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In situ formation of biphasic calcium phosphates and their biological performance *in vivo*

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Abstract

The co-precipitation technique has been applied to synthesize biphasic calcium phosphate (BCP). After annealing at 900 °C for 24 h, hydroxyapatite (HAp) and β-tricalcium phosphate (β-TCP) were obtained as a single phase at 1.67 and 1.5 Ca/P ratios, respectively. Between these two extremes, a whole range of BCP preparations could be synthesized by using this technique with an accurate control of starting reactants. The biological performance of BCP granulates with a specific content of 62% HAp and 38% β-TCP was investigated. After immersion in Hanks' balanced salt solution (HBSS) for 1 week, a precipitation started to be formed with individual small granules on the specimen surface. An MTT assay indicated that BCP granulates have no cytotoxic effects on MG-63 cells, and that they have good biocompatibility. An implantation experiment in mouse skulls revealed that BCP granulate provides a strong positive effect on bone formation *in vivo* in mice.

Keywords: Co-precipitation; Biphasic calcium phosphates; Granulates; Biocompatibility; Bone regeneration

1. Introduction

Calcium phosphate-based materials have received much attention as bone graft substitutes in dental and orthopedic reconstructive medicine because of their excellent biocompatibility, bioactivity, and osteoconduction characteristics [1–4]. Specifically, the most widely used calcium phosphate-based materials are hydroxyapatite [HAp, $Ca_{10}(PO_4)_6(OH)_2$] and β-tricalcium phosphate [β-TCP, $Ca_3(PO_4)_2$]. Despite their favorable biological properties, both materials have a number of drawbacks that reduce their clinical performance. *In vivo* and *in vitro* dissolution experiments [5,6] have indicated that the dissolution of HAp in the human body after implantation is too low to achieve the optimal formation of bone tissue. On the other hand, β-TCP shows fast release of Ca^{2+} and PO_4^{3-} ions when exposed to physiological fluids. This fast dissolution profile drastically reduces the surface area available for bone cell proliferation and, therefore, its application in the clinical setting is limited. An optimum bioresorbability was found when appropriately mixing both phases to give biphasic calcium phosphates (BCPs) [7,8]. Several researchers have recently attempted to develop BCPs comprising HAp and β-TCP as well using various synthetic routes, such as the blending of different calcium phosphates in solid state reactions [9], precipitation [6], liquid mix techniques [10], treatment of natural bone [8], spray pyrolysis [11], microwave [12], and combustion processing [13]. In addition to compositional requirements, a porous structure is necessary for improving bone regeneration as scaffolds for bone substitutes [14]. Therefore, several studies [15–17] have reported the preparation and evaluation of porous BCP scaffolds with 3 dimensional pore networks using different methods. Raynaud et al. [15] found that HAp/β-TCP dense scaffolds with 88/22 and 72/28 wt% ratios were degraded after 60 days of immersion, with the preferential dissolution of β-TCP grains. Lin et al. [16] stated that seed HAp was a prerequisite for precipitating an apatite layer on a more soluble second phase, such as TCP. Sanchez-Salcedo et al.

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[17] reported structural changes to BCP scaffolds after immersion in simulated body fluid (SBF) under different conditions, such as static and orbital stirring.

Recently, instead of scaffolds, granulate systems [18–21] for the delivery and application of drugs have been claimed to have enhanced bioavailability, predictable therapeutic response, greater efficacy and safety, and controlled and prolonged drug release time. Therefore, the development of granulate systems of calcium phosphate-based drugs with controlled drug release kinetics has increasingly become an important area of research, in view of the prevalent rates of infection in bone and dental surgery [20,21]. To date, several studies have reported the development of HAp granulates with irregular or spherical geometry using various techniques [18–21]. Various studies have been conducted using different polymers as matrices for the preparation of HAp microbeads [22–24]. For this reason, it could be valuable to find a simple and systematic way to produce BCP granulates with different proportions, depending on the characteristics required for the specific application.

With the aim of fabricating BCP granulates and *in vivo* culture of bone tissue engineered constructs, this study focused on the *in situ* preparation of BCP powders with precise proportions of HAp and β-TCP phases, based on the input Ca/P molar ratio, using the co-precipitation method. The success of this task would suggest the possibility of obtaining supplemental amounts of BCP powder with precise ratios of two components, depending on the characteristics required for the specific application, by controlling the input Ca/P molar ratio of starting reactants. The second aim was to prepare the BCP granulates using scaffolds synthesized from BCP powders with an appropriate ratio of HAp/β-TCP. Biocompatibility of the materials was then reported through biological *in vitro* and *in vivo* experiments using normal MG-63 cells and critical-sized calvarial bone defects in mice, respectively.

2. Materials and methods

BCP powders were synthesized by the co-precipitation method using reagent-grade calcium nitrate tetrahydrate [$Ca(NO_3)_2{\cdot}4H_2O$] and di-ammonium hydrogen orthophosphate [$(NH_4)_2HPO_4$] as the starting materials. The various input Ca/P molar ratios employed to prepare the different contents of HAp and β-TCP phases are detailed in Table 1. An appropriate amount of $Ca(NO_3)_2{\cdot}4H_2O$ was dissolved in deionized water by vigorously stirring at a rate of 1000 rpm. Different amounts of $(NH_4)_2HPO_4$ solution were slowly added individually to attain the Ca/P molar ratios shown in Table 1. The pH of the mixed solution was maintained at 10 by the addition of ammonium hydroxide (NH_4OH) solution. The solution was stirred constantly for 24 h by a mechanical stirrer, allowing the reaction to complete. The precipitated suspension was discharged from the reactor and allowed to settle for 24 h for the maturation of the precipitate. After 24 h, the precipitates were separated using vacuum filtration and were dried at 100 °C for 24 h in a drying oven (SFC-301, Dongwon Scientific Systems, Korea). The dried cakes were crushed to a fine powder, sieved through a mesh size of 200 (>75 μm) and used for the characterization and preparation of BCP scaffolds.

