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Effect of Varying HAP/TCP Ratios in Tape-cast Biphasic Calcium Phosphate Ceramics on Response In Vitro

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Abstract: The in-vitro response of the biphasic calcium phosphate (BCP) ceramics hydroxyapatite (HAP) and β-tricalcium phosphate (TCP) prepared by tape-casting was investigated. BCP ceramic slurries were prepared to HAP/β-TCP weight ratios of 75/25 (H75-T25), 50/50 (H50-T50), and 25/75 (H25-T75). Additionally, 100% HAP (H100) and 100% β-TCP (T100) were prepared as control. BCP ceramic sheets fabricated by tape-casting were sintered at 1200ºC for 4 h. After sintering at 1200ºC, thin-film X-ray diffraction confirmed that β-TCP in the BCP ceramics was partially transformed to α-TCP; HAP showed no change. Field-emission scanning electron microscope observation showed that the surfaces of H25-T75 and T100 were completely covered with precipitate after 90 d’ immersion in phosphate-buffered saline solution with pH 7.4 at 37ºC. After osteoblast-like (MC3T3-E1) cell cultivation for 7 d, H25-T75 showed a significantly higher cell number than H100, H75-T25, and H50-T50 (Fisher’s PLSD test, p<0.05). These results indicate that the determination of an optimal balance of biological stability and biodegradation rate is important to fabricating BCP ceramics because it directly affects the in-vitro response. Within the limitation of this study, it is concluded that BCP ceramics with a HAP/TCP ratio of 25/75 prepared by a tape-casting technique has high cellular activity.

Key words: Biphasic calcium phosphate, Hydroxyapatite, Tricalcium phosphate, Tape-casting, In vitro response

Introduction

Hydroxyapatite (Ca10(PO4)6(OH)2, HAP) is a well established primary osteoconductive bioceramic. Understanding the biological behavior of HAP is important, and particularly in relation to the measurement of biodegradable properties, because this provides direct means of assessing the effectiveness of bone reconstruction. The low biodegradation rate of HAP associated with inherent biological stability remains a hurdle because it frequently prevents natural bone ingrowth for extended periods. Meanwhile, β-tricalcium phosphate (Ca3(PO4)2, TCP) is a promising candidate because it is more readily dissolved in a biological setting. However, Some authors reported that the higher biodegradation rate of β-TCP may yield insufficient biological stability in vivo1). Therefore, the biphasic calcium phosphate (BCP) ceramics consisted of a mixture of HAP and β-TCP have drawn attention because the BCP may achieve a suitable biodegradation rate as well as biological stability than those of pure HAP or TCP2,3). In general, BCP ceramics have been prepared by mechanical mixing of HAP and TCP powders in desired quantities, or by chemical methods producing calcium-deficient HAP1,4). Precisely controlling the HAP/TCP ratio of BCP ceramics is difficult. We recently developed TCP sheets by tape-casting5). The latter consists of several steps: slurry preparation, vacuum defoaming, and casting. Slurry is mechanically prepared by mixing ceramic powder with a binder chemical during a ball milling process. Tape-casting is potentially attractive as a preparation method for BCP ceramics because it can readily produce the various ratios of BCP. Our latest work indicated that the in-vitro behavior of tape-cast TCP strongly depended upon sintering temperatures, so that sintering temperature is an important factor for successful biomaterial application of tape-cast TCP5). It is well known that the biological performances of BCP ceramics are dependent on HAP/TCP ratios6-9). In the present study, we prepared BCP ceramics by a tape-casting technique. The effect of varying HAP/TCP ratios, specifically 100/0, 75/25, 50/50, 25/75, and 0/100, on the in-vitro response of BCP ceramics was investigated.

Materials and Methods

Materials

BCP powders consisting of a mixture of HAP powder (HAP-
100 sintered product, Taihei Chemical Industries, Osaka, Japan) and β-TCP powder (β-TCP-100, Taihei) were prepared to HAP/β-TCP weight ratios of 100/0 (H100), 75/25 (H75-T25), 50/50 (H50-T50), 25/75 (H25-T75), and 0/100 (T100). BCP aqueous slurries were prepared by mixing 20 g BCP powder, 0.2 g dispersant (AQ-2559, Lion Company, Tokyo, Japan), 4 g double-distilled water (Wako Pure Chemical Industries Limited, Osaka, Japan), and 6 g ethanol (Wako) in an Al2O3 container with one of two different-sized Al2O3 balls (φ = 3 or 5 mm) for 24 h using a planetary ball mill (P5/2, Fritsch Japan Company Limited, Kanagawa, Japan). A binder (2.4 g, HB-500, Lion Company), plasticizer (1 g, PEG#600, Lion Company) and dispersing agent (0.04 g, 1020H, Lion Company) were added to the initially prepared BCP slurry. The resultant slurry was further mixed by ball milling for 2 h. BCP slurry was degassed by a rotary pump for 20–30 min.

