

In vitro bioactivity of novel tricalcium silicate ceramics

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Abstract In this study, bone-like apatite-formation ability of tricalcium silicate (Ca_3SiO_5) ceramics in simulated body fluid (SBF) was evaluated and the in vitro degradability was investigated by soaking in Ringer's solution. The effect of ionic products from Ca_3SiO_5 dissolution on osteoblasts proliferation was investigated. The result indicated that hydroxyapatite (HA) was formed on the surface of the Ca_3SiO_5 ceramics after soaking in SBF for 1 day, and Ca_3SiO_5 ceramics could degraded in Ringer's solution. The Si ions from Ca_3SiO_5 dissolution at certain concentration range significantly stimulated osteoblasts proliferation. Our results show that Ca_3SiO_5 ceramics possess bone-like apatite-formation ability and degradability, and can release soluble ionic products to stimulate cell proliferation.

1 Introduction

Biomaterials combining biodegradability and bioactivity, with the aim of developing materials that, once implanted, will help the body heal itself [1]. In the field of bone repair, bioactivity generally means the bone-bonding ability of materials. Bioactive materials like silicate glasses and glass-ceramics of specific compositions can directly bond to living bone tissue depositing an intervening biologically active apatite layer when embedded in human body [2, 3]. Bioactive glass containing calcium and silicon is biodegradable, and can form bone-like HA layer on its surface in simulated body environment [2]. In vivo, this bone-like HA plays an essential role in the formation, growth and maintenance of the bone tissue-biomaterial interface, and can increase the bonding intensity [4, 5]. In vitro, this HA can enhance cell adhesion and stimulate cell proliferation [6]. Recent studies showed that the released ions of bioactive glass have an effect on cell proliferation [7]. This was the new embodiment of bioactivity of the materials.

Tricalcium silicate (Ca_3SiO_5), which contains calcium and silicon, is a potential biomaterial, and in our previous studies, Ca_3SiO_5 powders [8] and ceramics [9] were synthesized and the results showed that the Ca_3SiO_5 was bioactive and the powders could induce bone-like apatite formation after soaking in SBF for 10 days [8]. However, the bioactivity (bone-like apatite-formation ability and the effect on cell proliferation) and degradability of Ca_3SiO_5 ceramics so far has not been investigated. The purpose of this study was to investigate the in vitro bioactivity and degradability of Ca_3SiO_5 ceramics. Bone-like apatite-formation ability of Ca_3SiO_5

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ceramics was evaluated by soaking in SBF, and the effect of the Ca and Si ions from Ca_3SiO_5 dissolution on cell proliferation was investigated using osteoblast culture.

2 Experimental

2.1 Preparation of tricalcium silicate ceramics

Ca_3SiO_5 ceramics were prepared according to the method as previously described [8]. The sintering was performed at 1500°C for 8 h in a high-temperature furnace at a heating rate of $2^\circ\text{C}/\text{min}$. The as-prepared Ca_3SiO_5 ceramics were investigated by SEM (JSM-6700F, JEOL, Tokyo, Japan) and XRD (Geigerflex, Rigaku Co., Japan) using monochromated CuK_α radiation.

2.2 Soaking in SBF

The SBF was prepared according to the procedure described by Kokubo [10]. The ion concentrations of the SBF are similar to those in human blood plasma. The Ca_3SiO_5 ceramic disks were soaked in the SBF solution for 1, 3 and 7 days with a surface area-to-volume ratio of 0.1 cm^{-1} [11] without refreshing the soaking medium. After the pre-selected soaking time, the ceramic disks were gently rinsed with deionized water to remove SBF solutions followed by drying at room temperature.

The surface structural and morphological variations of the ceramics before and after soaking in SBF were characterized by XRD and SEM. The concentrations of Ca, Si and P in the SBF solution after soaking were determined by ICP-AES (Varian Co., USA) and pH value of the soaking solution was measured using an electrolyte-type pH meter (pHS-2C, Jingke Leici Co., Shanghai, China).