Table 1
Ca/P molar ratio of samples prepared and expected and measured values showing contents of HAp and β-TCP.

HAp/β-TCP	Ca/P ratio					
	1.500	1.534	1.568	1.602	1.636	1.670
Expected value	0:100	20:80	40:60	60:40	80:20	100:0
Measured value	0:100	22:78	43:57	62:38	78:22	100:0

Macroporous BCP scaffolds with 3 dimensional interconnected pore structures were prepared by freeze-drying as previously reported by our group [25]. Aqueous slurries of the as-prepared BCP powder, with poly (methyl methacrylate) (PMMA) loading in the range of 30–50 vol%, were prepared using a small amount of ammonium polymethacrylate anionic dispersant (Darvan C, R.T. Vanderbilt Co., USA), polyvinyl alcohol (PVA, Sigma–Aldrich, Germany), glycerol (Sigma–Aldrich, Germany), Dynol 604 (Air Products, USA), and ball-milled in plastic jars for 24 h with zirconia balls as the medium. After ball-milling, well-dispersed and stable slurries were obtained. The as-received slurries were de-aired, and then poured into a cylindrical polyethylene mold (Ø 30 mm × *h* 15 mm). The freezing of the slurries was carried out in a cold chamber, where the temperature was −15 °C. The completely frozen samples were stripped out of the mold, and quickly transferred into the vacuum chamber of a freeze drier (Labconco 77540, Western Medics, USA), and the frozen samples were dried under a reduced vacuum for 24 h. As a result of the sublimation of the ice crystals, porous green bodies were obtained. The green compacts were placed in an alumina crucible and sintered at 1200 °C for 2 h in air. The obtained BCP scaffolds were crushed for granulation at the appropriate size.

An X-ray diffractometer (Rigaku D-max IIIV, Japan), at 30 kV and 20 mA with a scanning speed of 4°/min, was adopted to identify the phases of powders synthesized with different input Ca/P ratios. Diffraction patterns were prepared for each control sample, and the intensity ratio was calculated based on two unobscured peaks from each phase. The relative intensity ratio (RIR) of HAp/β-TCP was estimated using the intensity peaks of (2 1 1) and (0 2 1 0) of HAp and β-TCP, respectively, according to the formula $RIR = I_{\beta\text{-TCP}}/(I_{HAp} + I_{\beta\text{-TCP}})$ [26]. Various functional groups present in the prepared calcium phosphate powders were identified by Fourier transform infrared spectroscopy (FTIR) (Nicolet Magna IR 560, USA). Here 1 wt% of the powder was mixed and ground with 99% KBr. Tablets of 10-mm diameter were prepared for FTIR measurement by pressing the powder mixture at a load of 5 tons for 2 min and the spectrum was taken in the range of 400–4000 cm^{-1} with resolution 4 and 128 times scanning. The Ca/P ratios of the synthesized powders were determined using an energy-dispersive spectrometer (EDS; Noran, USA) and an inductively coupled plasma-optical emission spectrometer (ICP-AES, Model ICPS-7500; Shimadzu, Japan). The porosity of the sintered BCP scaffolds was determined by the Archimedes method, with deionized water as the immersion medium. Microstructural observations of the sintered samples

were conducted using a scanning electron microscope (SEM, JSM-5900 LV; JEOL, Japan).

Hanks' balanced salt solution (HBSS), an extracellular solution with an ionic composition similar to human blood plasma, was used as the supporting solution for the BCP granulates in an *in vitro* test. The simulated solution consisted of 8.00 g NaCl, 0.35 g $NaHCO_3$, 0.40 g KCl, 0.06 g KH_2PO_4, 0.10 g $MgCl_2{\cdot}6H_2O$, 0.14 g $CaCl_2$, 0.06 g $Na_2HPO_4{\cdot}2H_2O$, 0.06 g $MgSO_4{\cdot}7H_2O$, and 1.00 g glucose in 1000 ml distilled H_2O and had an initial pH of 7.4. The BCP granulates were immersed in 50 ml of HBSS without organic species (pH 7.4), at 37 °C in a Teflon-sealed polystyrene bottle for 1, 2, and 4 weeks. The solutions were refreshed every 2 days to expose the samples to fresh solutions. Following immersion for 1, 2, or 4 weeks, the BCP granulates were rinsed with double-distilled water to remove residual HBSS, and then immediately dried in a vacuum desiccator at 21 °C. The changes in the morphology of the BCP granulates following different periods of immersion in HSBB were analyzed using a scanning electron microscope (SEM, JSM-5900 LV; JEOL, Japan).

The cytotoxicity of the synthesized BCP granulates was assessed using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay [27]. The % cellular viability was determined with coherent dilutions of the samples following the standard testing protocol (ISO 10993-5) [28]. MG-63 cells (a human osteosarcoma cell line) were treated with BCP granulates for 24 h. MTT solution was added to each well (final concentration ~50 mg/ml). The cells were incubated at 37 °C for 3 h and dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. The absorbance of the samples was read at 570 nm in an ELISA reader (E-MAX Molecular Devices) against a reference wavelength of 490 nm.