Fabrication of BCP ceramic sheets by tape-casting technique

A doctor blade system (DP-150, Sayama Riken Corporation, Saitama, Japan) was filled with the obtained BCP slurry. Tape-casting was done on a batch-process type caster; the casting head and doctor blade traversed over a stationary floating glass slab, thereby discharging slurry onto the surface. The height of the blade was 0.6 mm, and the carrier speed was 60 cm/min. The width of the BCP ceramic sheet was 150 mm. The BCP ceramic sheet was dried at room temperature to remove solvent, and cut to specimen dimensions (10 mm long and 10 mm wide). The thickness of the BCP ceramic sheet was measured with a digital micrometer (CD-20CP, Mitutoyo, Kanagawa, Japan); it was approximately 0.3 mm and very uniform.

BCP ceramic sheets were sintered at a maximum temperature of 1200°C under atmospheric pressure in a furnace (MSFT-1520-P, Nikkato Corporation, Tokyo, Japan); the temperature was increased at a rate of 4°C/min to 1200°C, and hold time was then set to 4 h at 1200°C. Binder burnout was completed at pre-heating temperature of 500°C. After sintering, the apparent porosity of sintered BCP ceramics was measured in double-distilled water (Wako) in an Al2O3 container with one of the two different-sized Al2O3 balls (φ = 3 or 5 mm) for 24 h using a planetary ball mill (P5/2, Fritsch Japan Company Limited, Kanagawa, Japan). A binder (2.4 g, HB-500, Lion Company), plasticizer (1 g, PEG#600, Lion Company) and dispersing agent (0.04 g, 1020H, Lion Company) were added to the initially prepared BCP slurry. The resultant slurry was further mixed by ball milling for 2 h. BCP slurry was degassed by a rotary pump for 20–30 min.

Observation by field-emission scanning electron microscopy (FE-SEM)

Vacuum drying and platinum sputtering on the specimen surface was done. The surface appearance of sintered BCP ceramics was observed under a field-emission scanning electron microscope (JSM-6340F, JEOL, Tokyo, Japan) at an acceleration voltage of 5 kV.

Thin-film X-ray diffraction (TF-XRD)

Sintered BCP ceramics were characterized by TF-XRD (XRD-6100, Shimazu, Kyoto, Japan). The X-ray source was Cu, the power was 40 kV × 30 mA, and the scanning range was 20–40°.

Change in weight of sintered BCP ceramics in phosphate-buffered saline solution (PBSS)

Sintered BCP ceramics (10 mm long × 10 mm wide) were immersed for 90 d in 10 ml of PBSS (D-PBS(−), Wako) with pH 7.4 at 37°C in a teflon-sealed polystyrene bottle. The composition of PBSS is given in Table 1. PBSS was renewed every day to expose samples to fresh solution. The change in weight was determined by weighing the sample before and after immersion in PBSS. Before weighing, samples were dried with a gentle stream of dried air. Change in weight was calculated from the weight difference after 90 d of immersion, and expressed as a percentage (weight change divided by the sample weight before PBSS immersion). Experimental values are the average of three measurements (n = 3). The morphology of the sintered BCP ceramic surface after PBSS immersion was observed by FE-SEM. The crystallographic structure of the precipitates on the surfaces at 90 d was analyzed by TF-XRD which had an incidence angle of θ = 0.5°.

Cell culture of osteoblast-like cells

An osteoblastic cell line, MC3T3-E1, established from newborn mouse calvaria, was obtained from RIKEN Cell Bank (Tsukuba, Japan). Cells were cultured in a minimal essential medium (αMEM) (Gibco, Tokyo, Japan) containing 10% fetal bovine serum (FBS) (Gibco) and 1% antibiotic (penicillin, Gibco) in an atmosphere of 5% CO2 at 37°C. Cells were suspended in the medium at 2 ×104 cells/mL and used for cell cultivation. One milliliter of floating cells was plated onto each of the BCP ceramics, and incubated at 37°C in an atmosphere of 5% CO2 for 7 d.