2.3 The in vitro degradation of ceramics

For evaluation of degradation, the Ca_3SiO_5 ceramics (the relative density was 90.0%), hydroxyapatite (HA) (sintered at 1150°C for 2 h; the relative density was 89.5%) and β -tricalcium phosphate (β -TCP) (sintered at 1100°C for 2 h; the relative density was 88.5%) ceramics were soaked in Ringer's solution (pH 7.40) at 37°C in shaking water bath for 1, 4, 7, 10, 14 and 28 days with a surface area-to-volume ratio of 0.1 cm^{-1} , and the solution was refreshed and collected every day. After the set soaking time, the ceramics were dried at 60°C for 1 day, and the final weight of each sample was

accurately measured. The weight loss was expressed as percentage of the initial weight.

2.4 Cell culture

Osteoblasts were isolated by sequential trypsin-collagenase digestion on calvaria of neonatal (<2 days old) Sprague–Dawley rats as described elsewhere [12]. In short, the calvaria were excised under aseptic conditions and kept in ice-cold D-Hanks buffer. The fibrous layers of the periosteum were mechanically removed. To diminish fibroblastic contamination and cell debris, the calvaria were preincubated for 20 min with an enzyme solution (0.25% trypsin, 0.004 M ethylenediaminetetraacetic acid, EDTA) and the supernatant was discarded. After continuous enzyme treatment with 5 ml collagenase (Gibco) for 90 min, the supernatant was centrifuged (10 min at 1000 rpm; 250 g). The pellets were resuspended in the RPMI 1640 containing 15% fetal calf serum (FCS) and maintained at 37 in a humidified atmosphere of 95% air and 5% CO_2 . Media were changed every day until the cells reached confluence. In this study, only the cells at the 2nd and 5th passages were employed.

2.5 Effect of ionic dissolution products of Ca_3SiO_5 on osteoblasts

The dissolution extracts of Ca_3SiO_5 were prepared by adding Ca_3SiO_5 ceramics powders to Roswell Park Memorial Institute 1640 (RPMI 1640; Gibco, USA) cell culture medium according to International Standard Organization (ISO/EN 10993-5) [13]. The ratio of the Ca_3SiO_5 weight and the medium volume was 200 mg/ml. After incubation at 37°C for 24 h, the mixture was centrifuged and the supernatant was collected. Subsequently, the extract liquid was diluted with serum-free medium for further cell culture experiments. The ionic concentration of the extract liquid was measured by ICP-AES.

The cell suspension was adjusted to a density of $1 \times 10^4\text{ cell}\cdot\text{ml}^{-1}$, and 100 μl cell suspension was added to each well of a 96-well plate and incubated for 24 h. The culture medium was then removed and replaced by 50 μl of diluted extracts and 50 μl of RPMI 1640 medium supplemented with 20% FCS. The medium supplemented with 10% FCS without addition of extracts was used as a control. After incubating at 37°C and 5% CO_2 for 1, 3 and 7 days, 100 μl of 0.5 $\text{mg}\cdot\text{ml}^{-1}$ 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution was added and cultured for 4 h at 37°C . Then 100 μl dimethyl sulfoxide (DMSO) was added to each well, the plate was shaken

for 5 min, and the optical density (OD) at 590 nm was measured with an enzyme-linked immunoadsorbent assay (ELISA) plate reader (ELX800, Bio-TEK, USA).

Six samples per group were tested in the experiment, the values were expressed as mean \pm standard deviation (SD) and were analyzed using standard analysis of Student's *t*-test. A *p*-value <0.05 was considered statistically significant.

3 Results

3.1 XRD and microstructure

Figure 1 shows the XRD pattern of Ca_3SiO_5 ceramics sintered at 1500°C for 8 h. It was obvious that only tricalcium silicate peaks existed. Figure 3A, B show the SEM micrographs of surfaces of the Ca_3SiO_5 ceramics. It can be seen that most Ca_3SiO_5 particles were sintered, compact microcrystalline appearance with clear grain boundaries and some micropores were evident, which indicated that the sintered ceramics were not completely dense.