The BCP granulates were evaluated qualitatively *in vivo* using critical-sized calvarial bone defects in adult (6-week-old) severe combined immunodeficient (SCID) mice. The surgical procedures were performed in aseptic conditions under general anesthesia. Briefly, a linear incision (1 cm long) was made on the left side of the skull and the scalp was dissected to expose the calvaria. The periosteum was carefully peeled off and 2 lateral 4-mm-wide calvarial bone defects were performed in each animal using a 2-mm-diameter trephine bur using a slow-speed dental drill. To avoid tissue damage due to overheating, 0.9% saline was dripped onto the contact point between the bur and bone and great care was taken to avoid dura mater injury. BCP granulates with an appropriate size of between a few tenth and a few hundred micrometers were mixed with collagen extemporaneously in order to obtain an easy-to-handle paste. One defect was then used for filling with BCP granulates while the contralateral site was filled only with collagen, as a control group.

The animals were euthanized after 4 and 8 weeks by exposure to hyperbaric carbon dioxide. At each time point, the skulls were harvested and fixed in 4% paraformaldehyde for 12 h. Representative skulls stained with 1 mg/ml alizarin red for 12 h to view bone formation. Calvaria were X-rayed using a volumetric CT scanner (NFR-MXSCAN-G90: NanoFocusRay, Iksan, Korea) at 50 kVp, 65 μA, and 470-ms per frame and then decalcified overnight with decalcifying solution (10% EDTA). Samples were then trimmed, processed, and embedded in paraffin wax. A micro-CT image of mouse calvaria was taken using the CT scanner without having to move the animal's head position. Paraffin-embedded samples were sectioned at 10-μm thickness with a microtome. Sections were floated in a water bath at 40 °C, placed on poly-L-lysine-coated polysine microscope slides and baked at 37 °C overnight. For hematoxylin and eosin (H&E) staining, sections were dewaxed in xylene and rehydrated in ethanol baths. The sections slides were stained with H&E viewed under an optical microscope for histological observation.

3. Results

The thermal treatment applied to samples prepared with input Ca/P molar ratio of 1.602, to investigate the reaction between the constituting calcium phosphates, caused a modification in their proportions. X-ray diffraction (XRD) spectra of calcium phosphate powders, which were synthesized at input Ca/P molar ratio of 1.602, showed annealing at different temperatures as shown in Fig. 1. Up to 600 °C, most of the lines corresponding to HAp matched the standard JCPDS (File No. 74-0566) and the spectrum contained peak broadening, which could be due to a glassy state of the phosphate matrix. These poor crystalline degrees are a typical characteristic of apatite synthesized by a wet method [10]. Further treatment at higher temperatures caused the peak height of powders to increase substantially, with an associated narrowing of the peak corresponding to an increase in crystallinity for phases including HAp and β-TCP, respectively. It can be noticed that HAp was stable up to about 600 °C, after which dehydroxylation occurred leading to the formation β-TCP. After annealing at 900 °C, the final product shows biphasic calcium phosphates, and the HAp/β-TCP ratio was close to 62:38. Further heat treatment at 1000 °C provided a slight

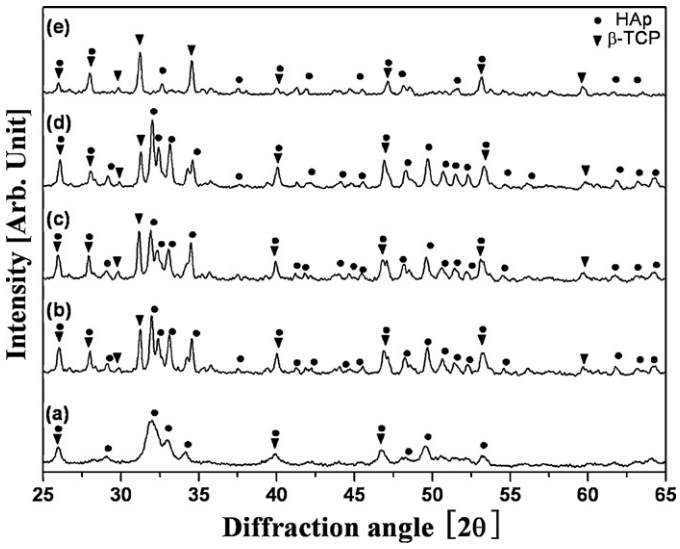

Fig. 1. XRD patterns of powders calcined at various temperatures; (a) 600 °C, (b) 700 °C, (c) 800 °C, (d) 900 °C, (e) 1000 °C.

increase in its crystallinity and in the β-TCP phase, but no evidence of the HAp phase. On the basis of these results, we focused on the samples annealed at 900 °C for 24 h due to the fact that it was the appropriate temperature at which the BCP with defined proportions of each of the calcium phosphates could be obtained. The effect of input Ca/P molar ratio on the variation in relative intensity of different phases after annealing at 900 °C for 24 h is shown in Fig. 2. Over the whole range of compositions, when the input Ca/P molar ratio was 1.67, the peaks corresponding to HAp were dominant with no evidence of other phases. At the other extreme, β-TCP was produced at an input Ca/P molar ratio of 1.5. Intermediate ratios provide BCPs with defined proportions of each of the calcium phosphates (HAp and β-TCP). Similar observations were explained in the literature, confirming the synthesized powders to be BCP when the input Ca/P molar ratios were in the range of 1.5–1.67 [10].

