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Fabrication of apatite-coated gypsum granules and histological evaluation using rabbits

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ABSTRACT

Calcium sulfate dihydrate (CSD) has been used as a material for bone augmentation for many years. However, rapid resorption of CSD sometimes results in insufficient formation of new bone. In the present study, the feasibility of fabricating apatite-coated CSD (Ap/CSD) was studied, with the hypothesis that the apatite coating would reduce the rate of CSD resorption and enhance the osteoconductivity. To prepare Ap/CSD granules, 300–400 μm CSD granules were immersed in Na_2HPO_4 solution at 20 °C or 60 °C, resulting in granules coated with apatitic precipitates. Granules were analyzed by microscopy and x-ray diffraction, and the dissolution rate into pH 7.5 Tris-HCl buffer solution was measured. Osteoconductivity was assessed by the release of calcium ions, and their ability to stimulate new bone formation was examined through reconstruction of rabbit distal femur bone defects. It was found that the apatite coating resulted in increased osteoconduction and a decreased dissolution rate compared with bare CSD granules. When rabbit distal femur bone defect was reconstructed with Ap/CSD, CSD and sintered hydroxyapatite (s-HAp), all samples demonstrated an excellent tissue response. Four weeks after surgery, a higher radio opacity was obtained for Ap/CSD when compared with CSD, indicating new bone formation. The s-HAp granules remained and thus, new bone formation was smallest even though s-HAp granules bonded with the bone. Ap/CSD that was fabricated at 60 °C induced greater bone formation than CSD. It is therefore concluded that Ap/CSD has good potential value as a bone augmentation material.

1. Introduction

Calcium sulfate dihydrate (CSD; $CaSO_4{\cdot}2H_2O$) has been used in clinics as an artificial bone augmentation material for many years [1–7]. The bioresorbable nature and release of calcium ions (Ca^{2+}) from CSD make it an ideal material for this application. It has been emphasized that Ca^{2+} availability is a key factor influencing new bone formation. Niklas et al. reported that Ca^{2+} is involved in intercellular signaling between osteoblasts and osteoclasts [8]. Marrow stromal cells differentiate to osteoclasts through the activation of mitogen-activated protein kinase (MAPK) signaling where calcineurin — which is modulated by Ca^{2+} — activates transcription factors of the nuclear factor of activated T-cells (NFAT) family, which induce osteoclastogenesis [9]. The activated osteoclasts resorbed old bone, and further activate osteoblasts. Osteoblasts have Ca^{2+} receptors which modulate differentiation, matrix formation and mineralization of the osteoblast [10]. Therefore, bone augmentation materials that release Ca^{2+} have advantages with respect to osteoconduction. However, it is essential that the release of Ca^{2+} is optimized, as calcium compounds that dissolve too rapidly would not be suitable materials for bone augmentation [11–13]. Although CSD has been used in clinics as a bone augmentation material, its rapid dissolution rate can be problematic [14–19]. Thus, regulation of the dissolution rate is considered to be an effective approach for the fabrication of improved CSD-based bone augmentation materials. It has been reported that the rate of the dissolution decreases when CSD is mixed with calcium phosphate (CaP) to produce CSD/CaP composite cement [20–23]. It can therefore be expected that in the case of granular bone augmentation material, the most effective method of regulating dissolution will be the use of CaP coating on CSD. Furthermore, the CaP coating may enhance the osteoconductivity of CSD granules, leading to increased osteogenesis.

Suzuki et al. reported that apatite (Ap) containing a small amount of anhydrous calcium phosphate (DCPA) that was produced from CSD and subsequently immersed in $(NH_4)_2HPO_4$ solution maintained the macroscopic structure of CSD [24]. This reaction is a compositional transformation based on dissolution-precipitation reaction. This suggests that CSD dissolved in the $(NH_4)_2HPO_4$ solution and released Ca^{2+} and SO_4^{2-} ions. The solution then became supersaturated with respect to

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Ap, leading to the precipitation of Ca^{2+} and PO_4^{3-}. The dissolution-precipitation reaction proceeds from the surface to the interior. Therefore, coating CSD with Ap, or the fabrication of core-shell granules should be possible through regulation of the dissolution-precipitation reaction.

In this study, the feasibility of fabricating Ap-coated gypsum by immersing CSD granules in disodium hydrogen phosphate [Na_2HPO_4] solution was evaluated. Furthermore, the potential of Ap/CSD as a bone augmentation material was assessed through measurement of the dissolution rate and histological studies.

2. Materials and methods

2.1. Preparation of calcium sulfate dihydrate granules

CSD granules were prepared by mixing improved dental stone powder (NEWFUJIROCK WHITE:GC corporation, Japan) with distilled water, at a water to powder mixing ratio of 0.2. The resulting set gypsum was crushed and sieved into 300–400 μm in the methanol.

