Strontium substituted calcium phosphate biphasic ceramics obtained by a powder precipitation method

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Strontium (Sr) substituted calcium phosphate ceramics were fabricated using a powder precipitation method. The Sr ions were added up to 8 mol % to replace the Ca ions during the powder preparation. Composition analysis showed that the added Sr was not fully incorporated within the as-precipitated apatite structure, presumably being washed out during the powder preparation. After calcination, the Sr containing powders were crystallized into apatite and tricalcium phosphate (TCP), that is, biphasic calcium phosphates were formed. The amount of TCP increased with increasing the Sr addition. The lattice parameters of the calcined powders increased gradually with Sr substitution in both the *a*- and *c*-axis. However, the obtained values deviated slightly from the calculated ones at higher Sr additions ($>$ 4%) due to the partial substitution of Sr ions. The microstructure of the sintered bodies changed with the Sr addition due to the formation of TCP. The Vickers hardness increased slightly from 5.2 to 5.5 MPa with increasing Sr addition, which was driven by the HA + TCP biphasic formation. The osteoblast-like cells cultured on the Sr-substituted biphasic sample spread and grew actively. The proliferation rate of the cells was higher in the samples containing more Sr. The alkaline phosphate activity of the cells was expressed to a higher degree with increasing Sr addition. These observations confirmed the enhanced cell viability and differentiation of the Sr-substituted biphasic calcium phosphate ceramics.

1. Introduction

Calcium phosphate ceramics, particularly hydroxyapatite [HA, $Ca_{10}(PO_4)_6(OH)_2$] and tricalcium phosphate [TCP, $Ca_3(PO_4)_2$], have attracted much attention for usage in the bone substitutes and dental implants due to their excellent biocompatibility with human hard tissues [1, 2]. Among the ionic groups consisting of the HA or TCP structure, Ca^{2+} can be replaced by various ions, such as Na^+, K^+, Mg^{2+}, and Sr^{2+} within the body fluid or during fabrication due to their similarity in chemical affinity and/or ionic radius [3, 4]. In reality, these ions affect the formation and resorption of bone *in vivo* and *in vitro* [5]. In particular, strontium ions (Sr^{2+}) have been examined for several decades in the field of osteoporosis treatment due to its inhibition of bone resorption and stimulation of bone formation [6, 7]. However, most of the work has demonstrated the effect of Sr-salt or Sr^{2+} ion dose on the rate of bone formation from a clinical or medical point of view [8–11]. Moreover, the reports on the chemical and crystallographic structure of the Sr-substituted hydroxyapatite [$(Ca, Sr)_{10}(PO_4)_6(OH)_2$] have been undertaken independently [12, 13]. Recently, there were a few reports on the fabrication of Sr-substituted calcium phosphates, explaining the structure changes with fabrication conditions or the growth and dissolution mechanisms *in vitro* [13–15]. However, most studies were restricted to the fabrication and structural investigation of the materials without a detailed evaluation of the mechanical or biological performances.

Therefore, in this study, we fabricated Sr-substituted calcium phosphates using a precipitation method with an alteration in the amount of Sr ions, and evaluated the structure and mechanical characteristics of the materials. In addition, the osteoblastic cellular responses of those fabricated materials were assessed in terms of proliferation rate and alkaline phosphatase (ALP) activity.

2. Materials and methods

2.1. Sample preparation

The Sr-containing calcium phosphates powders were fabricated by a precipitation method. Total 2 M of calcium nitrate tetrahydrate [$Ca(NO_3)_2 4H_2O$, Aldrich, WI, USA] together with strontium nitrate [$Sr(NO_3)_2$,

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Aldrich, WI, USA] in different ratios were dissolved in distilled water to prepare the Ca + Sr-containing solutions (molar ratios of [Sr]/[Ca + Sr] were set as 0%, 2%, 4%, and 8%). Another set of 1.2 M of ammonium hydrogen phosphate [$(NH_4)_2HPO_4$, Aldrich, WI, USA] was dissolved in distilled water to prepare the P-containing solutions. Each set of beakers was stirred with magnetic bar vigorously for 12 h to obtain clear solutions. Prior to adding the P-containing solution to the Ca + Sr containing solution, pH was pre-adjusted at $\sim$ 11 with ammonium hydroxide [NH_4OH, Aldrich, WI, USA] within the Ca + Sr-containing solution. While mixing the solutions, the pH was constantly adjusted to be $\sim$ 11. After additional stirring at 40 °C for 12 h, the mixtures were washed with ethanol and subsequently with distilled water twice by filtering under vacuum. The resulting filtercakes were oven dried at 80 °C for 24 h, then crushed and sieved to prepare the white-colored powders.

