Preparation of Sr-containing carbonate apatite as a bone substitute and its properties

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INTRODUCTION

Bone augmentation is a minimally invasive treatment option generally recommended to reverse bone loss or destruction caused by periodontal diseases or to enable dental implant placements. Bone reconstruction techniques include the use of autografts harvested from the patient’s own body or bone substitute materials. Autografts are long considered the gold standard among bone graft materials as they significantly reduce the risks of rejection and disease transmission. However, they are beleaguered with numerous problems such as harvest donor site morbidity (a consequence of invading healthy sites to harvest bone grafts) and limitations in the quantity of bone available1,2. Bone substitute materials were thus synthesized to minimize these complications. For example, hydroxyapatite (HAp) as an artificial biomaterial exhibits excellent tissue compatibility and osteoconductivity3. However, HAp bone substitute remains too stable at the implant site and resorbs very slowly. This heightens the risk of implant site infection due to the high affinity of oral bacteria and microorganisms for HAp surfaces.

Carbonate apatite (CAp) was developed as a resorbable bone substitute material—one that gradually resorbs and finally be replaced by newly formed bone4,5. HAp bioceramics are usually fabricated by sintering HAp powder compacts3,7. This procedure, however, is not appropriate for CAp which easily decomposes at temperatures above 600°C due to loss of carbonate ions. If low-temperature sintering were carried out to avoid decomposition, the synthesized product would have a low carbonate content and compromised mechanical strength6. Other disadvantages include increased crystallinity due to CO2 liberation, and high crystallinity impedes in vivo resorbability—which is supposed to be a highly acclaimed advantage of CAp.

We have proposed numerous novel methods to fabricate CAp blocks without the sintering process7–10. Using calcium carbonate as a precursor and via phosphatization in an appropriate phosphate solution, the precursor was transformed into CAp block with its original shape retained. Thus-synthesized CAp was found to have good resorbability and was eventually replaced by newly formed bone10. In vitro evaluation, however, revealed similar or slightly lower osteoconductivity when compared to HAp11. Therefore, the search is still on for a bone substitute material that promotes bone formation.

Strontium (Sr) ranelate is a newly developed drug for osteoporosis12; Sr2+ ions were found to promote bone formation and suppress bone resorption13. In vivo experiments using mice and rats showed that oral intake of Sr ranelate resulted in increased bone formation and decreased bone resorption, and hence improved bone mass12. In vitro experiments with osteoblasts showed that 2–5 ppm Sr in the culture medium promoted bone nodule formation and calcification, whereas 20–100 ppm Sr inhibited calcification14. Therefore, Sr has a dose-dependent effect on bone formation and calcification.

Sr2+ ion also reportedly substituted Ca2+ ion in HAp15. Several in vitro and in vivo evaluations16–19 have shown that Sr-containing HAp (SrHAp) could be a promising candidate for use as a bone substitute material. For Sr-containing carbonate apatite (SrCAp), studies on its suitability and potential as a bone substitute material are few and far between. CAp is a resorbable bone substitute. When endowed with the bone formation-promoting effect of Sr-incorporated CAp (SrCAp), the latter could be expected to provide rapid repair of bony defects.

In the present study, a non-sintering method was
used to prepare SrCAp blocks. Since an optimum Sr dose exists\(^{14,17}\), varying contents of Sr were used for SrCAp preparation. The effects of Sr content on CAp microstructure, osteoblast-like cell attachment and proliferation and alkaline phosphatase (ALP) activity on SrCAp surface were then investigated.

MATERIALS AND METHODS

SrCAp preparation

In our previous study, block-type CAp was prepared from a gypsum-calciite mixture after immersion in a sodium phosphate solution\(^{10}\). This preparation method was likewise applied to SrCAp in the present study, except that Sr-containing calcium carbonate was used instead of calcite.

To prepare Sr-containing calcium carbonate, sodium carbonate solution was added to a mixed solution of calcium and strontium chlorides of varied \(\text{Sr}/(\text{Sr+Ca})\) molar ratios (0 to 20 mol%) to precipitate carbonate. Precipitated carbonate was washed with deionized water several times and then dried. An amount of 40 wt% of calcium sulfate hemihydrate (\(\text{CaSO}_4 \cdot 1/2\text{H}_2\text{O}\)) was added to the prepared carbonate and mixed with deionized water at a water-to-powder ratio of 0.65. The mixed slurry was poured into a stainless steel mold (6 mm inner diameter, 3 mm thickness) and allowed to set at room temperature. For specimens to be used in cell study, a Teflon mold of 10 mm inner diameter and 1 mm thickness was used instead.