3.2 HA formation on Ca_3SiO_5 ceramics

Figure 2 shows the XRD patterns of Ca_3SiO_5 ceramics before and after soaking in the SBF solution. It can be seen that the characteristic peaks of Ca_3SiO_5 decreased within the first day of soaking. After 3 days of soaking, new peaks for calcium carbonate (CaCO_3) appeared due to the hydration of Ca_3SiO_5 [14], and peaks for Ca_3SiO_5 decreased further and the characteristic peaks

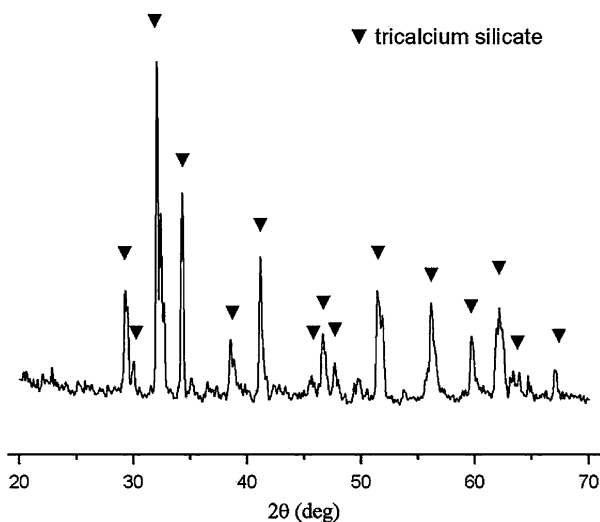


Fig. 1 XRD patterns of as-prepared tricalcium silicate ceramics

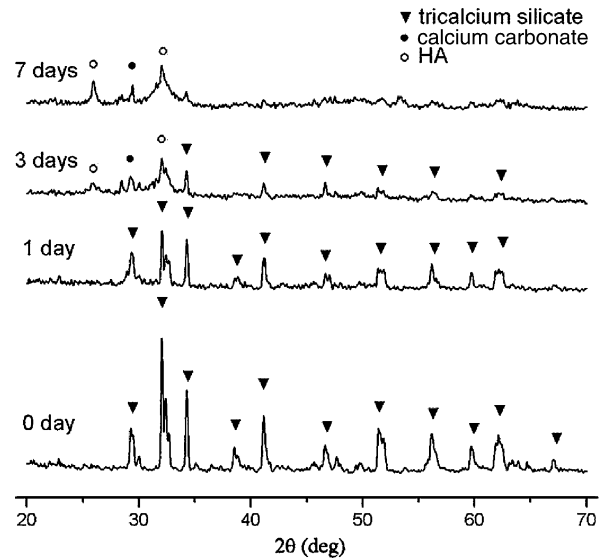


Fig. 2 XRD patterns of the tricalcium silicate ceramics before and after soaking in SBF