The dried powders were calcined at 900 °C for 1 h in air. After being molded in a disc shape and cold isostatically pressed (CIPed) at 150 MPa, each pellet was sintered at 1250 °C for 1 h in air at a heating and cooling rate of 10 °C/min and air cooling, respectively, to obtain disc samples for mechanical and biological tests.

The Sr-containing calcium phosphates specimens, obtained with Sr additions of 0, 2, 4, 8 mol % relative to Ca, were designated as CP, 2SrCP, 4SrCP, and 8SrCP, respectively.

2.2. Characterizations

The composition of each powder after drying and calcining at 900 °C was confirmed by inductively-coupled plasma atomic-emission spectroscopy (ICP–AES) analysis. The phase change of the powders was characterized by X-ray diffraction (XRD) patterns. The lattice parameter was calculated from the XRD results by using high purity Si powder (99.999%, Aldrich, WI, USA) as an internal standard to investigate the structural changes with Sr incorporation. In order to investigate the structural characteristics, Fourier-transform infrared (FT-IR) spectroscopy was used with KBr powder (Merck, USA) as a standard in the ratio of KBr/sample $\sim$ 10. The microstructure of the sintered specimen was observed with scanning electron microscopy (SEM) after polishing and thermal etching at 1100 °C for 30 min in Ar gas. The mechanical properties were estimated from the hardness test by indenting the polished samples at a load of 1.96 N with a Vickers indenter for 15 s. Five samples were tested for each condition.

2.3. *In vitro* cellular assay

The osteoblast-like cell lines (MG63 and HOS) were used to evaluate the proliferation and differentiation behaviors of the cells depending on the materials. Each cell line was cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc., MD, USA) supplemented with 10% fetal bovine serum (FBS, Life Technologies, Inc., MD, USA) [16].

The specimen for the cell test was prepared in a disc shape of dimensions with $\sim 11 \times 1\,mm^2$ (diameter $\times$ thickness) after polishing down to 1 μm followed by a sterilization in an autoclave at 121 °C for 20 min. The test samples were CP, 2SrCP, 4SrCP, and 8SrCP, with a cell culture dish (Thermanox; NUNC, IL, USA) as a control.

To measure the effect of each material on the proliferation of the MG63 cells, all specimens and the Thermanox control were placed in individual wells of a 24-well plate, and 3×10^4 cells/ml were plated onto the discs. The cells were cultured for three and seven days in an incubator humidified with 5% CO_2 in air at 37 °C. After detaching the cells with trypsin-EDTA solution (trypsinization), the living cells were counted using a hemocytometer (Superior Co., Germany). Each set of test was performed in triplicate, and the data were normalized by taking the surface area into consideration.

To observe the cell morphology after proliferation, SEM was performed after fixing the cells with 2.5% glutaraldehyde, dehydrating them with a series of graded ethanol (70, 90, and 100%, subsequently), and critical point drying with CO_2.

The isolated human osteosarcoma (HOS) cell line was used to determine the differentiation of the cells by measuring the ALP activity. The cells were plated at a density of 1×10^4 cells/ml in 24-well plate containing the specimens, and cultured for 10 days in the humidified incubator with 5% CO_2 at 37 °C. At harvest, the culture media was decanted, and the cell layers were washed with Hank's balanced salt solution (HBSS), followed by a trypsinization as described above. After centrifugation at 1200 rpm for 7 min, the cell pellets were washed with phosphate-buffered saline (PBS) solution and resuspended by vortexing in 200 μl of 0.1% Triton X-100. The pellets were disrupted further by freezing and thawing, each for 2 min, and this process was repeated seven times. After centrifugation at 13 000 rpm in a microcentrifuge for 15 min at 4 °C, the cell lysates were quantified using the BioRad DC protein assay kit (BioRad, Hercules, CA, USA) and assayed colorimetrically for the ALP activity using *p*-nitrophenyl phosphate as a substrate at pH 10.3 (Sigma, St. Louis, MO, USA). Each reaction was initiated with *p*-nitrophenyl phosphate and lasted for 60 min at 37 °C, then stopped by quenching on ice. The produced *p*-nitrophenol was measured at 410 nm using a spectrophotometer (UV-160A, Shimadzu, Kyoto, Japan). The *in vitro* cell tests were performed on six replicate samples.