All set specimens were immersed in 1 mol/L trisodium phosphate (\(\text{Na}_3\text{PO}_4\)) solution at 100°C for 7 days to transform into CAp. Six kinds of SrCAp specimens were prepared with the final molar ratios of \(\text{Sr}/(\text{Sr+Ca})\) ranging between 0 and 13.3 mol%. They are denoted as Sr0 (0 mol%), Sr1 (0.7 mol%), Sr2 (1.4 mol%), Sr3 (3.4 mol%), Sr4 (6.7 mol%), and Sr5 (13.3 mol%) in ascending order of Sr content.

X-ray diffraction (XRD) analysis and Fourier transform infrared (FT-IR) spectroscopy

XRD patterns and FT-IR spectra were obtained for each specimen before and after the phosphate treatment. XRD analysis was carried out using an X-ray diffractometer (RINT 2500V or MiniFlex II, Rigaku, Tokyo, Japan) at 40 kV and 60 mA or at 30 kV and 15 mA at a scan rate of 2°/min or 1°/min. Lattice constant for the SrCAp formed was calculated from several intense diffraction peaks using 2\(\theta\) between 20° and 50° by a least squares method. FT-IR spectra were obtained by KBr method using an FT-IR spectrometer (Alpha, Bruker, Tokyo, Japan). Carbonate content was determined using a method described by Featherstone based on the FT-IR spectra\(^{20}\).

Scanning electron microscope (SEM) surface observation

After platinum sputter coating at an accelerating voltage of 5 kV, the surfaces of specimens of varied Sr contents were observed using a SEM (JSM-6330F, Jeol, Tokyo, Japan).

Diametral tensile strength (DTS) measurement

Disk-shaped specimens of 6 mm diameter and 3 mm thickness were used for DTS measurement. Using a universal testing machine (AGS-J 500N, Shimadzu, Kyoto, Japan), DTS of each specimen was measured before and after phosphate treatment. Specimens were loaded in a diametral direction to fracture at a crosshead speed of 1 mm/min. DTS (MPa) was calculated using the following equation:

\[
\text{DTS} = \frac{2P}{\piDt}
\]

where \(P\) is maximum load at fracture (N), \(D\) is specimen diameter (mm), and \(t\) is specimen thickness (mm).

Osteoblast-like cell (MC3T3-E1) attachment and proliferation

1. Cell attachment

MC3T3-E1 cells were cultured in α-MEM (MEM Alpha, Gibco, NY, USA), which contained 10% fetal bovine serum and 1% antibiotics (Pen-Strep, Gibco), in a CO\(_2\) incubator at 37°C. After 5 days in primary culture, cells were detached using trypsin-EDTA (0.25% Trypsin-EDTA, Gibco). Detached cells were seeded on disk-shaped specimens (10 mm diameter, 1 mm thickness) in 48-well culture plates at a seeding density of 1×10\(^4\) cells/well.

After 3 h of incubation, specimen surfaces and plastic plates were rinsed three times with phosphate-buffered saline (PBS) to remove nonadherent cells. Adherent cells were detached from each specimen with 0.25% trypsin-EDTA solution and counted using a hemocytometer (Burker-Turk counting chamber, Erma Inc., Tokyo, Japan). All experiments were performed in triplicate.

2. Cell morphology

After cells were cultured on disk-shaped specimens for 3 h, the latter were gently washed with PBS. Cells were dehydrated through a graded series of ethanol, followed by critical-point drying. Morphologies of the attached cells on specimen surfaces were observed by SEM.

3. Cell proliferation

At a seeding density of 5,000 cells/well, MC3T3-E1 cells were cultured on specimen surfaces. Culture medium was refreshed after 24 h and every 2 days. For a period of up to 8 days, number of cells grown on the specimen surface was determined using MTT assay (Wako Pure Chemicals, Kyoto, Japan) according to manufacturer’s instructions.

ALP assay

Cell seeding and specimen preparation were carried out in the same manner as for cell proliferation evaluation. After culturing for 3, 6, and 9 days, specimen surfaces were rinsed three times with PBS. Cells layers were scraped into 250 μL of distilled water and homogenized.