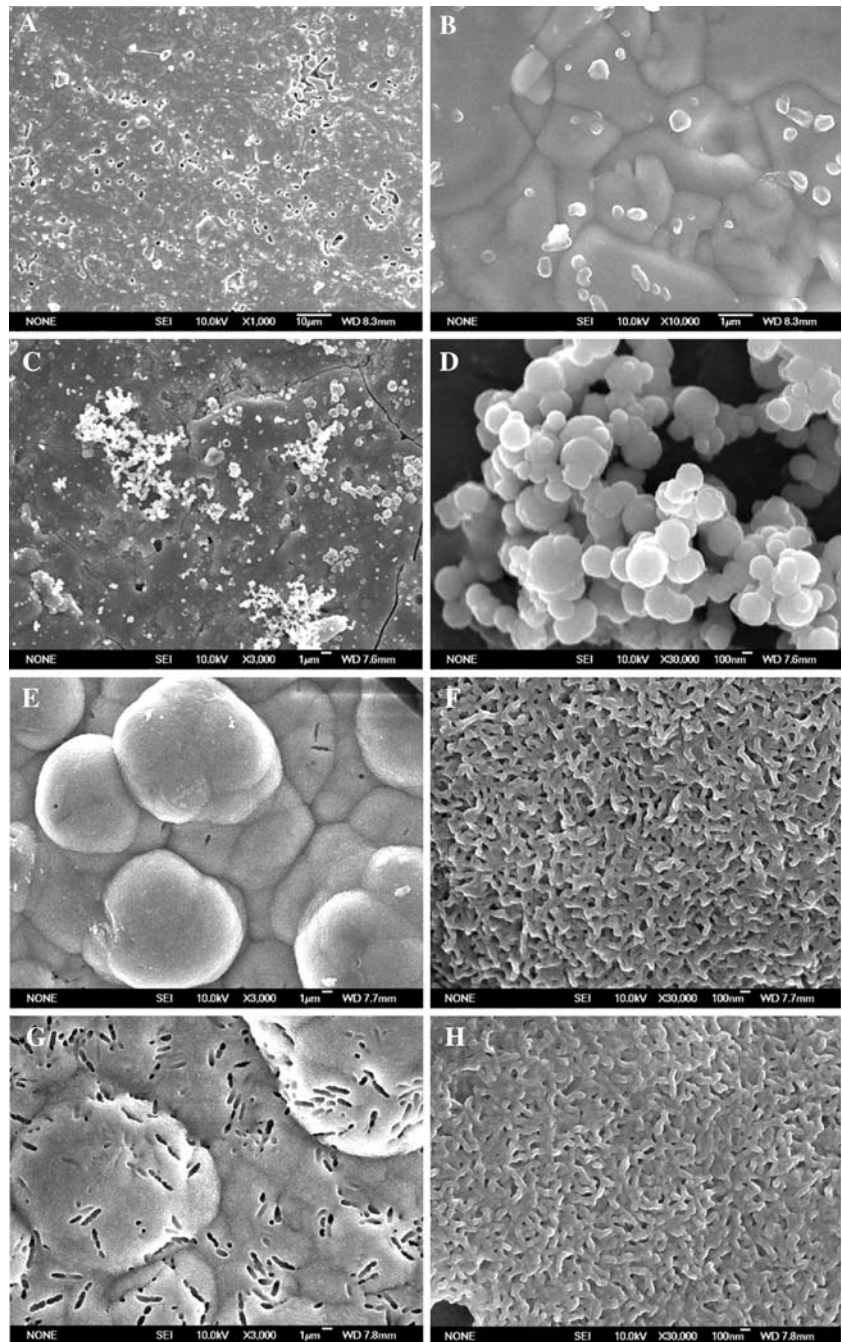
for HA appeared. After 7 days of soaking, peaks for Ca_3SiO_5 disappeared and the characteristic peaks for HA became the main constituent of the XRD patterns as the HA content increased and covered the surfaces of the ceramics, but peaks of CaCO_3 were still evident, which were formed from continued hydration of Ca_3SiO_5 .

Figure 3 shows SEM micrographs of the surfaces of Ca_3SiO_5 ceramics after soaking in SBF for various times. Before soaking, the Ca_3SiO_5 ceramic showed compact microcrystalline, clear crystal boundaries and some micropores (Fig. 3A, B). After soaking for 1 day, the crystal boundaries disappeared, cracks appeared, and tiny ball-like particles of HA were observed on the surface of the samples (Fig. 3C, D). After soaking for 3 days, a HA layer was formed on the ceramic surface (Fig. 3E). The higher magnification SEM micrograph showed that the particles of HA were lathlike and the size of the particles was about 50 nm in diameter and many of these particles formed agglomerates (Fig. 3F). After soaking for 7 days, some micropores were formed on the ceramic surface and the HA layer became compact (Fig. 3G, H).

3.3 The changes of ionic concentration and pH value of the SBF solution

Figure 4 shows the concentration changes of calcium, phosphorus and silicon and pH value of the SBF solution after soaking for various periods. After 1 day of soaking, the Ca and Si concentrations in the SBF solution increased with a corresponding decrease in the

Fig. 3 SEM micrographs of the surfaces of tricalcium silicate ceramics before (**A, B**) and after soaking in SBF solution for various times: (**C, D**) 1 day, (**E, F**) 3 days and (**G, H**) 7 days



P concentration. This reflected the dissolution of the Ca and Si accompanied with simultaneous uptake of P from the solution onto the paste, which corresponded to a quick increase of the pH value of the SBF solution and deposition of calcium phosphate. After prolonged soaking from 1 to 7 days, the Ca and Si concentration continued to increase with a corresponding decrease in the P concentration indicating that the P ion was used up to form HA.