2.4. Statistics

Statistical analysis was carried out on the mechanical and *in vitro* cellular data by using Student's *t*-test, and significance was considered at $p < 0.05$.

3. Results and discussion

Fig. 1 shows the XRD patterns of the calcium phosphate powders with various Sr additions after calcination at 900 °C for 1 h in air. All powders showed sharp and narrow apatite peaks. However, additional β-TCP peaks were formed in the powders containing Sr, and their intensity was more pronounced with increasing the Sr addition, that is, the TCP intensity was in the order of

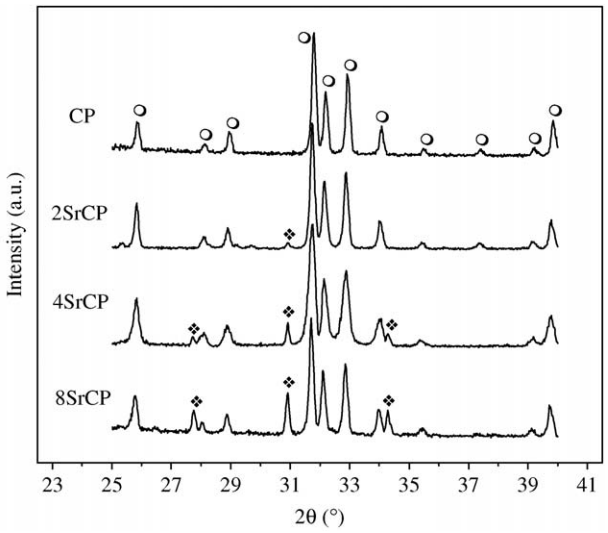

Figure 1 XRD patterns of the calcium phosphate powders calcined at 900 °C for 1 h in air. Legends are (❍) HA and (❖) β-TCP.

8SrCP > 4SrCP > 2SrCP > CP. The appearance of TCP in the apatite containing Sr has been reported previously, which was attributed to the non-stoichiometry of the apatite structure and the decomposition of apatite with further heat treatment [14].

Fig. 2 shows the FT-IR analysis of the calcined powders. In the powders with no Sr (CP), a typical HA structure containing sharp O–H and P–O bands was observed. The 2SrCP powder also showed a similar structure to the pure CP. However, in the powders containing relatively high Sr concentrations (4SrCP and 8SrCP), the apatite structures were quite different. The P–O bands at 1000–1100 cm^{-1} became broader, the O–H band at 550–650 cm^{-1} disappeared, and the O–H band at 3570 cm^{-1} was attenuated. Those changes in the apatite structure were attributed to the formation of TCP and consequently to the alteration in the bonding strength of O–H and P–O [17].

From the phase and structure analyses, it is confirmed that the Sr addition caused the apatite powder to be structurally non-stoichiometric and thermally unstable to form the TCP at elevated temperatures. It was previously

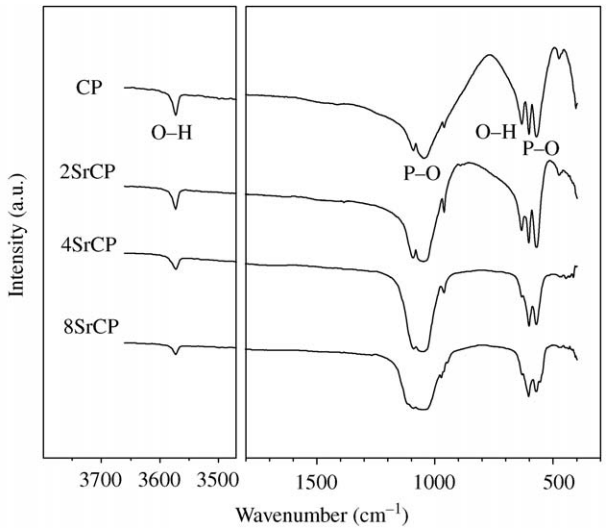

Figure 2 FT-IR spectroscope of the calcium phosphate powders calcined at 900 °C for 1 h in air.

reported that the non-stoichiometry of the apatite powder causes structural and compositional changes at elevated temperatures [18].