A cell-counting kit (Dojindo, Kumamoto, Japan) was used for measurement of cell number. After incubation, each BCP ceramic was moved to another well and washed three times using PBSS without Ca2+ and Mg2+ (Gibco) to remove non-adherent cells. Adherent cells were mixed with 1 mL of medium and 100 µL of reagent solution. Absorbance at 450 nm was measured after 1 h of incubation. The number of adherent cells was calculated from the activity of the original cell suspension. The results are the average of five measurements (n = 5). The results were examined with a one-way analysis of variance (ANOVA) and tested using Fisher’s PLSD test for multiple comparison among the means at p = 0.05.

<table>
<thead>
<tr>
<th>Composition</th>
<th>Grams/liter</th>
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<tr>
<td>NaCl</td>
<td>8.00</td>
</tr>
<tr>
<td>KCl</td>
<td>0.20</td>
</tr>
<tr>
<td>NaH2PO4</td>
<td>1.15</td>
</tr>
<tr>
<td>KH2PO4</td>
<td>0.20</td>
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Table 1 Composition of PBSS without organic species.
Results

Figure 1 shows FE-SEM images of the surface appearance of sintered BCP ceramics with varying HAP/TCP ratios. Average grain size increased with decreasing HAP/TCP ratio. The apparent porosity of sintered BCP ceramics with varying HAP/TCP ratios is shown in Figure 2. The apparent porosity of T100 was lower than that of the others. Figure 3 shows the TF-XRD spectra of sintered BCP ceramics with varying HAP/TCP ratios. HAP peaks gradually decreased and shifted to TCP peaks with decreasing HAP/TCP ratio. The spectra of sintered BCP ceramics containing β-TCP confirmed typical α-TCP peaks, though that of HAP showed no change. Figure 4 shows the results of the weight change of sintered BCP ceramics in PBSS. The weight changes of H100 and H75-T25 were negative, –0.72 % and –1.12 %, respectively, and indicated the dissolution of sample after immersion in PBSS. The weight changes of H50-T50, H25-T75, and T100 were positive, at 1.14 %, 2.88 %, and 1.75 %, respectively, indicating the increase of sample after immersion in PBSS. Figure 5a shows FE-SEM photographs of the surface appearances of sintered BCP ceramics after immersion in PBSS for 90 d. Precipitate formed on the surface of H50-T50, H25-T75, and T100; precipitation was not observed on the surface of H100 and H75-T25. The surfaces of H25-T75 and T100 were completely covered with precipitate. In the TF-XRD spectra of T100 after immersion in PBSS for 90d, TCP peaks disappeared, and peaks that could be assigned to apatite were observed as shown in Fig 5b. Figure 6 shows the number of osteoblast-like cells grown on the surface of various BCP ceramics after 7 d of cell cultivation. H100, H75-T25, H50-T50, H25-T75, and T100 had cell numbers of 11.2, 12.6, 13.0, 16.4, and 14.3 × 10⁴, respectively. The BCP ceramics of H25-T75 showed a significantly higher cell number than those of H100, H75-T25, and H50-T50 (p < 0.05). T100 also showed a significantly higher cell number than H100 (p < 0.05).

Discussion
FE-SEM images of the sintered BCP ceramic surface showed that grain size increased with decreasing HAP/TCP ratio (Figure 1). The grain size of T100 was significantly larger than that of the others. This could be explained from the results because the porosity of T100 was much lower than that of the others (Figure 4). Raynaud et al. reported that HAP had a homogeneous microstructure, with an average grain size of about 1 µm. The microstructure of sintered BCP ceramics showed significant differences depending on the composition of HAP and TCP. The authors also reported that BCP ceramics containing a high amount of TCP had coarse grains, though the microstructure of BCP ceramics was close to that of pure HAP, with small grains exhibiting a regular shape (up to 20% by weight of TCP). The microstructural observations of the sintered BCP ceramic surface from our study agreed with those reported by Raynaud et al. In TF-XRD spectra, the index of the two phases, HAP and TCP, shifted the HAP/TCP ratio. It was confirmed that the ratio of the intensities of the most intense diffraction peaks of the HAP phase compared with those of the most intense diffraction peaks of β-

![Graph](image)

Figure 4. Relationship between weight change of sintered BCP ceramics and HAP/TCP ratio after 90 d of immersion in PBSS.