3.4 Degradation of tricalcium silicate ceramics

Figure 5 shows the changes of the weight of Ca_3SiO_5 , HA and β -TCP ceramics in Ringer's solution. It was obvious that the weight loss of Ca_3SiO_5 and β -TCP ceramics increased with the increase of soaking time, while the weight loss of HA ceramics was nearly zero. The weight loss of Ca_3SiO_5 ceramics was slightly less than that of β -TCP ceramics after the same soaking

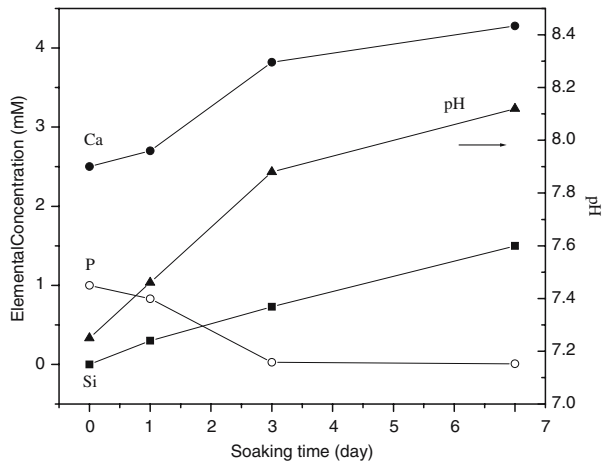


Fig. 4 Changes of Ca, Si and P concentrations and pH value of the SBF solution after soaking with the Ca_3SiO_5 ceramics for various times

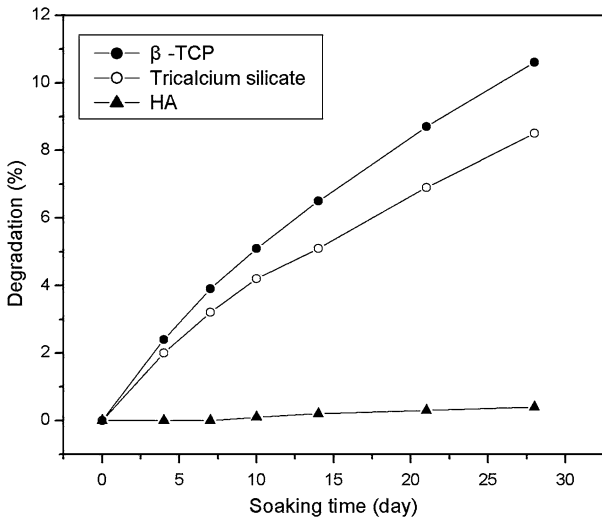


Fig. 5 Degradation of Ca_3SiO_5 , β -TCP and HA ceramics after soaking in Ringer's solution for various times

time. Our result indicated that Ca_3SiO_5 ceramics were soluble in Ringer's solution.

3.5 Effect of ionic products from Ca_3SiO_5 dissolution on osteoblasts

The dissolution of different concentrations of Ca and Si ions from Ca_3SiO_5 has an obvious effect on

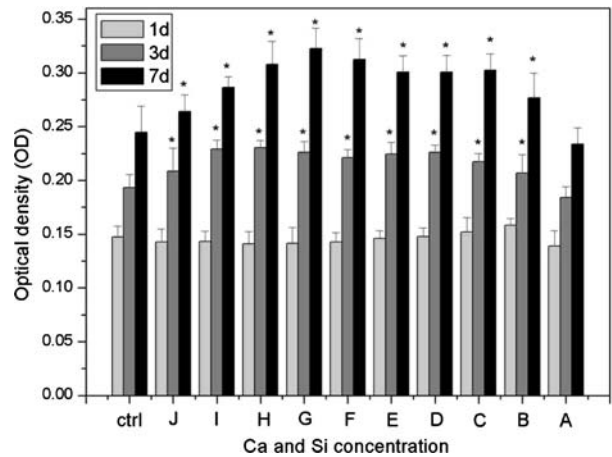


Fig. 6 Results of cell proliferation after culturing in dissolution extracts of the Ca_3SiO_5 powders for different periods. *, Denotes significant difference from the ctrl-, * $p < 0.05$

osteoblasts proliferation (Fig. 6), and the corresponding concentration of Ca and Si ions is shown in Table 1. It could be seen that Ca_3SiO_5 released Si ions in RPMI 1640 culture medium. The dissolution extracts of Ca_3SiO_5 at a certain Si ions concentration range of 0.00001 M–19.92 M stimulated osteoblasts proliferation after culturing osteoblasts for 7 days.