The chemical compositions of the precipitated and calcined powders obtained from the ICP–AES analysis are shown in Table I. When compared to the initial precursor contents, the (Ca + Sr)/P ratios were maintained as intended in the precipitated powders. Whilst, the Sr/(Ca + Sr) ratios in the precipitated powders were lower than those in the initial contents, and the discrepancy became larger as the Sr addition increased. Based on this observation, it is deduced that the increase in the Sr content caused the incomplete incorporation of Sr within the apatite structure and that the residuals were washed out. In practice, Sr incorporation within the HA structure was previously found to have a certain limit [13, 14]. After calcination at 900 °C for 1 h, both the (Ca + Sr)/P and Sr/(Ca + Sr) values changed little when compared to those in the precipitated powders.

The substitution of Sr ions within the apatite structure resulted in a change in the lattice parameters of the calcined powders, as represented in Fig. 3 after being quantified from XRD results. Both *a*- and *c*-axis increased rather steadily as the Sr concentration increased. It is known that the larger ionic radius of the Sr compared to Ca causes the apatite lattice to expand in both the *a*- and *c*-axis directions [3, 18]. The obtained values were quite similar to the reported ones. However, at the higher Sr concentration (over 4%), they were slightly lower than the calculated values (dashed lines) [3, 13, 18, 19]. Such a discrepancy in the lattice constants at the higher Sr concentrations was attributed to the incomplete substitution of the initial Sr ions within the apatite structure. As mentioned above, parts of the Sr ions were washed out during the powder preparation process. There is another possibility for the discrepancy, that is, a part of the Sr was substituted into the TCP structure, forming a $(Ca, Sr)_3(PO_4)_2$ structure [14].

The typical microstructures of the apatites sintered at 1250 °C for 1 h are shown in Fig. 4, after polishing and thermal etching at 1100 °C for 30 min. All the sintered samples were almost fully dense. In pure CP (Fig. 4(a)), the grains were in a regular and isotropic shape, and showed a bimodal distribution in size (approximately 2–3 and over 5 μm). Moreover, the grain boundaries were observed clearly. However, the 4SrCP had a quite different morphology as shown in Fig. 4(b). Compared to the pure CP, the grains were irregular in shape and the grain boundaries of the Sr-substituted specimen were not

TABLE I Chemical composition of each powder after drying and calcining at 900 °C for 1 h in air, which was calculated from ICP-AES analysis

Material	CP	2SrCP	4SrCP	8SrCP
(Ca + Sr)/P				
Initial	1.67	1.67	1.67	1.67
Precipitated	1.70	1.69	1.66	1.67
Calcined	1.69	1.71	1.65	1.65
Sr/(Ca + Sr)				
Initial	—	0.020	0.040	0.080
Precipitated	—	0.018	0.032	0.066
Calcined	—	0.019	0.030	0.067

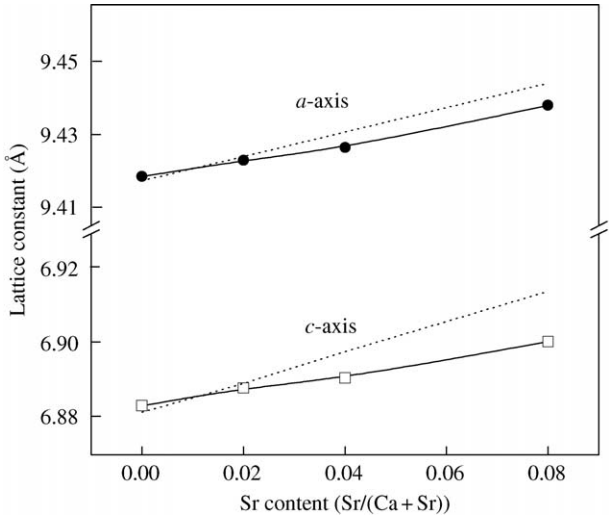

Figure 3 Lattice parameters of the calcium phosphate powders calcined at 900 °C for 1 h in air: The data was quantified from the XRD results using Si powder as an internal standard. The dashed lines represent calculated values.

clear. Such microstructures in the Sr-substituted specimens are believed to be due to the coexistence of HA and TCP structure and the consequent uneven thermal etching. The 2SrCP and 8SrCP specimens had similar microstructures with respect to pure CP and 4SrCP, respectively (data not shown).

