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: Ca_{10-a}(PO₄)_{6-b}(CO₃)_c], which is a carbonate included apatite, is an inorganic component of human bone with high osteoconductivity and bioresorbability¹⁻⁴. Artificially prepared granular type CO₃Ap has already been clinically used as an artificial bone substitute in dentistry in Japan^{5,6}. 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 cement⁷⁻⁹ or the composite of osteoconductive calcium phosphate and setting materials, such as calcium sulfate, have been developed¹⁰⁻¹². 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^{13,14}. 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: CaHPO₄·2H₂O] 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^{9,15}. Compared with the cases of calcium phosphate or composite cements, β-TCP granular cement advantageously becomes an interconnected porous block after setting^{13,14}. 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^{16,18}.

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 and made CO₃Ap granular cement (CO₃ApGC) 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 report¹¹.

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 (1076KSH, 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_3ApGC

For compositional analysis, the set CO_3ApGC 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 10° to 40° at a scanning rate of $6^\circ/\text{min}$. The amount of DCPD and CO_3Ap was calculated from calibration curves constructed by the measurements of the area ratio of DCPD and CO_3Ap .

Mechanical strength measurement of CO_3ApGC

After 24 h of drying at room temperature, the dynamic tensile strength (DTS) of the set CO_3ApGC 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-PO_4) or calcium hydroxide (Ca-H-PO_4) and demonstrated the setting ability of CO_3Ap granules after treating with the solutions. In cases with Na-H-PO_4 solutions as a liquid part, CO_3Ap 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_3Ap granules were set using the pH 1.5 Na-H-PO_4 solution as the liquid, the set CO_3ApGC easily collapsed and the shape was nonuniform compared with the CO_3ApGC set using H_3PO_4 solution (Fig. 1b). Alternatively, compared with Na-H-PO_4 solutions, Ca-H-PO_4 solutions can set CO_3Ap granules after mixing, regardless of the solution pH (Figs. 1e–g). As shown in the SEM observation of the CO_3ApGC surfaces prepared using Ca-H-PO_4 solutions, plate-like crystals were formed, interlocking the CO_3Ap granules and producing porous CO_3ApGC (Figs. 2a–h). Furthermore, μCT analysis of CO_3ApGC revealed that the pores in CO_3ApGC were interconnected (Figs. 2k–n). The morphology of CO_3ApGC was similar with previously reported $\beta\text{-TCPGC}^{13,14}$ and we expect the penetration of bone-related cells and tissues into the CO_3ApGC .

XRD analysis suggests that DCPD formation by mixing CO_3Ap granules with an acidic phosphate solution was accelerated by the presence of calcium ions and/or acidic pH. In the cases with Na-H-PO_4 solutions, DCPD formation decreased with increasing pH of the solution (Fig. 3a) and DCPD was barely observed at pH

Fig. 1 Images of CO_3Ap granules after 30 min of mixing with acidic phosphate solutions.

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-PO_4 solutions, the dissolution of CO_3Ap was suppressed and precipitation of DCPD was limited. Alternatively, in the cases with Ca-H-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-PO_4 solutions compared with using Na-H-PO_4 solutions (Fig. 3c).

The mechanical strength of CO_3ApGC after setting the CO_3Ap granules was improved by the presence of Ca^{2+} in the solutions. Comparisons cements prepared with the Na-H-PO_4 solution and Ca-H-PO_4 solution with pH 1.5 suggests that calcium ions improved the mechanical strength of CO_3ApGC even though the bulk porosity of CO_3ApGC was the same (Figs. 4a, b). Although there was no significant difference in the mechanical strength of CO_3ApGC 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_3ApGC (Fig. 4c), which was roughly proportional to the amount of

Fig. 2 Morphological analysis of CO₃Ap GC by (a–j) SEM and (k–n) μ CT. (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.

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 saturated¹⁾. Furthermore, the magnitude of the solubility product for DCPD is 10⁻⁷ (mol/L)² and the concentration of Ca²⁺ in Ca-H-PO₄ solutions is greater than 1 mol/L at pH 1.5, 2.0, and 2.5¹⁹⁾. Thus, in cases with Ca-H-PO₄ solutions as liquid phases, a sufficient amount of Ca²⁺ 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, Ca²⁺ was only supplied from CO₃Ap granules. With a high pH Na-H-PO₄ solution, dissolution of CO₃Ap and the concentration of Ca²⁺ in the solutions were limited because the amount of precipitated DCPD after

Fig. 4 (a) Mechanical strength, (b) bulk porosity of CO₃ApGC, and (c) the correlation of precipitated DCPD and mechanical strength. *, $p < 0.05$.

mixing CO₃Ap granules with Na-H-PO₄ solutions was smaller than after mixing the granules with Ca-H-PO₄ solutions. These results suggest that saturation of Ca²⁺ is a bottleneck to forming CO₃ApGC by a dissolution-precipitation reaction.

Previously, carbonate apatite cement prepared from the mixture of CaCO₃ and dicalcium phosphate anhydrous powder was reported²⁰. In this case, carbonate apatite block with independent pores (<5 μm) was formed after setting. Even though surface area of CO₃Ap cement is increased, it cannot be an advantage to penetrate bone related tissues. Compared with this type of CO₃Ap cement, we try to set CO₃Ap granules and CO₃ApGC after setting of CO₃Ap 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 formation²¹. Thus, CO₃ApGC with interconnected pores is also expected to improve the new bone formation after implantation in the bone defects.

The obtained CO₃ApGC was a biphasic-type bone substitute with DCPD and CO₃Ap and the effectiveness of biphasic bone substitutes has been reported by various researchers²²⁻²⁶. 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, CO₃Ap is highly osteoconductive and a suitable bioresorbable bone substitute compared with other calcium phosphate bone substitutes⁵) and DCPD has quick releasing ability of Ca²⁺ and PO₄³⁻ for bone regeneration²⁴. Based on these insights, we can expect that CO₃ApGC 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 CO₃ApGC using acidic phosphate solutions and evaluated the effect of pH and counter ions of the solutions on its formation. The presence of Ca²⁺ improved the precipitation of DCPD on the surface of CO₃Ap and increased the mechanical strength of CO₃ApGC even though the pH of the solutions was high. Although this is the first report demonstrating the setting ability of CO₃Ap 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 CO₃ApGC.

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