 Supernatants were assayed for ALP activity using an ALP B-test Wako kit (Wako Pure Chemicals) based on the Bessey-Lowry method. Homogenates (50 μL per homogenate) were incubated with the assay buffer at 37°C for 15 min, and absorbance was measured at 405

\[
\text{DTS} = \frac{2P}{\piDt}
\]
nm using a spectrophotometer. Total protein production of each specimen was measured using a protein assay kit (BCA™ Protein Assay Kit, Pierce Biotechnology Inc., IL, USA). Quantified ALP activity was expressed as ALP production per total protein unit (IU/µg/min). All experiments were performed in triplicate.

Statistical analysis
Results were expressed as mean±standard deviation (SD). Data were statistically analyzed using ANOVA at a significance level of 5% (p<0.05). Where applicable, post hoc analysis using Fisher’s PLSD method as a multiple comparison test was performed to detect significant differences between groups.

RESULTS

XRD analysis
Figures 1 and 2 show the XRD patterns of the set gypsum-and-carbonate mixture before and after phosphate treatment at 100°C for 7 days. Before treatment, XRD peaks assigned to gypsum and carbonate are observed. Gypsum was formed by hydration of hemihydrate, and no residual hemihydrate was found. CaCO₃ mostly existed as calcite, the most stable phase of CaCO₃, when Sr content was low. With increase in Sr content, vaterite—a metastable phase of CaCO₃—was also formed. At the highest Sr content (Sr5), aragonite-type CaCO₃ was exclusively formed instead of calcite.

After phosphate treatment, several new broad peaks corresponding to the apatite phase appeared. With Sr0 and Sr1, which contained 0 and 0.7 mol% Sr respectively, calcite peak still remained at 2θ of 29°. Amount of residual calcite in Sr0 and Sr1 was estimated to be about 15 wt% by quantitative XRD analysis using a standard sample containing known amounts of calcite and apatite. However, when Sr content reached and exceeded 1.4 mol% (Sr2–Sr5), CaCO₃ was completely transformed into the apatite phase.

FT-IR spectroscopy
Figure 3 shows the FT-IR spectra of the set gypsum-and-carbonate mixture after 7 days of phosphate treatment at 100°C. In all the obtained spectra, PO₄ absorption bands were observed at 1,000–1,100 cm⁻¹ and 550–600 cm⁻¹, which were assigned to PO₄³⁻ ion in the apatite lattice. Absorption band at 1,400–1,500 cm⁻¹ was assigned to CO₃²⁻ ion in the apatite lattice. Appearance of CO₃²⁻ peak indicated that the apatite phase was CaP. Two types of CaP exist with respect to the substitution site of CO₃²⁻ ion in the apatite lattice. One is A-type CaP, in which CO₃²⁻ groups substituted OH sites. For B-type CaP, CO₃²⁻ groups substituted the PO₄ sites. In the present study, the obtained spectra were typically those of B-type CaP, in which the absorption band of CO₃ had two maxima at 1,455 cm⁻¹ and 1,410 cm⁻¹.

CO₃ content was calculated from the absorbance ratio of CO₃ band at 1,410 cm⁻¹ to PO₄ band at 575 cm⁻¹ based on a method described by Featherstone et al. Figure 4 shows the change in CO₃ content from Sr0 to Sr5. Specimens with low Sr content (Sr0 and Sr1) had a
higher CO₃ content than those with high Sr content (Sr2–Sr5). The high CO₃ content in Sr0 and Sr1 was due to the presence of residual calcite as shown in XRD patterns in Fig. 2. Calcite content was estimated to be about 15 wt% in both Sr0 and Sr1, corresponding to about 9 wt% of CO₃ content. Therefore, the actual CO₃ content in the apatite phase of Sr0 and Sr1 was about 9 wt%. For Sr2 to Sr5, their CO₃ contents ranged between 11.4 and 13.6 wt%, with CO₃ content increasing slightly with increasing Sr content from Sr3 to Sr5.

DTS
Figure 5 shows the DTS results before and after phosphate treatment. Before treatment, DTS of Sr0 was about 1.9 MPa. Increase in Sr content resulted in decrease in DTS values, especially for specimens with a high Sr content (Sr3–Sr5).

After the treatment, DTS values decreased in all the specimens (p<0.05), ranging between 0.5 and 1 MPa. DTS values of Sr4 and Sr5 (with high Sr contents) were significantly lower than that of Sr3. Nonetheless, post-treatment strength was thought to be sufficient for its use as a bone substitute for dental implant placements.

SEM surface observation
Figure 6 shows the SEM images of the specimen surfaces of Sr0, Sr2, Sr3, and Sr5 before (upper) and after (lower) phosphate treatment. Before treatment, typical plate-like or needle-like gypsum crystals were seen on the surfaces of specimens.