The Vickers hardness of the sintered specimens was tested by indentation at a load of 1.96 N, as shown in

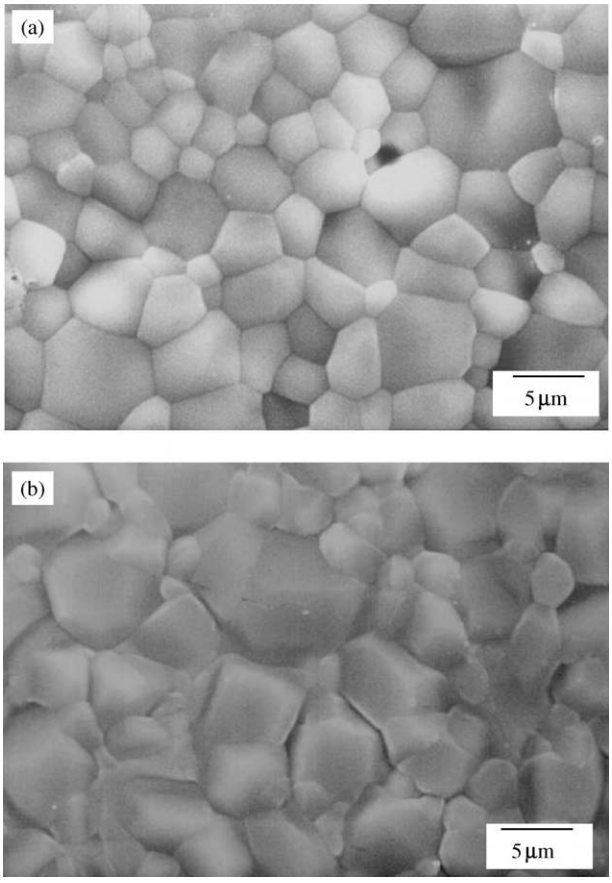

Figure 4 SEM of the calcium phosphate ceramics sintered at 1250 °C for 1 h in air: (a) CP and (b) 4SrCP. Microstructure observed after polishing and thermal etching at 1100 °C for 30 min in Ar gas.

TABLE II Vickers hardness of the calcium phosphate ceramics after sintering at 1200 °C for 1 h in air, tested at an indentation load of 1.96 N for 15 s (mean ± 1 SD, $n = 5$)

Materials	CP	2SrCP	4SrCP	8SrCP
Hardness (GPa)	5.23 (± 0.09)	5.21 (± 0.13)	5.43 (± 0.20)	5.48 (± 0.19)

Table II. Compared to the pure CP, there was little difference in the values (~ 5.2 GPa) at low Sr addition (2SrCP), but slight increases (~ 5.5 GPa) were observed at higher Sr concentrations (4SrCP and 8SrCP). This appears to be correlated with TCP formation. In practice, a HA + TCP biphasic formation is known to increase the mechanical properties of pure HA [20, 21]. However, the difference between samples was not statistically significant.

The cellular responses to the calcium phosphate samples were assessed by the proliferation and differentiation behaviors of osteoblastic cells (MG63 and HOS). Fig. 5 shows the typical morphologies of the proliferated MG63 cells on pure CP (a and b) and 4SrCP specimens (c and d) after culturing for three days. At low magnification, lots of cells were observed to proliferate favorably on both CP and 4SrCP surfaces, covering most parts of the surfaces. At higher magnification, the cell membranes were in intimate contact with and well-flattened on the surface with cytoplasmatic extensions. For the other samples (2SrCP and 8SrCP), there were no significant morphological differences.

The proliferation rates of the MG63 cells on each sample are represented in Fig. 6 after culturing for three and seven days. The cells cultured for seven days multiplied actively compared to those cultured for three days, confirming the favorable environment of the materials for cell proliferation. The samples containing higher Sr concentrations (4SrCP and 8SrCP) appeared to show slightly higher proliferation rates than those of pure CP, but not significantly different ($p > 0.05$).