FTIR spectra of the powders produced with different input Ca/P molar ratios are shown in Fig. 3. The overall spectra appear to have mainly two modes corresponding to characteristic PO_4^{3-} and OH^- groups (Fig. 3). The bands at 570 cm^{-1} and 600 cm^{-1} were assigned to the O–P–O bending mode, 1090 and 1040 cm^{-1} to the asymmetric stretching mode and 962 cm^{-1} to symmetric stretching vibration [29]. The intensity of absorption of these bands was not much changed with an increase in the input Ca/P ratio. However, the intensity of absorption for the peak at 3600 cm^{-1} assigned to the OH^- group decreased with decreasing input Ca/P molar ratio. This progressive reduction of the hydroxyl group could be attributed to transition from HAp to β-TCP when decreasing the input Ca/P molar ratio. There is no

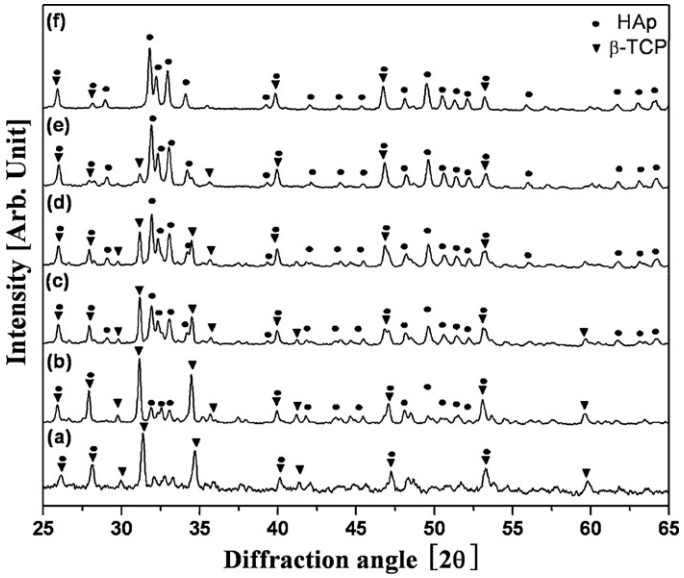

Fig. 2. XRD patterns of powders synthesized with different input Ca/P ratios; (a) 1.500, (b) 1.534, (c) 1.568, (d) 1.602, (e) 1.636 and (f) 1.670. The heat treatment was carried out at 900 °C.

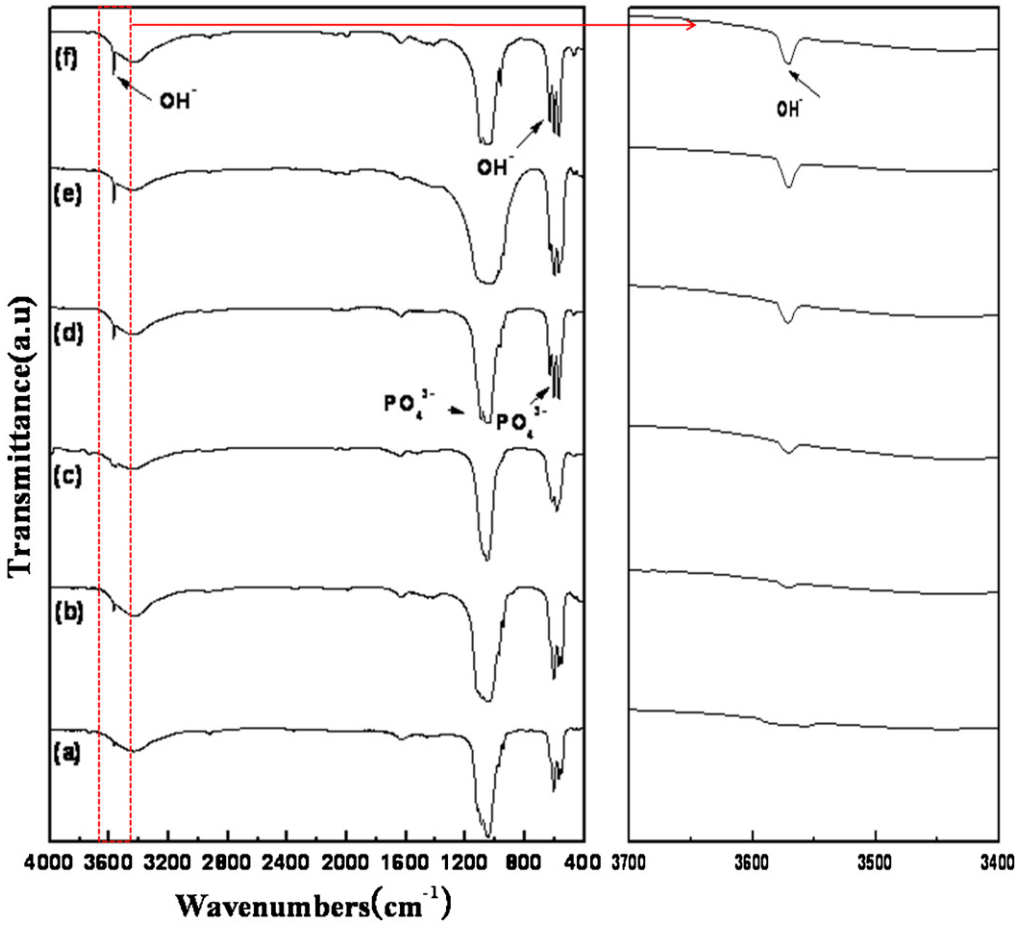

Fig. 3. FTIR spectra of powders synthesized with different input Ca/P ratios; (a) 1.500, (b) 1.534, (c) 1.568, (d) 1.602, (e) 1.636, and (f) 1.670.

