Reinforcement of carbonate apatite bone substitutes with carbonate apatite by Ca salt introduction

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Reinforcement of carbonate apatite (CO3Ap) block with CO3Ap was studied to increase the mechanical strength of CO3Ap block and widen applicable clinical fields. First, calcium salt was introduced to the micropores of CO3Ap block. Then, calcium salt was carbonated to form calcite inside the micropores of CO3Ap block by exposing CO3Ap block to carbon dioxide at the second step. On the third step, the CO3Ap block was immersed in Na2HPO4 aqueous solution. In this process, calcite inside the micropores of CO3Ap block transformed to CO3Ap on the dissolution–precipitation reaction, and the newly formed CO3Ap crystals entangled not only along the newly formed CO3Ap but also on CO3Ap crystals of the existing CO3Ap block. As a result of bonding between newly formed CO3Ap and the existing CO3Ap block, the mechanical strength of the CO3Ap block increased approximately 1.5 times higher when compared to that before the treatment.

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1. Introduction

The reconstruction of bone defects is a common challenge in the medical and dental fields. Though the gold standard for the reconstruction of bone defects is autograft, there are serious shortcomings including invasion to healthy site, limitation of the morphology and amount of collectable bone.1,2) As such, various calcium phosphate compounds have been used as bone substitutes.3) Of those compounds, hydroxyapatite (HA: Ca10(PO4)6(OH)2) is the most popular because of its superior biocompatibility and osteoconductivity.4) However, sintered HA is stable in the bone defect and would not be replaced to bone.5) Since bone plays not only mechanical role but also biological roles, replacement of artificial bone substitute to bone similar to autograft is strongly desired in some clinical cases. Key differences between sintered HA and bone apatite are chemical composition and crystallinity.6,7) Bone apatite contains 7% of carbonate ion in its apatitic crystal structure3) and thus should be called carbonate apatite (CO3Ap). Also crystallinity of bone apatite is low. On the other hand, sintered HA is stoichiometric HA and its crystallinity is high. For the osteoelastic resorption and resultant replacement to bone, carbonate contents in apatic structure is important since solubility of CO3Ap under weak acidic condition or the acidic condition formed in Howship’s lacunae is proportional to the carbonate content in apatic structure.8) With respect to crystallinity, lower crystallinity is preferable for higher solubility.

Although sintering is widely used to fabricate ceramics block, CO3Ap is unstable at high temperature. Upon sintering of CO3Ap, CO3 is liberated, and it decomposes to other calcium phosphates. Therefore, carbonate-free stoichiometric HA is used as a starting materials and it is sintered to fabricate block or granular HAp products. To fabricate low crystalline CO3Ap block, we proposed a new method based on dissolution–precipitation reaction using a precursor. Lin et al. employed low-crystalline calcite (CaCO3) block as a precursor. The calcite block can be prepared by exposing calcium hydroxide (Ca(OH)2) compact to carbon dioxide.8,9) The calcite block thus prepared would not be washed out even when it was immersed in liquid. Then, the calcite block was immersed in 1 mol/L Na2HPO4 solution at 60°C for 14 days. Based on dissolution–precipitation reaction, calcite was transformed to CO3Ap block without changing its microscopic structure.10,11) CO3Ap block thus prepared had approximately 25% porosity and its diametral tensile strength (DTS) was approximately 6 MPa. Although mechanical strength of CO3Ap block is not enough for its use in load-bearing area, mechanical strength of CO3Ap block is enough for its use in non-load-bearing area. CO3Ap granule implanted in the parietal bone defect of rat was found to be replaced to bone whereas sintered HAp granule remained in the defect.12) Wakae et al. attempted to fabricate an artificial bone replacement which has not only chemical composition but also structure similar to cancellous bone. Initially α-tricalcium phosphate (α-TCP; α-Ca3(PO4)2) foam was prepared using polyurethane foam as a template.13,14) Then, the α-TCP foam was immersed in ammonium carbonate solution. At this process, α-TCP foam transformed to CO3Ap foam based on dissolution–
precipitation reaction, keeping the three-dimensional fully interconnected porous structure. Fully interconnected porous structure of CO3Ap foam is ideal for cells and tissue penetration. However, large porosity of the CO3Ap foam, 90—95%, results in limited mechanical strength. The α-TCP foam has hollow structure since polyurethane foam used as a template is burned out during the sintering process. CO3Ap foam also has hollow structure since compositional change due to dissolution—precipitation proceeded without changing its macroscopic structure. Of course, hollow structure of the CO3Ap foam would not contribute to the cells and tissue penetration.