2.2. Treatment of calcium sulfate dihydrate granules

One gram of CSD granules were immersed in 100 mL of 1.0 mol/L Na_2HPO_4 solution at 20 and 60 °C for 1, 3 and 7 days. The pH of the Na_2HPO_4 solution was monitored during the reaction using a pH meter (LAQUA twin, HORIBA Ltd., Japan). The granules were washed with distilled water and dried at room temperature for at least 24 h.

2.3. Microscopic analysis of calcium sulfate dihydrate granules

Surface morphologies of the granules were examined using scanning electron microscopy (SEM; S-3400 N, Hitachi High Technologies Co., Tokyo, Japan) under an accelerating voltage of 10 kV after being coated with gold palladium. The samples were coated using a magnetron spattering machine (MSP-1 *S*, Vacuum Device Co., Japan). An energy dispersive X-ray spectrometer (EDAX; Genesis XM4, New Jersey, USA) attached to the SEM was used to analyze the depth profile of the samples. CSD plate with 0.35 mm thickness was immersed in the 1.0 mol/L Na_2HPO_4 solution in the same way as the granules. The cross-sectional surface of the plate was observed by SEM with an acceleration voltage of 10 kV. Line analyses of calcium, sulfur and phosphorus were performed using EDAX software.

2.4. Surface component analysis

Surface component analysis was performed by attenuated total reflection with fourier transform infrared spectroscopy (ATR-FTIR; FT/IR-6200, JASCO Corp., Tokyo, Japan) and X-ray photoelectron spectroscopy (XPS; K-alpha, ThermoFisher Scientific, East Grinstead, UK). The XPS machine was calibrated using Au $4f_{7/2}$ with a binding energy (BE) of 83.96 eV and Ag $3d_{5/2}$ with a BE of 368.21 eV.

2.5. X-ray diffraction analysis

For compositional analysis, the granules were ground into a fine powder and evaluated with an X-ray diffractometer (XRD; DB Advance, Bruker AXS GmbH, Harlsruhe, Germany) operated at 40 kV and 40 mA. The diffraction angle was continuously scanned in the 2θ range from 10° to 40° at a scanning rate of 2°/min, or from 20° to 35° at a scanning rate of 0.17°/min.

2.6. Calcium and phosphate content of the granules

Granules (0.05 g) were dissolved in 5 mL of 2% HNO_3 solution and diluted 500-fold with 2% HNO_3 solution. The Ca and PO_4 concentrations of the solution were measured using inductively coupled plasma optical emission spectrometry (ICP-OES; Optima 7300 DV, Perkin Elmer Inc., USA), and the PiBlue phosphate assay kit (Bio Assay Systems ltd., USA), respectively. The wt% of coated Ap (Ca/P = 1.67) on the CSD granules was calculated from the amount of dissolved Ca and PO_4.

2.7. Rate of release of calcium ions from Ap/CSD granules

The rate of Ca^{2+} release from the granules was measured by immersing the Ap/CSD granules in 0.05 mol/L Tris-HCl buffer (pH 7.5) at 25 °C for up to 7 days. The Ca^{2+} concentration of the solution was then evaluated by ICP-OES and the granule weight measured.

2.8. Animal implantation

All animal experiments were conducted under the approval of the ethical committee of animal experimentation at the Kyushu University (approval number: A29-396-0). Male Japanese White Rabbits (3.0–3.5 kg) were used in this study. Eight rabbits were divided into four groups for treatment with: CSD, Ap/CSD prepared at 20 °C for 7 days, Ap/CSD prepared at 60 °C for 7 days, or sintered hydroxyapatite (s-Hap; control). The fur of both legs was shaved and disinfected with iodine. Ketamine hydrochloride (30 mg/kg) and xylazine hydrochloride (5.0 mg/kg) were used for anesthesia. Lidocaine was injected at 2 wt% to stop the bleeding and provide additional intraoperative analgesia. Bone cavities of 6 mm diameter and 3 mm depth were prepared in the cortical bones of both femurs by drilling with a trephine bur. Granules were put into the cavity and the skin flap closed with suturing. Gentamicin sulfate (GENTACIN®, MSD Co., Japan) was injected to prevent infection. Four weeks after implantation the animals were sacrificed using intravenous injection of ketamine (30 mg/kg) and xylazine (5.0 mg/kg), and the distal femur was collected along with the surrounding tissue.

2.9. Microcomputed tomography

The remaining granules and new bone formation in the bone defect were evaluated using microcomputed tomography (μCT; Skyscan 1075 KHS, Kontich, Belgium) using a source voltage of 59 kV, a source current of 169 μA and a 0.5-mm aluminum filter. The samples were scanned using 18 μm voxel resolution scanning mode, and the scanner's built-in software was used for 3D reconstruction. The CT-positive volume in the bone defect area was calculated by the CT analyzer, with a threshold decided from the result of μCT immediately following implantation.