The differentiation behavior of the proliferated cells was assessed by their alkaline phosphatase (ALP) activity, as shown in Fig. 7. The ALP was used as a marker for the differentiation behavior of the cells, relatively at an initial period [24, 25]. The HOS cells on the calcium phosphate samples showed higher ALP expression levels compared to those on the plastic culture dish after culturing for 10 days. With the Sr addition increasing, the ALP expression level became significantly higher on 4SrCP and 8SrCP as compared to CP ($p < 0.05$), suggesting that the proliferated cells on the Sr-substituted calcium phosphates underwent a differentiation step to a higher degree at least at an initial step.

The improved proliferation and differentiation of osteoblast-like cells on the Sr-substituted calcium phosphates were attributed to the combined effects, that is, not only to the Sr ions itself but also to the TCP formation. As was observed, the increase in the Sr concentration resulted in the formation of TCP. In practice, many studies have reported the positive role of Sr ions in the bone forming processes both *in vitro* and *in vivo* [5–11]. Since the calcium phosphate ceramics are not inert biologically, the Sr ions released from the Sr-

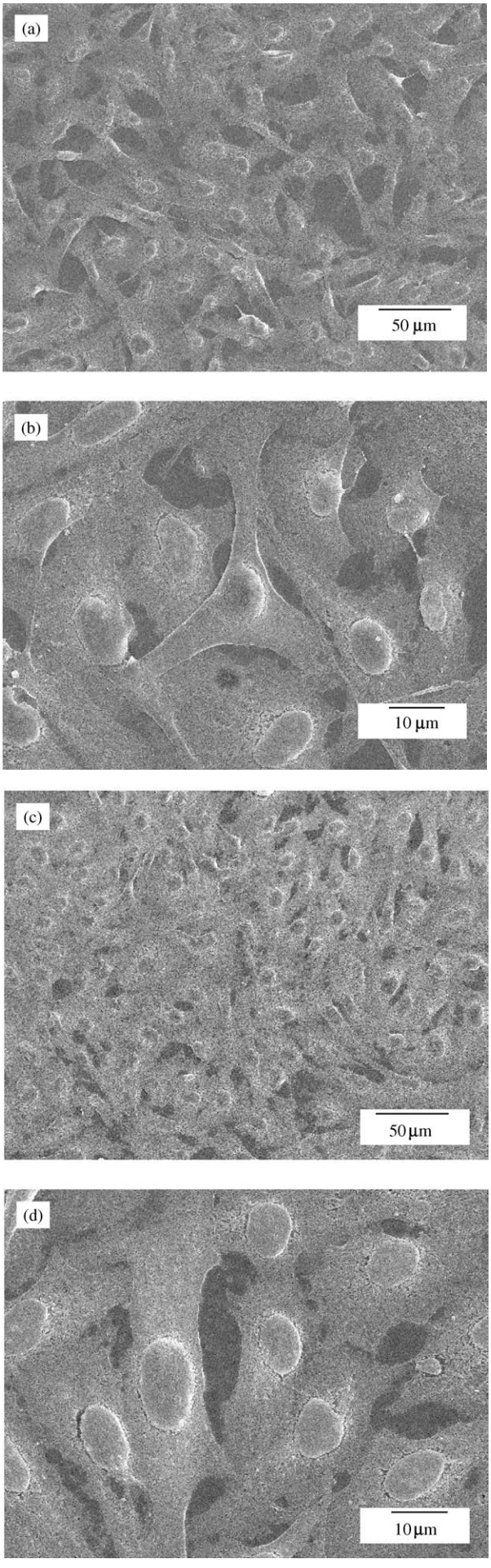

Figure 5 SEM of the MG63 cells proliferated on various calcium phosphate ceramics after culture for three days: The cells on (a and b) CP and (c and d) 4SrCP.