After treatment, some gypsum crystals still retained their original morphologies, but the whole surface was generally covered with many fine granular crystals. XRD analysis revealed that the crystalline phase had transformed into the apatite phase. Therefore, these fine crystals were SrCAp containing varied amounts of Sr. With increase in Sr content, round-shaped crystalline particle with increased size were also observed inside the specimen, although these SEM images were not shown.

Cell attachment
Figure 7 shows the cell attachment results after 3 h of
incubation. Initial cell attachment was significantly better in specimens Sr2 to Sr5 than in specimens Sr0 and Sr1 \( (p<0.05) \). This result suggested that Sr benefited cell attachment on SrCAp surfaces.

**Cell morphology**
Figure 8 shows the morphologies of cells attached to the specimen surfaces of Sr0, Sr2, Sr3, and Sr5. Generally, the attached cells had a polygonal shape with filopoidal extensions — and the latter seemed to be spreading considerably. No differences in cell morphology were observed among the different Sr contents.

**Cell proliferation**
Figure 9 shows the cell proliferation results at days 2 and 8. No significant differences in cell proliferation were observed among the different Sr contents. However, Sr5 with the highest Sr content exhibited a significantly lower proliferation rate at day 8 than Sr0 which contained no Sr.

**ALP activity**
Figure 10 shows the ALP activity value per total protein unit of Sr0 to Sr5 after 3, 6, and 9 days of culturing. At day 9, Sr-containing specimens exhibited significantly higher ALP activity levels than the control specimen without Sr \( (p<0.05) \). ALP activity tended to increase with increase in Sr content and peaked at 3.4 mol% Sr (Sr3). Increase in Sr content above 3.4 mol% (Sr4 and Sr5) resulted in decreased ALP activity. This result well corresponded with the cell attachment results (Fig. 7). Similarly, cell proliferation rate peaked in Sr3 after 8 days of culturing and that it decreased when Sr content exceeded 3.4 mol% (Fig. 9).

**DISCUSSION**

**Effect of Sr on microstructure**
To synthesize a mixed carbonate containing both Ca and Sr, co-precipitation method was used to ensure a homogeneous distribution of Sr\(^{2+}\) ion in the formed
Fig. 8 Morphologies of MC3T3-E1 cells attached to the SrCAp surfaces: (a) Sr0; (b) Sr2; (c) Sr3; and (d) Sr5.

Fig. 9 MC3T3-E1 cell proliferation results at day 2 and day 8, as determined using MTT assay. *: *p*<0.05.

Fig. 10 ALP assay results for MC3T3-E1 cells cultured on the surfaces of Sr0 to Sr5. *: *p*<0.05.
carbonate in atomic scale. In the present study, vaterite was also formed in some of the mixed carbonate specimens. Although calcite is the most stable phase among the three polymorphic modifications of CaCO$_3$ at room temperature and normal pressure, aragonite and vaterite (the other two polymorphic modifications) are often formed as by-products through the precipitation process of solutions containing Ca$^{2+}$ and CO$_3^{2-}$ ions. Phase formation is influenced by several preparation conditions such as temperature, concentration of reactant solutions, and mixing rate$^{23}$. However, Sr$^{2+}$ ion notably stabilizes the aragonite phase, one of the metastable phases in CaCO$_3$$^{24}$. In the present study, vaterite or aragonite was formed together with calcite in Sr-containing specimens. At the highest Sr content, however, only aragonite was formed as shown in XRD patterns before the phosphate treatment (Fig. 1).

Synthesized CaCO$_3$ was added to calcium sulfate hemihydrate and mixed with water at a water-to-powder ratio of 0.65. Hardened mass was immersed in 1 mol/L Na$_3$PO$_4$ solution at 100°C for 7 days to transform into SrCAp. During phosphate treatment, the hardened mass maintained its original shape and was eventually fully transformed into the apatite phase. In Sr0 and Sr1, which contained 0 and 0.7 mol% Sr respectively, residual unreacted calcite amounted to about 15 wt%. At higher Sr contents, the nucleation-catalyzing effect of Sr caused the specimens to be completely converted into the apatite phase. It has been reported that strontium catalyzed nucleation and accelerated the kinetics of SrHAp formation from solutions supersaturated with respect to strontium$^{25}$.

