Influence of pH and ion components in the liquid phase on the setting reaction of carbonate apatite granules

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Carbonate apatite (CO₃Ap) is an inorganic component of bone and replaces by natural bone after implantation into the bone defect. Because of this unique characteristic, CO₃Ap granules have been used in the dental field. However, washing out of granules from the bone defect area is an issue. The aim of this study was to set CO₃Ap granules by mixing CO₃Ap granules with acidic phosphate solutions and evaluate the influence of the pH and ion components of the solutions. When Na⁺ was the counter ion, the amount of precipitated dicalcium phosphate dihydrate (DCPD) was small and the setting ability disappeared with increasing pH of the solutions. Alternatively, when the counter ion was Ca²⁺, the amount of precipitated DCPD was high and the setting ability was observed even at high pH. These results suggest the presence of Ca²⁺ in the acidic phosphate solution is a key for fabricating CO₃Ap granular cement.

Keywords: Carbonate apatite, Granular cement, Bone substitute, Dissolution-precipitation reaction

INTRODUCTION

Carbonate apatite [CO₃Ap: Ca₁₀₋₅(PO₄)₆₋₅(CO₃)₁₋₅], which is a carbonate included apatite, is an inorganic component of bone with high osteoconductivity and bioresorbability. Artificially prepared granular type CO₃Ap has already been clinically used as an artificial bone substitute in dentistry in Japan. Although the granules can be implanted in a bone defect of any shape, washing out of granules from the bone defect is a problem for achieving a maximum therapeutic effect. Cement-type bone substitutes are a key to addressing this issue. For instance, calcium phosphate cement or the composite of osteoconductive calcium phosphate and setting materials, such as calcium sulfate, have been developed. These cements can set and become solid in the defect area after implantation. Alternatively, for granules, Fukuda et al. fabricated beta-tricalcium phosphate (β-TCP) granular cement by mixing β-TCP granules with an acidic phosphate solution. The acidic phosphate solution dissolves the surface of β-TCP granules and after saturating with respect to the stable calcium phosphate in acidic condition, dicalcium phosphate dihydrate [DCPD: Ca(H₂PO₄)₂•2H₂O] is precipitated on the surface of the granules. DCPD is a plate-like crystal and the DCPD crystals on the surface of β-TCP granules enable interlocking of the granules. Furthermore, DCPD has been used for bone tissue regeneration as a brushite cement. Compared with the cases of calcium phosphate or composite cements, β-TCP granular cement advantageously becomes an interconnected porous block after setting. Interconnected pores in the bone substitute are key to fabricating recent novel bone substitutes that allow for the penetration of body fluid into the block.

Setting method of CO₃Ap granules is also in demand. From the insight of previous report, in dissolution-precipitation reaction, pH for dissolution and saturation of the ions for precipitation should be important points to set the CO₃Ap granules. In this report, we tried to set CO₃Ap granules by mixing CO₃Ap granules with acidic phosphate solutions and evaluated the influence of the solution pH and ion components in the solutions on the setting properties of CO₃ApGC.

MATERIALS AND METHODS

Materials
All chemicals were purchased from Fujifilm Wako Pure Chemical (Osaka, Japan).
CO₃Ap granules with a size of 300–600 μm were prepared according to a previous report.

Preparation of acidic phosphate solutions
A sodium based phosphate solution (Na-H-PO₄) was prepared by mixing a 1 mol/L NaH₂PO₄ solution and 1 mol/L H₃PO₄ solution and adjusting the pH to 1.5, 2.0, and 2.5. A calcium based phosphate solution (Ca-H-PO₄) was prepared by adding Ca(OH)₂ powder to H₃PO₄ solutions, the phosphate concentration was adjusted to 1 mol/L, and the pH was adjusted to 1.5, 2.0, and 2.5.

Setting of CO₃ApGC
CO₃Ap granules were mixed with 1 mol/L H₃PO₄ (pH 0.9) and a sodium or calcium-based phosphate solution at an L/G ratio of 0.5 and transferred a split acrylic mold (φ6 mm×3 mm). The mixture of granules and solution was removed from the split mold after 30 min.

Morphological analysis of CO₃ApGC
The morphology of set CO₃ApGC was observed by
scanning electron microscopy (SEM; S3400N, Hitachi High Technologies, Tokyo, Japan) at an accelerating voltage of 15 kV after coating the samples with a gold-palladium alloy by magnetron sputtering (MSP-1s, Vacuum Device, Ibaraki, Japan). X-ray micro-computed tomography (107KSH, SkyScan, Kontich, Belgium) using an X-ray voltage of 50 kV, 0.5 μm aluminum filter, and pixel size of 18 μm was also used.