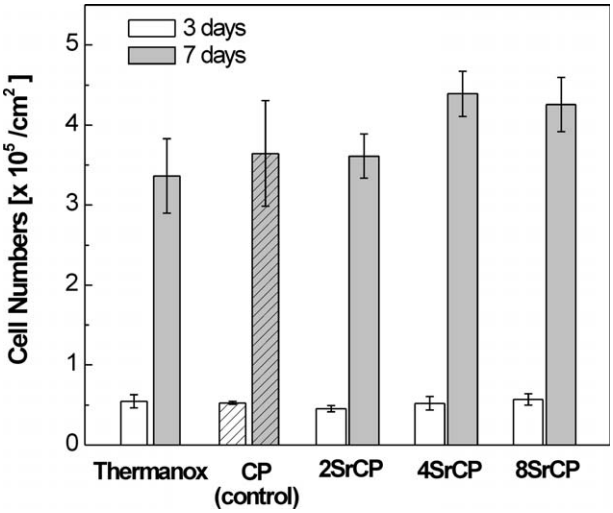

Figure 6 Proliferation rates of MG63 cells-lines on various calcium phosphate ceramics after culturing for three and seven days (mean $\pm$ 1 SD, $n = 6$).

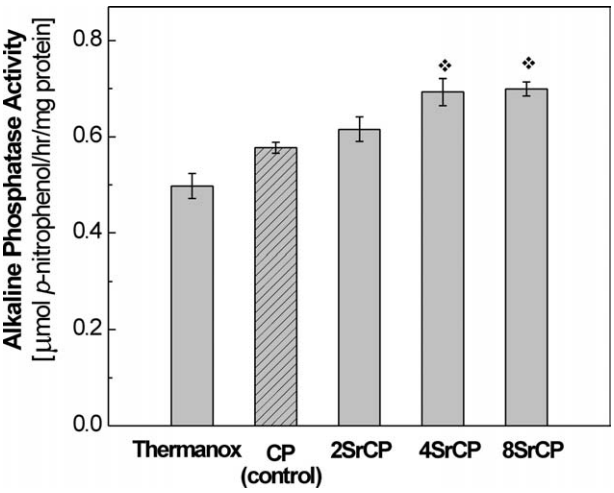

Figure 7 Alkaline phosphatase activity of the HOS cells-lines on various calcium phosphate ceramics after culturing for 10 days (mean $\pm$ 1 SD, $n = 6$). The activity on 4SrCP and 8SrCP was significantly higher than that on CP (❖; $p < 0.05$).

substituted apatite samples are supposed to interact with the cells and affect the cell functions positively. In addition, the formation of biphasic calcium phosphates (HA + TCP) caused by Sr addition can affect the cell response. From the *in vivo* studies, the biphasic ceramic has been reported to enhance earlier bone healing than HA alone because of its enhanced bioactivity [24–27]. Therefore, the biphasic formation driven by the Sr ion substitution is considered to be favorable for the biological properties. Even though the exact roles of Sr ions and TCP formation are not clear at this point, the Sr-substituted calcium phosphates fabricated in this study are regarded as promising biomaterials for hard tissue engineering from the viewpoint of *in vitro* cellular responses in the proliferation and differentiation steps. In order to confirm the exact effect of the Sr ion and the TCP formation separately, further studies on the fabrication of Sr containing apatite ceramic without the existence of a TCP phase or on synthesizing the HA + TCP biphasic ceramic without the addition of Sr ions are needed. Moreover, other protein-regulated differentiation tests and eventually *in vivo* experiment remains as further

studies for a comprehensive understanding of the biocompatibility of the Sr-substitute calcium phosphate ceramics.

4. Conclusions

Powder precipitation method was used to obtain Sr-substituted calcium phosphate bioceramics. The Sr ions added up to 8 mol % to replace the Ca ions were partially washed out during the powder preparation. All the powders exhibited highly crystalline apatite characteristics after calcination at 900 °C, however, additional β-TCP form appeared in the Sr-substituted powders, and the intensity increased with increasing Sr addition. With the substitution of Sr, the lattice parameters (both *a*- and *c*-axis) of the calcined powders increased steadily. A Sr addition over 4 mol % changed the microstructure of the sintered bodies from round grains with apparent grain boundaries to faceted grains with ambiguous grain boundaries. Moreover, the Vickers hardness increased slightly from $\sim$ 5.2 to 5.5 GPa. The *in vitro* cellular response showed that the Sr incorporation increased the proliferation rate and differentiation (ALP activity) of the osteoblast-like cells, confirming the enhanced cell viability and functionality of the Sr-substituted calcium phosphate ceramics.