2.10. Histological evaluation

The harvested femurs were fixed with 10 wt% formalin neutral buffer solution (Wako Pure Chemical). Samples were submitted to decalcification and set into paraffin blocks for histological analysis. The samples were sectioned, and slides were stained with hematoxylin and eosin (H-E) and examined under a microscope (BZ-X710, KEYENCE, Osaka, Japan).

3. Results

Fig. 1 shows typical SEM images of CSD granules prior to immersion in 1.0 mol/L Na_2HPO_4 solution (a, b), after immersion at 20 °C for 7 days (c, d), and after immersion at 60 °C for 7 days (e, f). No macroscopic differences were observed between the samples examined before and after immersion in Na_2HPO_4 solution, nor were any differences seen with altered solution temperature. However, at high magnification it was observed that the surface morphologies of the samples were different before and after immersion in Na_2HPO_4 solution (Fig. 1b, d and f). Prior to immersion, CSD granules showed relatively large needle-like crystals. In contrast, the surfaces of CSD granules that had

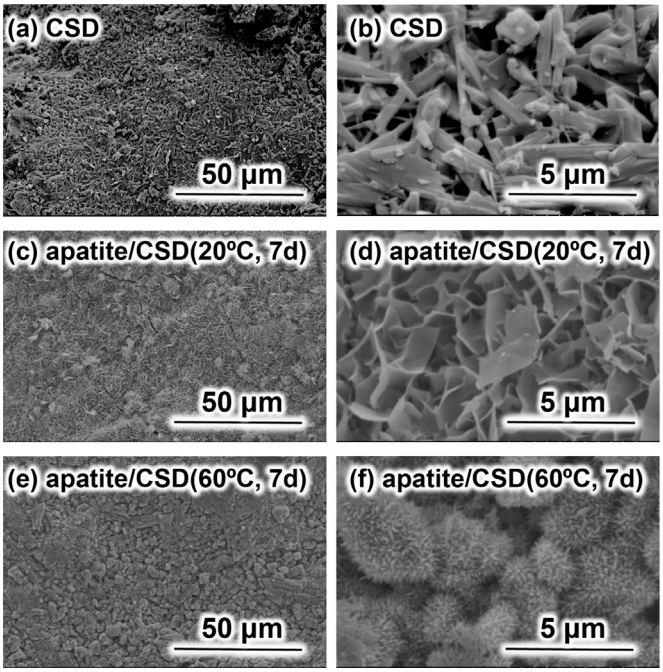

Fig. 1. SEM image of the CSD granules before (a, b) and after immersion in (c, d) 1.0 mol/L Na_2HPO_4 solution at 20 °C for 7 days and (e, f) 1.0 mol/L Na_2HPO_4 solution at 60 °C for 7 days. The image was observed with low (a, c, e) and high (b, d, f) magnification.

been immersed in Na_2HPO_4 were covered with small plate-like crystals. The crystal size was smaller when CSD granules were immersed in Na_2HPO_4 solution at 60 °C compared with Na_2HPO_4 solution at 20 °C.

The XRD patterns of the prepared granules were examined for the presence of a peak that could be attributed to apatite (2θ = 25.9°, 31.8°). This peak was not observed in the XRD spectrum of untreated CSD granules (Fig. 2a and i) but was present in the spectra of CSD granules that had been immersed in 1.0 mol/L Na_2HPO_4 solution at 20 °C or 60 °C for 1 day or 7 days (Fig. 2b–e, j and k, Fig. S1). The XRD patterns of standard CSD (f), calcium sulfate hemihydrate (CSH, g) and hydroxyapatite (HAp, h, l) are also shown for comparison.

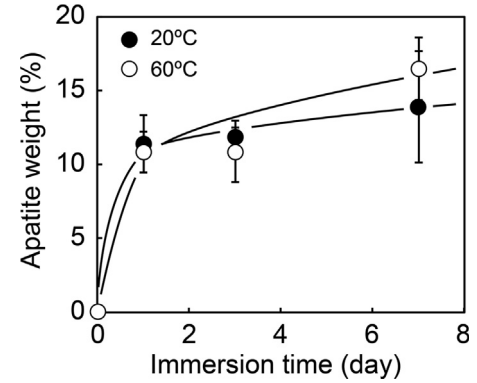

Fig. 3. Ap weight in the Ap/CSD granules fabricated by immersing CSD granules in 1 mol/L Na_2HPO_4 solution at 20 °C and 60 °C for 1, 3, and 7 days. Results are presented as the mean ± standard deviation (n = 3).

As shown in Fig. 3, the formed Ap weight rate of the granules that had been immersed in Na_2HPO_4 solution at 60 °C was slightly higher than that of the granules immersed in the solution at 20 °C. However, the difference was not considered to be significant.