On the other hand, it is well known that mechanical strength is governed by the porosity. In other words, reduction of porosity is useful to increase the mechanical strength. In the present study, therefore, we evaluated the feasibility of increasing the mechanical strength by decreasing the porosity of CO3Ap block. To reduce the porosity without changing its composition, the pores were attempted to be filled with additional CO3Ap precipitates. In short, calcium salt solution was introduced inside the pores of CO3Ap block, followed by carbonation and phosphatization to form additional CO3Ap. The resultant CO3Ap block was characterized in terms of density, porosity, composition and mechanical strength.

2. Experimental procedure

2.1 Preparation of CO3Ap block

CO3Ap block was prepared as described previously.14) In short, 0.2 g of calcium hydroxide powder (Ca(OH)2; Wako Pure Chemical Industries Ltd., Japan) was placed in a stainless steel mold and pressed uniaxially with an oil pressurized press machine (Riken Seiki Co. Ltd., Japan) under 2 MPa. The Ca(OH)2 compact (9 mm in diameter and 2 mm in thickness) was exposed to water vapor—saturated carbon dioxide (CO2) for 14 days at room temperature for carbonation. Calcite (CaCO3) block thus prepared was supplied for phosphatization process. In other words, calcite block was placed in 1 mol/L Na2HPO4 solution kept at 60°C for 14 days. The resultant specimens were carefully rinsed with distilled water and dried at 60°C for 12 hours.

2.2 Precipitation of additional CO3Ap in the micropores of CO3Ap block

At first, CO3Ap block was immersed in 1 mol/L calcium lactate solution to introduce calcium lactate into the micropores of CO3Ap block. For the introduction of large amount of calcium lactate, the vessel containing calcium lactate and CO3Ap block was evacuated using a water aspirator, followed by exposure to atmospheric pressure as shown in Fig. 1. The specimens were taken out from the solution then dried at 60°C for 12 hours. Second step is the carbonation of calcium lactate in the micropores of CO3Ap block. For the carbonation, the specimens were exposed to CO2 at room temperature for 14 days. Third step is the phosphatization. In this process, the specimens were immersed in 1 mol/L Na2HPO4 solution at 60°C for 14 days. Weight change was monitored at each treatment step.

2.3 Characterization of the specimens

The apparent density and apparent porosity was calculated using Archimedes’ principle. For each specimen, dry weight (w1), weight-in-kerosene (w2) and weight-containing-kerosene (w3) were measured and the following formulas were used.

\[
\text{Apparent density (g/cm}^3) = \frac{w_1 \times D}{w_1 - w_2}
\]

\[
\text{Apparent porosity (\%)} = \frac{w_3 - w_1}{w_3 - w_2} \times 100
\]

The average value was calculated from at least three specimens. The morphology of fractured surface of the specimens was characterized by means of scanning electron microscope (SEM; JSM 5400LV, JEOL, Japan) at an accelerating voltage of 15 kV after gold coating. For the compositional analysis, the specimens were characterized by powder X-ray diffraction (XRD). The XRD patterns were recorded with a vertically mounted diffractometer system (RINT 2500V, Rigaku, Japan) using counter-monochromatized CuKα radiation generated at 40 kV and 100 mA. The specimens were scanned from 20 to 40° in 2θ under a continuous mode at a scanning rate of 2°/min. Fourier transformation infrared spectroscopy (FT-IR) analysis was also performed for compositional analysis with an FT-IR spectrometer (Spectrum 2000LX; Perkin-Elmer Co. Ltd., USA).