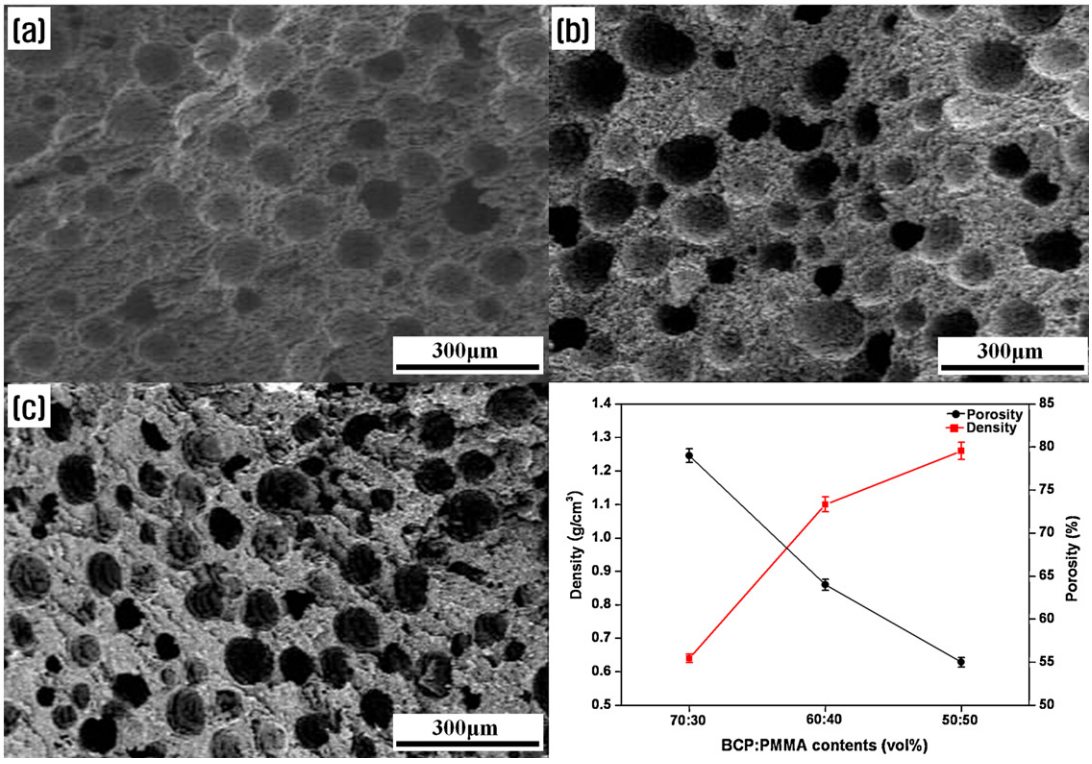

Fig. 4. SEM micrographs, density and porosity of porous BCP scaffolds with BCP:PMMA contents; (a) 70:30, (b) 60:40, (c) 50:50 (vol%).

clear evidence for the absorption peak at 880 cm^{-1} assigned to the P–O–H vibration in the HPO_4^{2-} group in these samples, as could be detected for samples with a state of calcium deficient HAp. These results are in good agreement with XRD results presented in Figs. 1 and 2. The FTIR and XRD data demonstrate a gradual variation in the HAp/β-TCP ratio starting from β-TCP as a single phase and ending with an HAp phase.

From an application point of view, the success of these biphasic products as bone substitutes lies in the combination of a higher solubility of β-TCP and the superior biocompatibility of HAp. To date, the 60/40 (in weight ratio of HAp/β-TCP) BCPs are the best known materials [30,31]. However, different applications in diverse parts of the organism require different resorption times and this depends on the relationship between HAp and β-TCP in these mixtures. With regard to the application to bone substitutes, porous BCP granulates were prepared by using the obtained (62/38) BCP powders. Before preparing the BCP granulates, the macroporous BCP scaffolds with appropriate pore size and interconnecting pore structure were fabricated by the freeze-drying method as demonstrated in earlier experimental procedure. Fig. 4 shows the microstructures, density, and porosity of the BCP scaffolds, prepared using slurries with different PMMA contents in the range of 30–50 vol%. As the content of BCP powders in the slurry increased, the porosity and density of scaffolds decreased and increased, respectively. These phenomena can be attributed to the fact that the pores become interconnected with denser and thicker pore walls on increasing the BCP content of the slurry

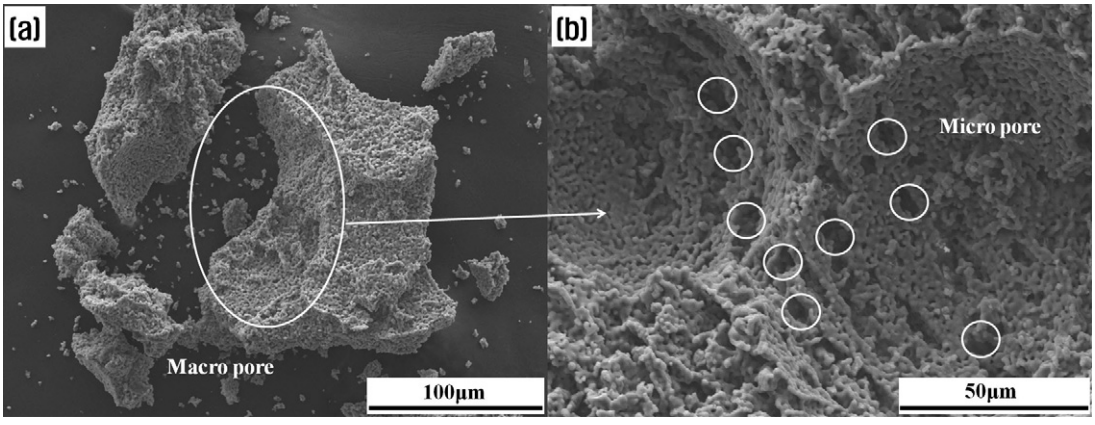

Fig. 5. Scanning electron microscopy observation of (a) individual BCP granulates, (b) high magnification (×700). The large macro-sized pore on the free surface of granulate consisted of many micro-sized (<20 μm) pores.