**Compositional analysis of CO$_3$ApGC**

For compositional analysis, the set CO$_3$ApGC was ground into a fine powder and evaluated using an X-ray diffractometer (D8 Advance diffractometer, Bruker AXS, Karlsruhe, Germany) operated at 40 kV and 40 mA. The diffraction angle was continuously scanned in the 2θ range of 10º to 40º at a scanning rate of 6º/min. The amount of DCPD and CO$_3$Ap was calculated from calibration curves constructed by the measurements of the area ratio of DCPD and CO$_3$Ap.

**Mechanical strength measurement of CO$_3$ApGC**

After 24 h of drying at room temperature, the dynamic tensile strength (DTS) of the set CO$_3$ApGC was measured by a universal testing machine (Autograph AGS-J, Shimadzu, Kyoto, Japan). A load was applied to the samples at a crosshead speed of 1 mm/min to crush the samples.

**RESULTS**

First, we prepared two types of acidic phosphate solutions with pH adjusted by sodium phosphate (Na-H$_2$PO$_4$) or calcium hydroxide (Ca-H$_2$PO$_4$) and demonstrated the setting ability of CO$_3$Ap granules after treating with the solutions. In cases with Na-H$_2$PO$_4$ solutions as a liquid part, CO$_3$Ap granules can be set only using the solution at pH 1.5, not at pH of 2.0 or 2.5 after mixing granules with the solutions for 30 min (Figs. 1b–e). However, even though the CO$_3$Ap granules were set using the pH 1.5 Na-H$_2$PO$_4$ solution as the liquid, the set CO$_3$ApGC easily collapsed and the shape was nonuniform compared with the CO$_3$ApGC set using H$_3$PO$_4$ solution (Fig. 1b). Alternatively, compared with Na-H$_2$PO$_4$ solutions, Ca-H$_2$PO$_4$ solutions can set CO$_3$Ap granules after mixing, regardless of the solution pH (Figs. 1e–g). As shown in the SEM observation of the CO$_3$ApGC surfaces prepared using Ca-H$_2$PO$_4$ solutions, plate-like crystals were formed, interlocking the CO$_3$Ap granules and producing porous CO$_3$ApGC (Figs. 2a–h). Furthermore, μCT analysis of CO$_3$ApGC revealed that the pores in CO$_3$ApGC were interconnected (Figs. 2k–n). The morphology of CO$_3$ApGC was easily collapsed and produced porous CO$_3$ApGC (Figs. 2a–h).

XRD analysis suggests that DCPD formation by mixing CO$_3$Ap granules with an acidic phosphate solution was accelerated by the presence of calcium ions and/or acidic pH. In the cases with Na-H$_2$PO$_4$ solutions, DCPD formation decreased with increasing pH of the solution (Fig. 3a) and DCPD was barely observed at pH greater than 2.0. It is well known that the solubility of apatite decreases with an increase in pH. Thus, at higher pH Na-H$_2$PO$_4$ solutions, the dissolution of CO$_3$Ap was suppressed and precipitation of DCPD was limited. Alternatively, in the cases with Ca-H$_2$PO$_4$ solutions, pH dependence of the formation of DCPD was not observed (Fig. 3b). Moreover, at the same pH, the amount of DCPD was drastically increased using Ca-H$_2$PO$_4$ solutions compared with using Na-H$_2$PO$_4$ solutions (Fig. 3c).

The mechanical strength of CO$_3$ApGC after setting the CO$_3$Ap granules was improved by the presence of Ca$^{2+}$ in the solutions. Comparisons cements prepared with the Na-H$_2$PO$_4$ solution and Ca-H$_2$PO$_4$ solution with pH 1.5 suggests that calcium ions improved the mechanical strength of CO$_3$ApGC even though the bulk porosity of CO$_3$ApGC was the same (Figs. 4a, b). Although there was no significant difference in the mechanical strength of CO$_3$ApGC prepared by different pH of the solutions, the tendency was the same regarding the amount of DCPD. Thus, a significant amount of precipitated DCPD induced the high mechanical strength of CO$_3$ApGC (Fig. 4c), which was roughly proportional to the amount of
precipitated DCPD. CO₃ApGC did not set for DCPD precipitated up to 7.6%. CO₃Ap set at 10.6% precipitated DCPD or more. In cases with Ca-H-PO₄ solutions, the amount of precipitated DCPD was greater than in cases with Na-H-PO₄ solutions and the mechanical strength of CO₃ApGC was increased. This meant that a large amount of the precipitated DCPD should be interlocked, as observed by SEM (Fig. 2), creating a granular cement block without any change in porosity.