Mechanical strength of specimens was evaluated in terms of diametral tensile strength (DTS). The diameter and height of each specimen were measured with a micrometer (156-101; Mitsutoyo Co. Ltd., Japan). Then, the samples were crashed down at a crosshead speed of 1 mm/min using a universal testing machine (IS5000, Shimadzu Co., Japan). The DTS value with SD at each treatment step was calculated as average of at least five specimens.

3. Results

Table 1 summarizes the apparent density and apparent porosity of CO3Ap blocks before and after each treatment. Apparent density increased, whereas apparent porosity decreased after each treatment step. Figure 2 shows the cross-sectional SEM images for CO3Ap block before and after CO3Ap precipitation treatments. CO3Ap block had granular and sparse structure which was composed of inter spaces between connected CO3Ap granules. After CO3Ap precipitation treatment, open spaces between grains were filled with fine CO3Ap precipitations (Figs. 2(b) and (d)). Figure 3 shows the XRD patterns of CO3Ap.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Apparent density (g/cm³)</th>
<th>Apparent porosity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before treatment</td>
<td>2.19 ± 0.15</td>
<td>25.4 ± 0.6</td>
</tr>
<tr>
<td>After calcium lactate introduction</td>
<td>2.46 ± 0.06</td>
<td>19.8 ± 1.8</td>
</tr>
<tr>
<td>After carbonation</td>
<td>2.52 ± 0.05</td>
<td>19.6 ± 1.3</td>
</tr>
<tr>
<td>After phosphatization</td>
<td>2.91 ± 0.56</td>
<td>6.3 ± 4.2</td>
</tr>
</tbody>
</table>

Fig. 1. Schematic illustration of apparatus for Ca lactate introduction.
blocks before (Fig. 3(a)) and after calcium lactate treatment (Fig. 3(b)), followed by carbonation (Fig. 3(c)) and phosphatization (Fig. 3(d)). Although XRD pattern for calcium lactate could not be detected after calcium lactate treatment, increment and decrement of calcite peak were observed after carbonation and phosphatization, respectively. Figure 4 shows the FT-IR spectra of CO3Ap blocks before (Fig. 4(a)) and after calcium lactate treatment (Fig. 4(b)), followed by carbonation (Fig. 4(c)) and phosphatization (Fig. 4(d)). In all specimens, typical spectra assigned to CO3Ap were detected. Besides, similar results with XRD measurement were acquired, that is, spectra for calcium lactate could not be detected even if after calcium lactate treatment (Fig. 4(b)). Figure 5 summarizes DTS of CO3Ap block before (Fig. 5(a)) and after calcium lactate treatment (Fig. 5(b)), followed by carbonation (Fig. 5(c)) and phosphatization (Fig. 5(d)). There were no significant differences among the DTS values of original CO3Ap block, CO3Ap block after calcium lactate introduction and CO3Ap block after calcite formation. In contrast, DTS value of CO3Ap block after phosphatization was significant ($p < 0.01$) and approximately 1.5 times higher when compared to that of non-treated CO3Ap block.

4. Discussion

The method employed for the reinforcement of CO3Ap block is basically the same as Lin's method except for the introduction of calcium salt into the micropores of the CO3Ap block. In the present study, calcium lactate was chosen as an example of Ca salts. Ca salt with high solubility and high Ca content may be the requirements for the choice of Ca salt, therefore, the stability of the Ca salt should be emphasized. In the preliminary study, calcium chloride was used instead of calcium lactate. In fact, calcium chloride has higher solubility and higher Ca content even when compared to calcium lactate. However, calcium chloride is hygroscopic and tends to absorb moisture in air so it’s difficult to dry and thus was not proper for this purpose.
To introduce larger amount of Ca salt as well as homogeneously inside the CO$_3$Ap block, CO$_3$Ap block immersed in calcium lactate solution was evacuated with aspirator and then, exposed to atmosphere as shown in Fig. 1.