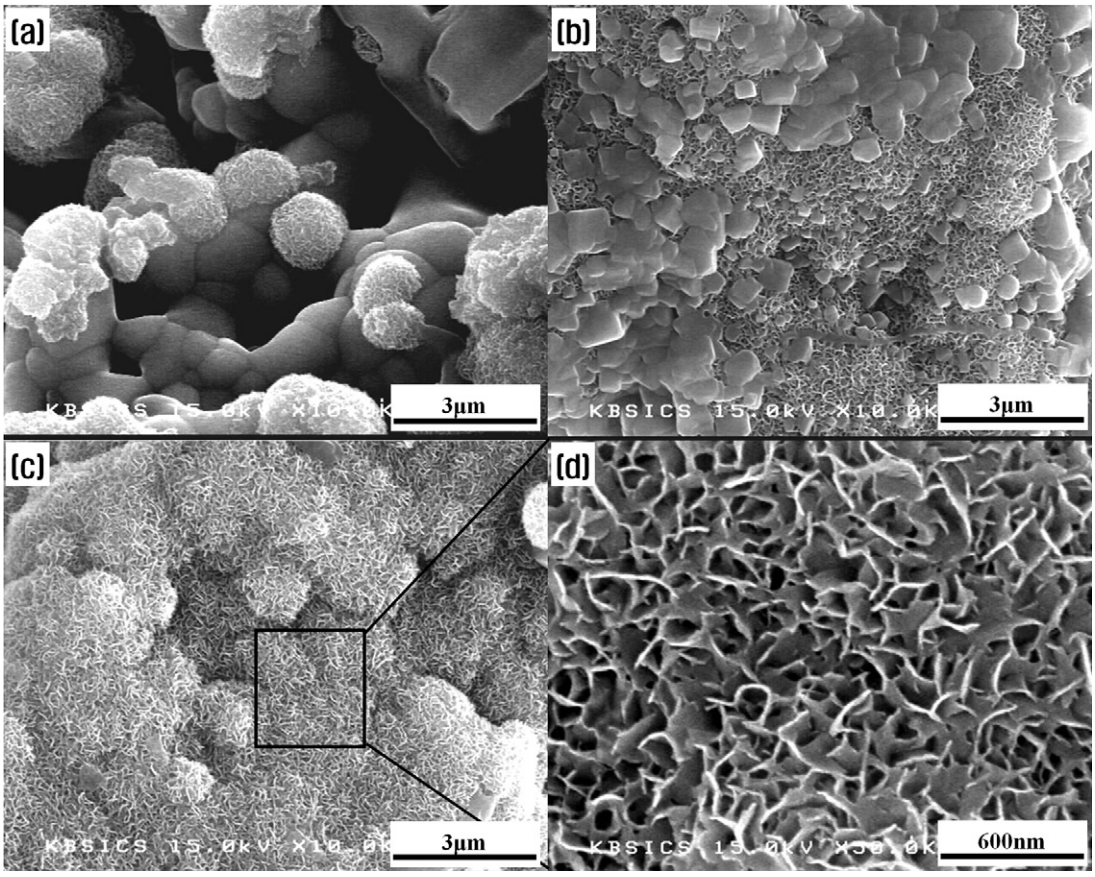

Fig. 6. SEM morphologies of BCP granulates after immersion in HBSS for (a) 1 week, (b) 2 weeks, and (c) 4 weeks, (d) high magnification of (c).

[32]. Porosity is, in general, based on the presence of open pores, which is in turn related to properties such as permeability and surface area of the porous structure. Therefore, high porosity usually provides a high surface area/volume ratio, and thus favors cell adhesion to the scaffold and promotes bone tissue regeneration. To obtain BCP granulates with an appropriate size, the BCP scaffolds fabricated by the freeze-drying method were crushed in the mortar. As shown in Fig. 5,