The pH of the solution was monitored during the course of the immersion and decreased slightly with increased immersion time (Fig. 4). However, the final pH was above 8.6 where apatite was the most stable phase.

The SEM images and EDAX analysis profile of the CSD plate cross section revealed that the plate consisted of homogeneous crystals (Fig. 5). The homogenous structure was also confirmed by the EDAX line analysis for calcium and sulfur, which showed the content of both elements to be the same at the surface and inside the CSD plate. In contrast, after immersion in Na_2HPO_4 solution, the CSD plate showed a "sandwich" structure. The crystal structure of the inside the plate was unchanged following immersion, however, the surface became coated with small plate-like crystals. These crystals were comparable to those observed on the CSD granules that had been immersed in Na_2HPO_4 solution (Fig. 1). The appearance of a sandwich structure suggests that Ap/CSD granules will have been successfully fabricated after immersion in Na_2HPO_4 solution. The EDAX line profile of the CSD plate showed the content of calcium and sulfur to be higher inside compared with the

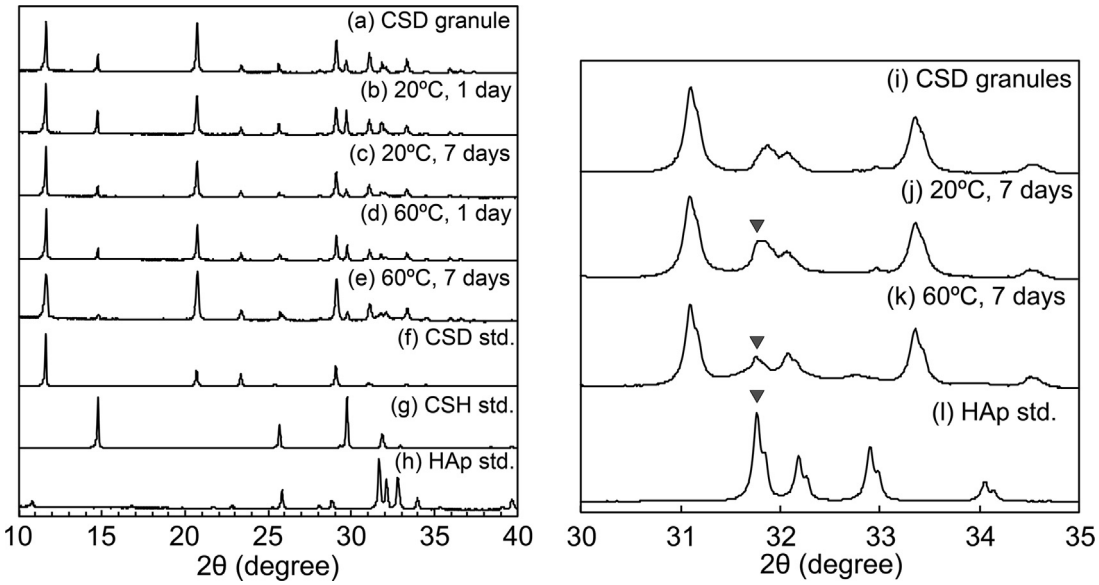

Fig. 2. Typical powder XRD patterns of: (a, i) CSD granules, (b) CSD granules after immersion at 20 °C for 1 day, (c, j) CSD granules after immersion at 20 °C for 7 days, (d) CSD granules after immersion at 60 °C for 1 day, and (e, k) CSD granules after immersion at 60 °C for 7 days. XRD patterns of standard (f) CSD, (g) CSH, and (h, l) HAp are shown for reference.

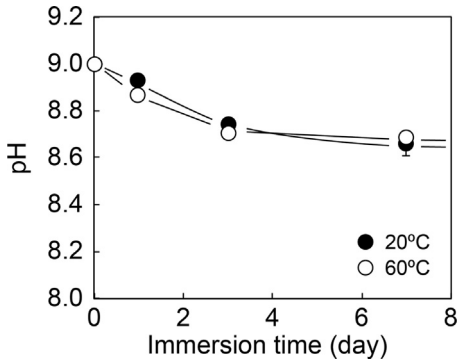

Fig. 4. pH change of the 1.0 mol/L Na_2HPO_4 solution after immersion of CSD granules for 1, 3, and 7 days. Results are presented as the mean ± standard deviation (n = 3).