Figure 11 shows the changes in a- and c-axes of the hexagonal SrCAp lattice with different Sr contents. Lattice parameters of SrHAp$^{15}$ were also shown in the figure for comparison. The a- and c-axes of stoichiometric HAp were reported to be 0.9421 nm and 0.6881 nm respectively$^{26}$. Carbonate content of B-type CaP specimens obtained in this study was about 9–12 wt%. Carbonate inclusion in the HAp lattice resulted in decreased a-axis parameter and increased c-axis parameter$^{27}$. At lower Sr contents (up to 3.4 mol%), the a-axis decreased with increase in Sr content. This decrease in a-axis parameter was probably caused by a difference in carbonate contents among the specimens. At higher Sr contents (above 3.4 mol%), the a-axis parameter increased with increase in Sr content because of Sr substitutions of Ca in CaP lattice. As shown in Fig. 11 for specimens with higher Sr contents, Sr substitution of Ca in HAp lattice increased both the a- and c-axes. This was because Sr$^{2+}$ ion (0.113 nm) has a larger ionic radius than Ca$^{2+}$ ion (0.099 nm). In the present study, simultaneous substitutions by Sr and CO$_3$ made the changes in lattice parameters slightly complicated. At higher Sr contents of 3.4 mol% and above (Sr3 to Sr5), the carbonate content tended to increase with increase in Sr content. It was reported that increase in Sr content favored CO$_3$ substitution because of lattice strain caused by Sr incorporation$^{15}$. The loosened lattice network could thus accommodate more carbonate ions$^{25}$.

**Effect of Sr on DTS**

Before phosphate treatment, DTS values of specimens ranged between 0.7 and 1.9 MPa. After phosphate treatment, DTS values were about 0.5–1 MPa. The DTS values obtained in this study were slightly lower than those reported by Zaman et al.$^{10}$. It should be pointed out that Zaman et al. prepared CaP from a set gypsum- and calcite mixture at a water-to-powder ratio of 0.510, which was slightly lower than that used in the present study. We used a water-to-powder ratio of 0.65 to ensure satisfactory workability when preparing the specimens.
If we had used a lower water-to-powder ratio, higher strengths might have been attained. However, considerations on particle size and distribution of CaCO₃ should be taken into account and thus further study is needed.

Effect of Sr on cell activities
Sr favored cell attachment to specimen surfaces although cell numbers on all specimen surfaces were lower than that of plastic dishes used as a control (Fig. 7). Interestingly, Sr did not show a clear effect on cell proliferation (Fig. 9) although it did seem to inhibit proliferation on Sr5 which had the highest Sr content. It has been reported that osteoblasts proliferated very slowly on calcium phosphate apatite surfaces because of poor adhesion signaling in osteoblast-like cells on these surfaces. This explained why lower cell numbers were observed on Sr0 to Sr5 than on plastic control in this study.

Sila-Asna et al. reported that Sr ranelate in the culture media did not affect the proliferation of osteoblast derived from human mesenchymal stem cell but promote ALP activity in the concentration of Sr between 0.2107 and 210.07 µg/mL. Capuccini et al. also reported that at Sr contents ranging between 0 and 7 mol%, there were no significant differences in the proliferation of human osteoblast-like cells, MG63, on SrHAp specimens. However, in SrHAp specimens containing 3 and 7 mol% of Sr, significantly higher ALP activity was observed.

With Sr contents of 0, 8.7, 38.0 and 100 mol%, Zhang et al. investigated effects of Sr on proliferation and ALP activity of MG63 cells on SrHAp. Proliferation and ALP activity were significantly higher in SrHAp containing 8.7 mol% of Sr. These results agreed with those obtained in this study. In the present study, ALP activity peaked at Sr content of 3.4 mol% but declined at higher Sr content levels. Differences in Sr concentration in the vicinity of the cell probably accounted for this result.

CONCLUSIONS

Disk-shaped SrCapped specimens of varying contents of 0–13.3 mol% were successfully prepared using phosphate treatment of a set gypsum-and-carbonate mixture at 100°C for 7 days. XRD analysis showed that Sr²⁺ ion substituted Ca²⁺ ion in the apatite lattice. Carbonate content of SrCapped was about 12 wt%, although it was slightly lower at lower Sr contents. Surface observation by SEM revealed that fine granular apatite crystals were formed, although some gypsum crystals still retained their original plate-like or needle-like morphologies. Sr inclusion improved the attachment of osteoblast-like cells on carbonate apatite surfaces. Cell proliferation was not significantly affected by Sr at low content levels, but seemed to be inhibited at the highest Sr content. ALP activity reached a peak in SrCapped containing 3.4 mol% of Sr. SrCapped containing 3.4 mol% of Sr appeared to be a promising bone substitute material with good resorbability and osteoconductivity.

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