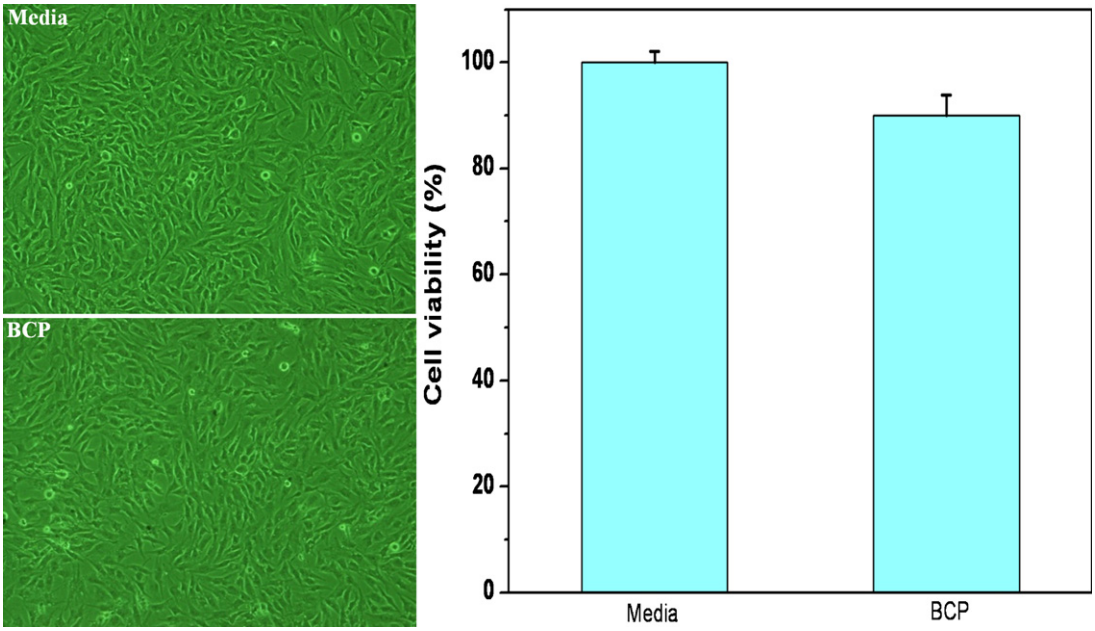

Fig. 7. Effect of BCP on cell viability in human osteosarcoma MG-63 cells. Cells were incubated with BCP granulates for 24 h. Cell viability was determined by light microscopy and MTT assay. Values are means ± SD of three independent experiments.

each BCP granulate consists of macropores with a diameter of approximately a few hundred micrometer and micropores with a diameter of less than 10 μm.

Fig. 6 shows the typical features of precipitation on specimens after immersion in HBSS for 1 week, 2 weeks, and 4 weeks. After immersion in HBSS for 1 week, the precipitation starts to be formed with individual small granules on each BCP granulate. With increase of soaking time, the granules gradually grow together to form a dense layer on the overall BCP granulate surface. At higher magnification, images revealed that each granule consists of a large number of tiny flake-like crystals [Fig. 6(d)]. These results are consistent with previous experiments in which observation with transmission electron microscopy (TEM) reveals the formation of a nanocrystalline apatite phase after immersion in SBF [17]. However, the amount and crystallinity of the newly formed apatite phase are dependent on the test conditions. An MTT assay was used to evaluate the cytotoxicity of the tissue engineering materials, and the cell viability value can provide an indication of cell growth and proliferation on different test groups. In this experiment, the human osteosarcoma MG-63 cells were treated with BCP for 24 h and are shown in Fig. 7. The results show that treatment with BCP exhibited no cytotoxic effects on the MG-63 cells; BCP treatment did not result in cell necrosis and detachment from the culture plates, as viewed by light microscopy.

Defects with oval size were filled with two types of implant material. As shown in Fig. 8, one site was filled with collagen, which is a group of naturally occurring proteins. BCP granulates mixed with collagen were inserted on the opposite site. During the *in vivo* experiment, mice remained in good health and did not show any wound complications. At explantation, no inflammatory signs or adverse tissue reactions were seen. After 4 weeks *in vivo*, X-ray analysis showed that the site implanted with BCP granulates produced, to some extent, high-density mineralization in the area of the defect, whereas the area covered with collagen was still not connected. These features were more obvious after 8 weeks of implantation, as shown in Fig. 8. After 8 weeks of implantation, the area inserted with BCP granulates had formed sufficient bone to span most of