4 Discussion

It is a common notion that bonelike HA plays an essential role in the formation, growth and maintenance of the tissue-biomaterial interface, and this HA layer can be reproduced in vitro in SBF [15]. Ca_3SiO_5 is analogous with CaSiO_3 and Ca_2SiO_4 in component, and they all contain calcium and silicon in different ratios. Previous studies [3, 16] showed that CaSiO_3 and Ca_2SiO_4 ceramics were bioactive and could induce formation of HA on their surfaces when soaked in SBF. Our results suggest that Ca_3SiO_5 ceramics also has excellent in vitro bioactivity. It is clear to see from the XRD, SEM and ICP-AES analysis that the Ca_3SiO_5 ceramics also developed a layer of HA on their surface when exposed to SBF. Based on all these results, we may assume that calcium silicate ceramics with different Ca/Si ratio might all be able to induce

Table 1 Ionic concentration (mM) of the extract from the Ca_3SiO_5 powders in the culture medium

	RPMI 1640	A	B	C	D	E	F	G	H	I	J
Ca	0.424	0.230	0.327	0.376	0.400	0.412	0.418	0.423	0.424	0.424	0.424
Si	0	39.84	19.92	9.96	4.98	2.49	1.25	0.13	0.013	0.0013	0.00001

formation of HA, but more work need to be done to confirm this assumption.

ICP analysis showed that the Ca and Si concentrations in SBF increased with an increase of soaking time. The increase in the Ca and Si concentrations was attributed to the dissolution of the Ca and Si ions from the Ca_3SiO_5 ceramics. Although the formation of the bone-like apatite layer consumed some Ca ions, the Ca ions dissolution from Ca_3SiO_5 ceramics were more than those consumed. The ion exchange of Ca^{2+} in the Ca_3SiO_5 ceramics with H^+ in SBF attributed to an increase in pH of SBF at the early stage of soaking, which resulted in the formation of a hydrated silica layer on the surfaces of the ceramics and provided favorable sites for phosphate nucleation. The decrease in P concentration was attributed to the formation of amorphous calcium phosphate and the subsequent formation of HA by incorporating OH-ions from SBF, which resulted in a pH decrease after prolonged soaking [16]. The profile of the changes of Ca, Si, and P ion concentrations, pH in SBF and the formation of the silica-rich layer were similar to that of the bioactive CaSiO_3 ceramics [16, 18]. All these results suggested that the mechanism of bone-like apatite formation on the surfaces of Ca_3SiO_5 ceramics might be similar to that of the bioactive CaSiO_3 ceramics. Our results suggest that Ca_3SiO_5 ceramics are not only bioactive, but also degradable. It can degrade in Ringer's solution at a rate slightly slower than that of β -TCP ceramics. However, more in vitro and in vivo degradation studies need to be conducted to confirm the degradation properties of the Ca_3SiO_5 ceramics.

Previous studies showed that the bioactive glass could release Ca and Si ions. The released Si ions promoted mineralized nodule formation of human primary osteoblasts [19, 20], and stimulated osteoblasts proliferation and gene expression [21–24]. Other studies showed that the released Ca ions could induce osteoblast proliferation and chemotaxis through binding to a G-protein coupled extracellular calcium sensing receptor [24]. In our study, the Si ions from Ca_3SiO_5 dissolution at certain concentration range significantly stimulated osteoblasts proliferation. All these studies suggest that some inorganic ions, such as Ca and Si ions, together possess the ability of stimulating cells proliferation, and this stimulatory effect might be considered as one of the evaluation criterion for bioactivity of the materials. However, further investigations are required to identify the mechanisms of the specific stimulatory effects of different ions and ion combinations

5 Conclusions

In vitro study showed that Ca_3SiO_5 ceramics could induce the formation of HA after soaking in SBF, and the mechanism of bone-like apatite formation on Ca_3SiO_5 ceramics was similar to that of the CaSiO_3 ceramics. In addition, Ca_3SiO_5 ceramics could degrade in simulated body environment, and the Si and Ca ions released from Ca_3SiO_5 ceramics at certain concentration range significantly stimulated osteoblasts proliferation. Our results suggest that Ca_3SiO_5 ceramics possessed excellent bioactivity and degradability, and may be used as a bioactive bone defect filling material. However, further in vivo studies are required to explore the applicability of this ceramics as implant materials.

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