Fig.5. High-resolution transmission electron microscopy (HR-TEM) of the cross-sectional shape of crystals showed that hydroxyapatite was slender and hexagonal, while CO$_3$Ap changed gradu-
Detailed Analyses of CO₃ApATites

Fig. 3 Fourier transmission infrared spectra of CO₃Aps with different CO₃ contents.

Fig. 4 Expanded FT-IR absorption bands mostly due to CO₃³⁻ ion, of which the peaks can be observed at about 1410-1450 cm⁻¹.

ally from thin to indistinct (Fig. 6). At high magnification, a crystal lattice image and defects were observed.

Fig. 7 shows the SEM photos after one-week incubation of osteoblasts on the surface of each CO₃Aps-collagen sponge. Osteoblasts deformed to expand and cover the sponge sample. They expanded their projections and appeared to adhere well to the sponge surface.

Fig. 5 Scanning electron micrographs of CO₃Aps (A), together with their magnified micrographs of CO₃Aps (B).
DISCUSSION

Many researchers have investigated the physicochemical properties of CO$_3$Aps\textsuperscript{4,5,7–9}; however, claims and observations from these researches are yet to be confirmed. Over the past decade, advancements in chemical analytical tools have made more accurate chemical analyses to be possible. Most notably, ICP analysis has been developed to enable detailed analysis to be performed.

In this study, we were able to obtain more reliable data of P concentration. Fortuitously too, Ca and P data obtained using ICP were relatively similar to those obtained previously by atomic absorption and spectrophotometry\textsuperscript{8}. Calcium and phosphate contents were lower than those of stoichiometrical values due to the formation of Ca- and P-deficient apatites in relation to crystallinity. As for the chemical analysis of carbonate concentration, it still depended on the Conway method\textsuperscript{20}. Although this is a reliable method, it does not provide high accuracy especially at low concentrations. At higher concentrations, P+CO$_3$ content exceeded the stoichiometrical value of 6 mmol/g at which all CO$_3^{2−}$ ions are substituted into PO$_4^{3−}$ positions. A few explanations might be proffered for the obtained P+CO$_3$ value, although their effects were very small – namely, partial substitution of CO$_3^{2−}$ into OH$^−$ positions, CO$_3^{2−}$ adsorption on the crystal surface, and/or error of synthesis and chemical analysis.

LeGeros\textsuperscript{7} and Okazaki et al.\textsuperscript{8} found that by synthesizing at different temperatures, crystallinity decreased with increasing carbonate content. They also showed that the a-axis dimension decreased with the substitution of CO$_3^{2−}$ ions into PO$_4^{3−}$ positions in the

Fig. 6 High-resolution transmission electron micrographs of CO$_3$Aps (A), together with their magnified micrographs (B).

Fig. 7 Scanning electron micrographs of osteoblasts cultured onto CO$_3$Ap-collagen sponges with different CO$_3$ contents after 1-wk incubation.
case of apatite crystals synthesized under aqueous conditions, while the c-axis dimension increased with the substitution of CO$_3^{2-}$ ions into OH$^-$ positions in the case of apatite crystals synthesized under dry conditions at high temperature$^7$. To investigate the crystallinity of biomimetic apatites as biomaterials, apatites were synthesized under aqueous conditions. As a result, a shift of the (300) reflection peak to a high angle direction was recognized, indicating shrinkage of the crystal lattice. Therefore, the substitution of CO$_3^{2-}$ ions into the PO$_4^{3-}$ positions accounted for the shrinkage in the a-axis dimension.

The crystallinity of CO$_3$apatites synthesized in this study was lower than that of CO$_3$apatites synthesized at higher temperatures such as 80°C$^7$-$^9)$. It was observed that a relatively small amount of CO$_3$ could significantly affect the physicochemical properties. As shown in Figs. 5 and 6, both crystal size and shape changed dramatically. Disorder or lattice imperfection increased with increased substituting CO$_3^{2-}$ ions. Gradually, it became hard to maintain the hexagonal shape, which became distorted due to the difference between the ionic radius of PO$_4^{3-}$ (0.228 nm) and that of CO$_3^{2-}$ (0.198 nm)$^{35}$. In this study, with the purpose of applying large-amount production to biomaterials, the calcium concentration in the supplied solution was much higher than that in previous studies$^{35}$. Therefore, according to the calcium concentration in the supplied solution, a higher carbonate concentration was also supplied. Consequently, the amount of carbonate content in the apatite crystals differed with each synthesis experiment due to different calcium concentrations in the supplied solutions. In other words, CO$_3$ contents could not be compared by simply using the CO$_3$/P ratio due to differences arising from the supplied solutions. In the case of high calcium concentration in the supplied solution, and hence an increase in absolute carbonate concentration, the uptake of CO$_3^{2-}$ ions in the apatite crystal would definitely increase and this uptake is not stoichiometric.

Based on the results obtained in the present study, it could be said that crystallinity and chemical composition significantly influence the applicability of CO$_3$apatites as biomaterials. This is because crystallinity and chemical composition are important factors during bone regeneration in relation to bone apatite dissolution by osteoclasts and bone formation by osteoblasts. In conclusion, when taking into account the results of chemical analysis and X-ray diffraction, CO$_3$Ap$_{90}$ appeared to be similar to bone and is thus suitable for application as a biomaterial. To further explore its potential as a biomaterial, biological evaluation of CO$_3$Ap is currently in progress.

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