Increase in apparent density listed in Table 1 showed the introduction of calcium lactate into the micropores in CO$_3$Ap block. XRD and FT–IR analyses could not detect the introduction of calcium lactate. This may be caused by the limited amount of introduced calcium lactate and/or the precipitation of amorphous calcium lactate. After the introduction of calcium lactate into CO$_3$Ap block, the CO$_3$Ap block was exposed to carbon dioxide. At this second step, calcium lactate was carbonated to form calcite as confirmed by XRD. CO$_3$Ap block used in the present study contained small amount of unreacted calcite. Although unreacted calcite is known to be converted to CO$_3$Ap with time, CO$_3$Ap block was used as it is since the purpose of this study can be done even by using CO$_3$Ap block containing small amount of calcite. After the carbonation process, increase in XRD patterns and FT–IR spectra corresponding to calcite was confirmed as shown in Fig. 3(c) and Fig. 4(c). On the other hand, no significant increase in apparent density was observed for this process (Table 1). Transformation of calcium lactate to calcite theoretically should increase apparent density since no elimination of lactate was made. However the change is limited and thus may not be detected by the apparent density measurement. At this step, no increase in mechanical strength was observed as shown in Fig. 5. Although the detailed mechanism has not been clarified yet with respect to the unincorporated mechanical strength, absence of bonding between the calcite and CO$_3$Ap may be the reason. In other words, calcite contained in CO$_3$Ap block act as dust with respect to the mechanical strength. At the third step, calcite transformed to CO$_3$Ap as shown by the decrease in peaks assigned to calcite (Fig. 3(d) and Fig. 4(d)). At this step, apparent density increased by taking phosphate ion from the solution (Table 1). SEM image also support precipitation of additional CO$_3$Ap in the microstructure of CO$_3$Ap block. DTS value increased approximately 1.5 times higher when compared to that before the treatment. Upon immersion of CO$_3$Ap block containing calcite in Na$_2$HPO$_4$ solution, the calcite dissolves to supply Ca$^{2+}$ and CO$_3$$^{2-}$ since the Na$_2$HPO$_4$ solution is under saturated with respect to calcite. However the resultant solution would be supersaturated with respect to CO$_3$Ap. Therefore, calcite transforms to CO$_3$Ap based on dissolution–precipitation reaction. Although the detailed mechanism has not been clarified yet in the present study, precipitation reaction of CO$_3$Ap as secondary crystal might occur on the surface of CO$_3$Ap block since the existing CO$_3$Ap crystals on the surface of CO$_3$Ap block can be taken as primary crystals. Of course, this is a key for the increased mechanical strength.

In the present study, CO$_3$Ap block was reinforced with additional CO$_3$Ap precipitation for simplicity. Basically, this method should be applicable for other artificial bone substitutes including CO$_3$Ap foam as long as their chemical composition is CO$_3$Ap. Key advantage for CO$_3$Ap block is its osteoelastic resorption and resulting replacement to bone. Fortunately, chemical composition of CO$_3$Ap reinforced CO$_3$Ap block is the same with CO$_3$Ap block. Therefore, the same response to cells and tissues i.e., osteoelastic resorption and resulting replacement to bone are expected for CO$_3$Ap reinforced CO$_3$Ap block. On the other hand, micropores in CO$_3$Ap block which might be concerning osteoelastic resorption were disappeared in this method. However, the mechanical property takes precedence over the resorbability in some clinical uses, especially for load-bearing area. Of course, in vivo behaviors of CO$_3$Ap reinforced CO$_3$Ap block should be confirmed based on cells and histological studies in the future.

5. Conclusions

CO$_3$Ap block was found to be reinforced with CO$_3$Ap based on further precipitation of CO$_3$Ap crystals inside the micropores of CO$_3$Ap block. The reinforcement consists of three steps. Initial step is the introduction of calcium salt in the micropores. The calcium salt was transformed to calcite based on the exposure to CO$_2$ at the second step. At the third step, calcite was phosphatized to transfer to CO$_3$Ap. Increase in mechanical strength of CO$_3$Ap block may be explained by the bonding mechanism between newly formed CO$_3$Ap crystals and CO$_3$Ap matrix.

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References