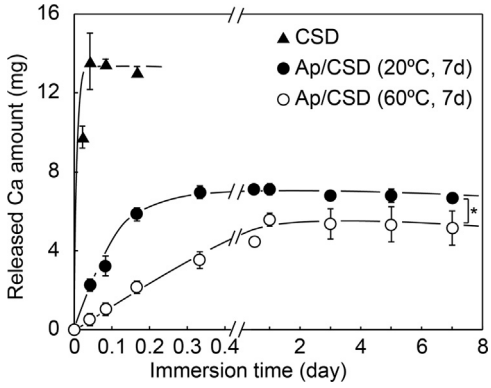

Fig. 6. Ca^{2+} release rate in the Tris-HCl buffer solution after immersion (pH 7.5) of CSD and ApCSD granules at 25 °C for 1, 2, 4, 8, and 12 h and 1, 3, 5, and 7 days. Results are presented as the mean ± standard deviation (n = 3, $^*p < 0.05$).

surface of the plate. Phosphorus was only observed at the surface of the plate, further confirming that Ap crystals were coated onto the CSD.

Furthermore, ATR-FTIR and XPS analysis suggested that the apatite coating on the surface of CSD did not contain sulfur ions. Fig. S2 shows the ATR-FTIR spectra of CSD (a), Ap/CSD fabricated by immersing CSD granules in 1 mol/L Na_2HPO_4 solution for 7 days at (b) 20 °C and (c) 60 °C, and (d) hydroxyapatite. The peak of SO_4^{2-} (1150 cm^{-1}) [25] disappeared following immersion in Na_2HPO_4 solution, suggesting that the surface layer of Ap/CSD did not contain sulfur ions. Highly sensitive XPS analysis also confirmed the absence of sulfur on the surface of Ap/CSD (Fig. S3). Although the sulfate ion can be substituted in Ap layer [26] and Fig. 5 shows that the sulfur seemed to be detected in Ap layer of Ap/CSD, ATR-FTIR and XPS results indicated that the surface of Ap/CSD was Ap without sulfate.