![Images](image)

Figure 5. (a) FE-SEM images showing surface appearance of sintered BCP ceramics after 90 d of immersion in PBSS; (b) TF-XRD spectra of T100 after 90 d of immersion in PBSS. This indicates apatite peaks at 2θ values of 25.72º and 31.60º.

The weight change of the BCP ceramics after 90 d of immersion ranged from –1.12 % to 2.88 %. As the HAP/TCP ratio decreased, the weight increased, and sign reversal from “–” to “+” was observed.
Na$_2$HPO$_4$. They reported that the weight of TCP increased after buffered Ringer’s solution (PBRS) composed of NaCl, KCl, and influenced by the composition of PBSS (Table 1). Kohri et al.$^1)$ (Figure 5a) observed that many apatite crystals were incorporated in apatite microcrystals on the surface of BCP ceramics after PBSS immersion was noted (Figure 5a). This was attributed to apatite, and was probably the surface of BCP ceramics after PBSS immersion decreased, the appearance of precipitated crystal on the surface of BCP ceramics after PBSS immersion was noted (Figure 5a). This was attributed to apatite, and was probably influenced by the composition of PBSS (Table 1). Kohri et al.$^1)$ investigated the in-vitro stability of BCP ceramics in phosphate-buffered Ringer’s solution (PBRS) composed of NaCl, KCl, and Na$_2$HPO$_4$. They reported that the weight of TCP increased after immersion in PBRS containing many kinds of supersaturation. The authors argued that fine needle-like apatite crystals were observed on the surface of BCP and pure β-TCP after 12 wk of immersion in PBRS, whereas HAP showed no change. Therefore, we assumed that the increase in weight of H50-T50, H25-T75, and T100 was largely due to crystal deposition on the surfaces of samples. Apatite precipitation in biological fluids is an important determinant in the bioactivity of BCP ceramics. In particular, the enrichment of calcium and phosphorous ions in the microenvironment seems to promote apatite formation. In addition to these, other supersaturations (e.g., Mg$^{2+}$, Na$^+$, CO$_3^{2-}$) from biological fluids are incorporated in apatite microcrystals on the surface of bioceramics. The PBSS used in the present study contained PO$_4^{3-}$ ions, which originated from Na$_2$HPO$_4$ and KH$_2$PO$_4$ reagents without Ca$^{2+}$, Mg$^{2+}$, or CO$_3^{2-}$ ions, in comparison with physiological buffered solution (e.g., Hanks’ balanced salt solution)$^{10}$. Many apatite crystals on the surfaces of H25-T75 and T100 after immersion in PBSS were observed. This indicates that the formation of apatite on the surface of calcium phosphates is related to its biodegradation, rather than precipitation of the inorganic components in PBSS. LeGeros et al.$^3)$ reported that the abundance of apatite microcrystals on BCP surfaces was related to the HAP/β-TCP ratio of BCP ceramics: the lower the ratio, the more abundant the crystals. This is attributed to the preferential dissolution of the more soluble β-TCP component, and is in agreement with our PBSS examination results. It could be concluded that there were many apatite formations on the surfaces of the samples with a high amount of TCP (e.g., H25-T75 and T100) because of its rapid dissolution.

Only H25-T75 showed higher cell proliferation than pure HAP or TCP (Figure 6); other reports agree with the cellular responses in our study.$^{18,7}$ Yamada et al.$^7$ investigated the osteoclastic resorption of BCP ceramics with various HAP/TCP ratios prepared by an aqueous precipitation method. The authors reported that osteoclastic resorption was highest for BCP ceramics with a HAP/TCP ratio of 25/75 compared with those with HAP-rich samples. This implied that the biological stability achieved with incorporated 25% HAP of H25-T75 is crucial in cellular activities, as well as its higher biodegradation rate associated with 75% TCP. The determination of an optimal balance of biological stability and biodegradation rate is important to fabricating BCP ceramics because it directly affects the in-vitro response (e.g., formation of apatite crystals and cellular activity).

Within the limitation of this study, it is concluded that BCP ceramics with a HAP/TCP ratio of 25/75 prepared by a tape-casting technique has high cellular activity.

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References