**DISCUSSION**

CO₃Ap granules mixing with acidic phosphate solution were set by the dissolution-precipitation reaction. The pH of the solutions relates to the dissolution step of CO₃Ap and the ion concentration relates to the precipitation step of DCPD. It is well known that DCPD is a stable phase of calcium phosphate in acidic conditions and is precipitated when the concentration of calcium and phosphate ions in the solution is saturated\(^1\). Furthermore, the magnitude of the solubility product for DCPD is \(10^{-7}\) (mol/L)\(^2\) and the concentration of Ca\(^{2+}\) in Ca-H-PO₄ solutions is greater than 1 mol/L at pH 1.5, 2.0, and 2.5\(^\text{19}\). Thus, in cases with Ca-H-PO₄ solutions as liquid phases, a sufficient amount of Ca\(^{2+}\) in the solutions induced significant precipitation of DCPD and there was no correlation between DCPD formation and the solution pH. Alternatively, in the cases with Na-H-PO₄ solutions, Ca\(^{2+}\) was only supplied from CO₃Ap granules. With a high pH Na-H-PO₄ solution, dissolution of CO₃Ap and the concentration of Ca\(^{2+}\) in the solutions were limited because the amount of precipitated DCPD after

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**Fig. 2** Morphological analysis of CO₃Ap GC by (a–j) SEM and (k–n) μCT. (a, f) CO₃Ap granules, CO₃ApGC surfaces prepared by (b, g, k) H₃PO₄, (c, h, j) Ca-H-PO₄ at pH 1.5, (d, i, m) Ca-H-PO₄ at pH 2.0, and (e, j, n) Ca-H-PO₄ at pH 2.5.

**Fig. 3** XRD pattern of CO₃ApGC prepared by (a) Na-H-PO₄ solutions and (b) Ca-H-PO₄ solutions. (c) The amount of DCPD in CO₃ApGC at each pH calculated from the XRD results.
mixing CO\textsubscript{3}Ap granules with Na-H-PO\textsubscript{4} solutions was smaller than after mixing the granules with Ca-H-PO\textsubscript{4} solutions. These results suggest that saturation of Ca\textsuperscript{2+} is a bottleneck to forming CO\textsubscript{3}ApGC by a dissolution-precipitation reaction.

Previously, carbonate apatite cement prepared from the mixture of CaCO\textsubscript{3} and dicalcium phosphate anhydrous powder was reported\textsuperscript{20}. In this case, carbonate apatite block with independent pores (<5 μm) was formed after setting. Even though surface area of CO\textsubscript{3}Ap cement is increased, it cannot be an advantage to penetrate bone related tissues. Compared with this type of CO\textsubscript{3}Ap cement, we try to set CO\textsubscript{3}Ap granules and CO\textsubscript{3}ApGC after setting of CO\textsubscript{3}Ap granules has interconnected pores (>100 μm). We previously confirm that the pores in the block, which diameter is around 100 μm, improve the penetration of bone related tissues and new bone formation\textsuperscript{21}. Thus, CO\textsubscript{3}ApGC with interconnected pores is also expected to improve the new bone formation after implantation in the bone defects.

The obtained CO\textsubscript{3}ApGC was a biphasic-type bone substitute with DCPD and CO\textsubscript{3}Ap and the effectiveness of biphasic bone substitutes has been reported by various researchers\textsuperscript{22-26}. In these cases, different characteristics, such as solubility and osteoconductivity of the different calcium phosphate, induce improvement in the osteoblastic activity and new bone formation. In our case, CO\textsubscript{3}Ap is highly osteoconductive and a suitable bioresorbable bone substitute compared with other calcium phosphate bone substitutes\textsuperscript{5} and DCPD has quick releasing ability of Ca\textsuperscript{2+} and PO\textsubscript{4}\textsuperscript{3-} for bone regeneration\textsuperscript{24}. Based on these insights, we can expect that CO\textsubscript{3}ApGC has setting ability in bone defect area, which was the preferential objective of this study, as well as the potential to improve new bone formation.

In summary, we developed a preparation method of CO\textsubscript{3}ApGC using acidic phosphate solutions and evaluated the effect of pH and counter ions of the solutions on its formation. The presence of Ca\textsuperscript{2+} improved the precipitation of DCPD on the surface of CO\textsubscript{3}Ap and increased the mechanical strength of CO\textsubscript{3}ApGC even though the pH of the solutions was high. Although this is the first report demonstrating the setting ability of CO\textsubscript{3}Ap granules, acidic pH of the liquids may be causing the damage to surrounding tissues. In the future, we should evaluate the new bone formation and inflammation after implantation of CO\textsubscript{3}ApGC.

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