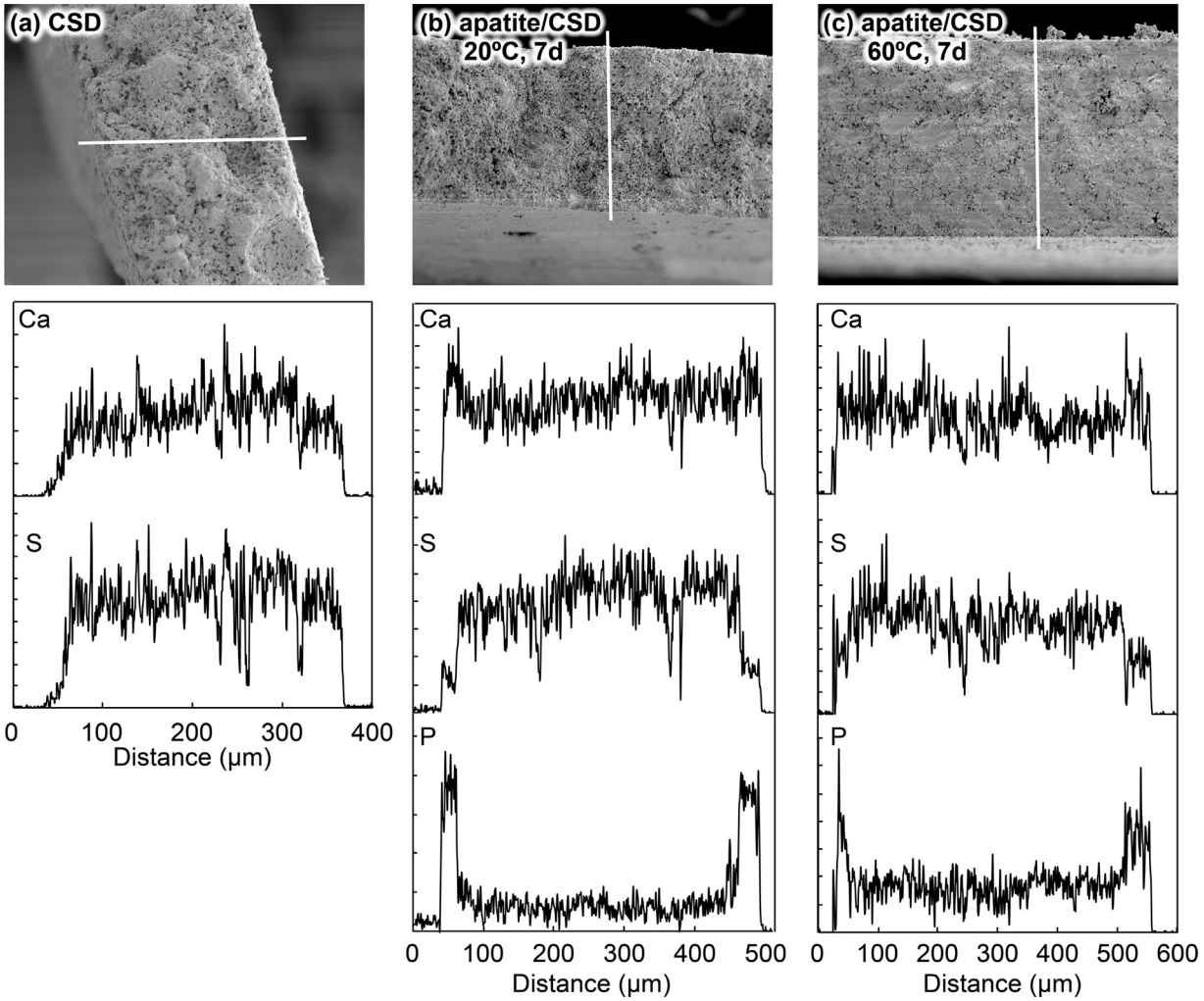

Fig. 5. SEM/EDAX line analysis of CSD plate (a) before and after immersion in (b) 1.0 mol/L Na_2HPO_4 at 20 °C for 7 days and (c) 1.0 mol/L Na_2HPO_4 at 60 °C for 7 days.

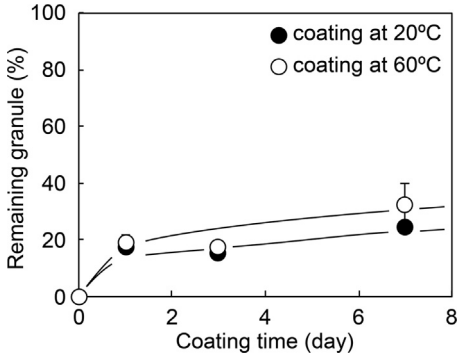

Fig. 7. Remaining amount of Ap/CSD granules after immersing in Tris-HCl buffer solution (pH 7.5) at 25 °C for 7 days. Results are presented as the mean ± standard deviation (n = 3).

Rapid release of Ca^{2+} was observed when CSD granules were immersed in Tris-HCl buffer solution at 25 °C up to 7 days (Fig. 6). In contrast, the release of Ca^{2+} was reduced when the granules were coated with Ap. Ap/CSD that were fabricated by immersion in Na_2HPO_4 solution at 60 °C showed decreased Ca^{2+} release compared with Ap/CSD granules fabricated by immersion at 20 °C.

It was found that untreated CSD granules dissolved completely within 1 h when immersed in a Tris-HCl buffer solution at 25 °C. The rate of dissolution of Ap/CSD granules fabricated at both 20 °C and 60 °C was significantly lower than that of untreated CSD granules, and incomplete dissolution observed even after 7 days (Fig. 7).

Typical μCT images of the granules immediately following implantation and 4 weeks after are shown in Fig. 8. When the bone defect was filled with s-Hap granules, density of the CT-positive value was high. The lowest CT-positive value was observed when CSD granules, or Ap/CSD granules fabricated by immersion in Na_2HPO_4 solution at 20 °C were administered. When the bone defect was filled with Ap/CSD granules fabricated by immersion in Na_2HPO_4 solution at 60 °C, the CT-positive value was found to be between those of untreated CSD and s-HAp.

Histological imaging of the granules 4 weeks after implantation revealed that all samples induced excellent tissue responses (Fig. 9). For most samples, no granules remained at the bone defect, except in the case of s-HAp where granules were observed.

The percentage of CT-positive volume in the bone defect area was calculated from the results of μCT analysis (Fig. 10). The highest CT-positive volume was observed in the case of Ap/CSD granules fabricated by immersion in Na_2HPO_4 solution at 60 °C.

4. Discussion

Calcium sulfate has a long history of clinical use as a bone substitute because of its bioresorbable and Ca^{2+} releasing properties. In recent studies, the potential for calcium sulfate as a vehicle for local delivery of drugs such as antibiotics and growth factors has been investigated [27–31]. Although calcium sulfate has unique properties suitable for a bone augmentation material, the problems of rapid resorption remain. Therefore, regulation of Ca^{2+} release from CSD granules is necessary.

The results obtained in this study demonstrate that Ap/CSD granules can be fabricated by simple immersion of CSD granules in a solution of Na_2HPO_4. Previously, Suzuki et al. reported that set gypsum or a CSD block transformed into an Ap-DCPA mixed-composite block when immersed in $(NH_4)_2HPO_4$ solution at 100 °C for 48 h [24]. The resulting block maintained the macroscopic structure of the original CSD block. Basically, dissolution-precipitation reaction will occur when CSD granules is immersed in a phosphate salt solution. (Eq. (1–3)).