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References

1. L. L. HENCH, *J. Am. Ceram. Soc.* **74** (1991) 1487.
2. R. Z. LEGEROS, *Prog. Cryst. Grow. Char.* **4** (1981) 1.
3. J. C. ELLIOT, in ''Studies in Inorganic Chemistry 18: Structure and Chemistry of the Apatites and Other Calcium Orthophosphates'' (Elsevier, Amsterdam, 1994) p. 111.
4. D. K. SMITH, in ''Hydroxyapatite and Related Materials'', edited by P. W. Brown and B. Constantz (CRC Press, London, 1994) p. 29.
5. F. S. KAPLAN, W. L. HAYES, T. M. KEAVENY, A. BOSKEY, T. A. EINHORN and J. P. IANNOTTI, in ''Orthopedic Basic Research'', edited by S. P. Simon (American Academy of Orthopedic Surgeons, 1994) p. 127.
6. E. SHORR and A. C. CARTER, *Bull. Hosp. Joint. Dis. Orthop. Inst.* **13** (1952) 59.
7. F. E. MCSASLIN and J. M. JANES, *Proc. Staff. Meet. Mayo. Clin.* **34** (1959) 329.
8. P. J. MARIE, M. T. GARBA, M. HOTT and L. MIRAVET, *Miner. Electrolyte Metab.* **11** (1985) 5.
9. P. J. MARIE and M. HOTT, *Metabolism* **35** (1986) 547.
10. E. F. FERRARO, R. CARR and K. ZIMMERMAN, *Calcif. Tissue. Int.* **35** (1983) 258.
11. E. CANALIS, M. HOTT, P. DELOFFRE, Y. TSOUDEROS and P. J. MARIE, *Bone* **18** (1996) 517.
12. K. SUDARSANAN and R. A. YOUNG, *Acta. Cryst.* **B28** (1972) 3668.
13. J. CHRISTOFFERSEN, N. KOLTHOFF and O. BARENHOLDT, *Bone* **20** (1997) 47.
14. L. LEROUX and J. L. LACOUT, *J. Mater. Res.* **16** (2001) 171.
15. D. DONAZZON, G. DECHAMBRE and J. L. LACOUT, *Ann. Chim. Sci. Mat.* **23** (1998) 53.
16. H.-W. KIM, Y.-J. NOH, Y.-H. KOH and H.-E. KIM, *J. Mater. Sci.: Mater. Med.* **14** (2003) 899.
17. J. C. RENDON-ANGELES, K. YANAGISAWA, N. ISHIZAWA and S. OISHI, *Chem. Mater.* **12** (2000) 2143.
18. R. Z. LEGEROS, R. KIJKOWSKA, M. TUNG and J. P. LEGEROS, in Proceedings of the 5th International Symposium, edited by R. W. Fearnhead (Elsevier Science Ltd., 1989) p. 393.
19. R. L. COLLIN, *J. Am. Chem. Soc.* **81** (1959) 5275.
20. S. RAYNAUD, E. CHAMPION, J. P. LAFON and D. BERNACHE-ASSOLLANT, *Biomaterials* **23** (2002) 1081.
21. A. ROYER, J. C. VIGUIE, M. HEUGHEBAERT and J. C. HEUGHEBAERT, *J. Mater. Sci.: Mater. Med.* **4** (1993) 76.
22. N. ALI, N. N. J. ROWE and N. M. TEICH, *J. Bone Mine. Res.* **11** (1996) 512.
23. S. OZAWA and S. KASUGAI, *Biomaterials* **17** (1996) 23.
24. R. F. ELLINGER, E. B. NERY and K. L. LYNCH, *Int. J. Periodont. Restor. Dent.* **3** (1986) 223.
25. N. PASSUTI, G. DACULSI, J. M. ROGEZ, S. MARTIN and J. V. BAINVEL, *Clin. Orthop. Rel. Res.* **248** (1989) 169.
26. G. DACULSI, M. BAGOT D'ARC, P. CORLIEU and M. GERSDORFF, *Ann. Otol. Rhinol Laryngol.* **101** (1990) 669.
27. D. C. MOORE, M. W. CHAPMAN and D. MANSKE, *Clin. Orthop. Res.* **5** (1987) 356.

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