$$CaSO_4{\cdot}2H_2O \rightarrow 2Ca^{2+} + SO_4^{2-} + 2H_2O \quad (1)$$

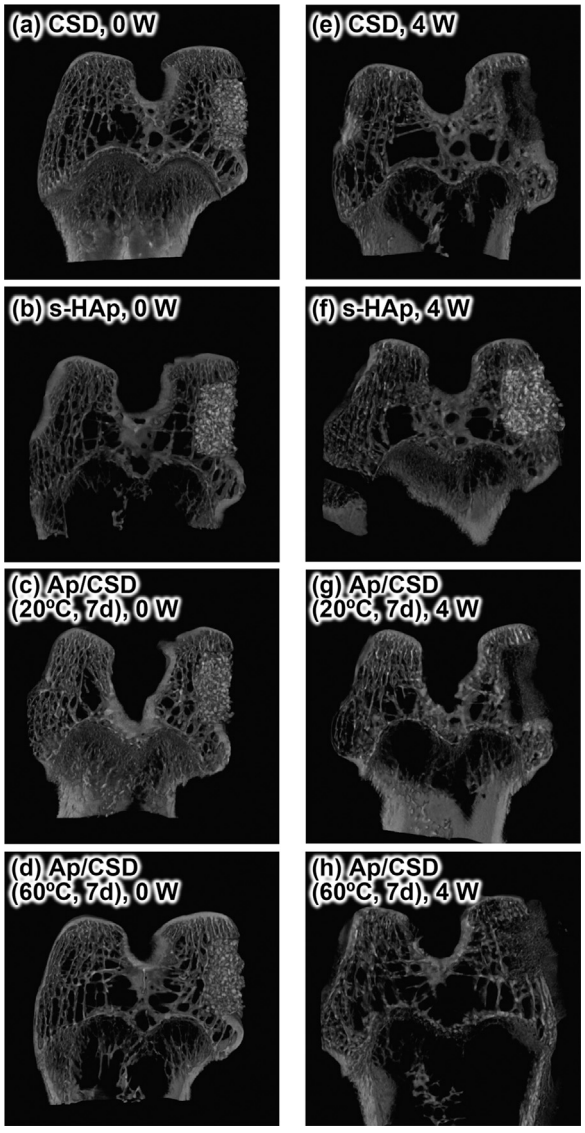

Fig. 8. μCT images of rabbit femur 4 weeks after implantation of (a) CSD granules, (b) s-HAp granules, (c) Ap/CSD granules prepared in 1.0 mol/L Na_2HPO_4 solution at 20 °C for 7 days, and (d) Ap/CSD granules prepared in 1.0 mol/L Na_2HPO_4 solution at 60 °C for 7 days.

$$Na_2HPO_4 \rightarrow 2Na^+ + H^+ + PO_4^{3-} \quad (2)$$

$$10CaSO_4{\cdot}2H_2O + 6Na_2HPO_4 \rightarrow 10Ca^{2+} + 10SO_4^{2-} + 20H_2O + 12Na^+ + 6H^+ + 6PO_4^{3-} \rightarrow Ca_{10}(PO_4)_6(OH)_2 + 12Na^+ + 8H^+ + 10SO_4^{2-} + 20H_2O \quad (3)$$

CSD partially dissolves and releases Ca^{2+} and SO_4^{2-} (Eq. (1)). The Na_2HPO_4 solution, which contains PO_4^{3-} (Eq. (2)), will become supersaturated with respect to Ap. Thus, Ap will precipitate on the surface of CSD (Eq. (3)). Precipitation of Ap crystals results in under-saturation of CSD in the Na_2HPO_4 solution, leading to the dissolution of CSD. As a result of the dissolution-precipitation reaction, CSD transforms to Ap, maintaining its original macroscopic structure. It should be noted that Ap is a more alkaline salt than CSD, therefore the measured pH of the solution will decrease with the progression of Ap crystal formation.

A key difference between Suzuki's study and this study is the ratio of CSD and phosphate salt solution. In this study, 1.0 g of CSD granules were immersed in 100 mL of 1.0 mol/L Na_2HPO_4 solution. Therefore, the molar ratio of Na_2HPO_4 to CSD was 17.1. In Suzuki's study, 1.3 g

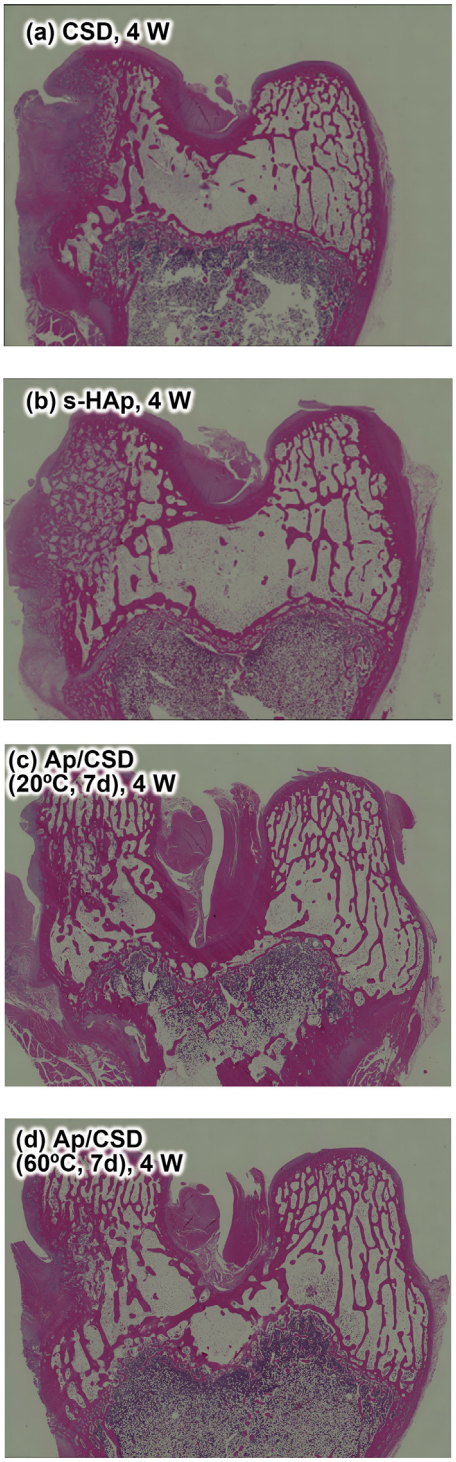

Fig. 9. Histological analysis of rabbit femur 4 weeks after implantation of (a) CSD granules, (b) s-HAp granules, (c) Ap/CSD granules prepared in 1.0 mol/L Na_2HPO_4 at 20 °C for 7 days, and (d) Ap/CSD granules prepared in 1.0 mol/L Na_2HPO_4 at 60 °C for 7 days.

CSD blocks were immersed in 15 mL of 1.0 mol/L $(NH_4)_2HPO_4$ solution. Therefore, the molar ratio of $(NH_4)_2HPO_4$ to CSD was 2.0. Suzuki's study aimed to completely transform CSD to Ap. To this end, Suzuki et al. reduced the pH to 4.8 during fabrication, and so the resulting material was not pure Ap but an Ap-DCPA composite block. In this study, the aim was to produce pure Ap at the surface of CSD granules only, therefore a larger volume of Na_2HPO_4 solution was used in order to minimize the pH drop. The result was the successful coating of Ap

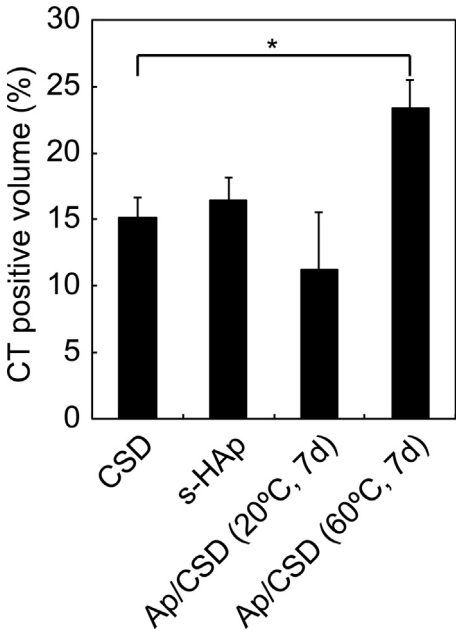

Fig. 10. Percentage of CT-positive volume in bone defect area at 4 weeks post-implantation, based on the μCT results. Results are presented as the mean ± standard deviation (n = 2, $^*p < 0.05$).

onto the surface of the granules.

Although the dissolution of Ap/CSD granules was suppressed in buffer solution at pH 7.5 (Fig. 6), some dissolution still occurred. If the CSD was completely coated with Ap crystals, no dissolution would occur and the granules would be equivalent to Ap granules. It therefore appears that the structure of the Ap-coated CSD granules (Fig. 1) allows diffusion of the solution through the Ap coating and subsequent diffusion of dissolved CSD into the solution.

Both μCT and H-E staining results suggested that Ap/CSD granules had higher onsteoconductivities than CSD alone. Granules that were fabricated at 60 °C significantly improved osteoconductivity compared with untreated CSD granules. Furthermore, while s-HAp granules still remained in the bone defect area at 4 weeks after implantation, both samples of Ap/CSD granules dissolved completely. This suggests that the inclusion of CSD in the granules led to improved replacement of granules with bone.

5. Conclusion

The fabrication of Ap/CSD granules by simple immersion of CSD granules in Na_2HPO_4 solution was achieved in this study. Ap coating on the CSD granules did not suppress the dissolution of CSD granules completely but partially. When rabbit's femur bone defect was reconstructed with Ap/CSD granules, most of the granules replaced to new bone 4 weeks after surgery whereas s-HAp granules remained at the bone defect, and CSD granules just disappeared without new bone formation. It is therefore concluded that Ap/CSD has good potential to be an improved CSD-based bone augmentation material.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ceramint.2018.08.021.

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