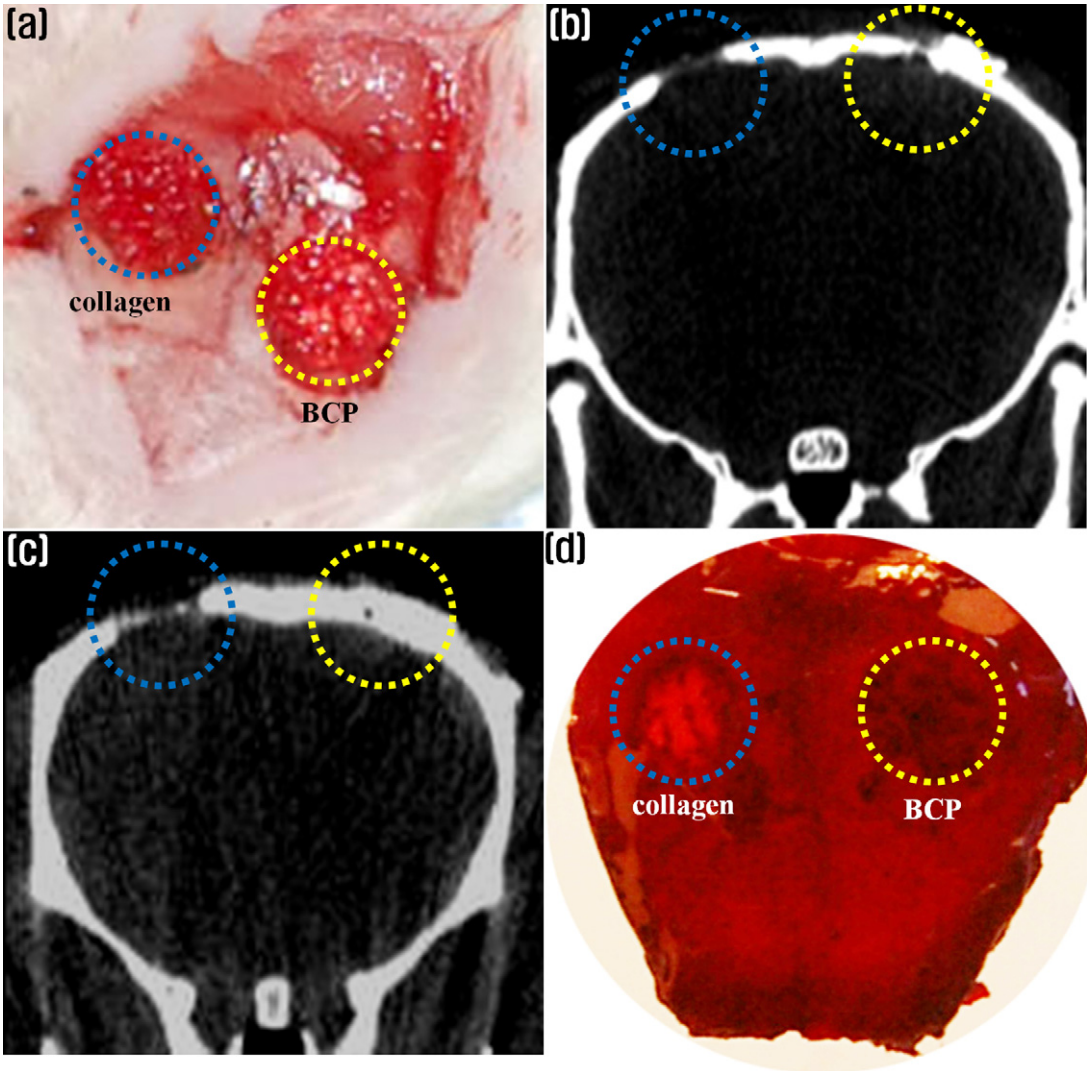

Fig. 8. Photography image of (a) the mouse skull after implantation. Micro-computed tomography images of a mouse skull after (b) 4 weeks and (c) 8 weeks. Alizarin red analysis of a mouse skull also showed (d) after 8 weeks.

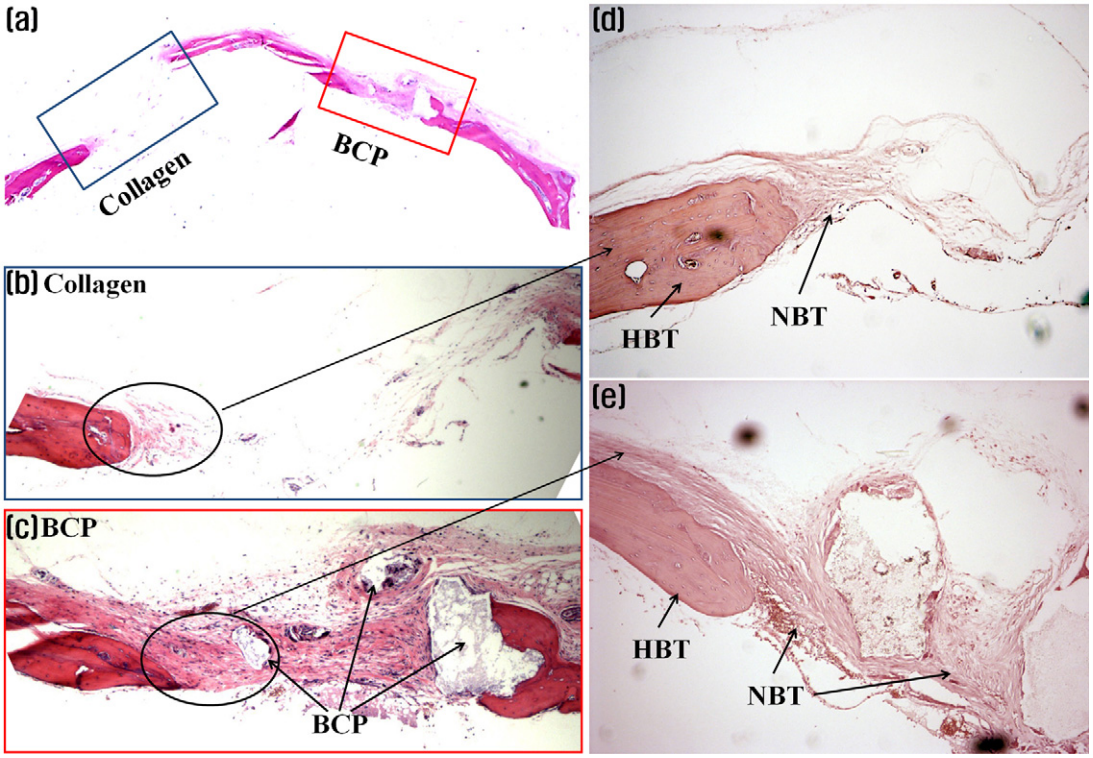

Fig. 9. Histological analysis of (a) the mouse skull after 8 weeks, (b) implanted site with collagen, (d) high magnification of (b), (c) implanted site with BCP granulates, (e) high magnification of (c). HBT, host bone tissue; NBT, new bone tissue.

the defect, even though the site implanted with collagen had just started to heal with new generation bone. Alizarin red staining localized bone formation to areas of mineralization onto the implanted site with BCP granulates, as opposed to the area filled with collagen materials. H&E staining showed characteristic bone morphology on the implanted sites (Fig. 9). After 8 weeks, the appearance and shape of the site implanted with collagen showed no obvious change, although slight connection between the material and surrounding tissue was observed. On the other hand, an obvious connection between material and tissue occurred in proximity to BCP granulates in test sites. On the basis of Fig. 9(e), newly formed bone was observed in test sites with a persistence of BCP granulates. These *in vivo* results displayed osteoconductive properties of materials after 4 weeks healing and absence of foreign body reaction around BCP granulates.

4. Discussion

Using the co-precipitation method, BCPs with specific HAp/β-TCP content were designed and synthesized, by controlling the input Ca/P molar ratio in the precursor solution. The present experiments suggest that this synthetic method could make it possible to easily prepare not only single phase HAp and β-TCP but also biphasic calcium phosphates over a wide range of compositions, as has been schematized in Fig. 10. This graph represents the weight content of HAp and β-TCP (y axis) for different initial Ca/P molar ratios (x axis) annealed at 900 °C. The

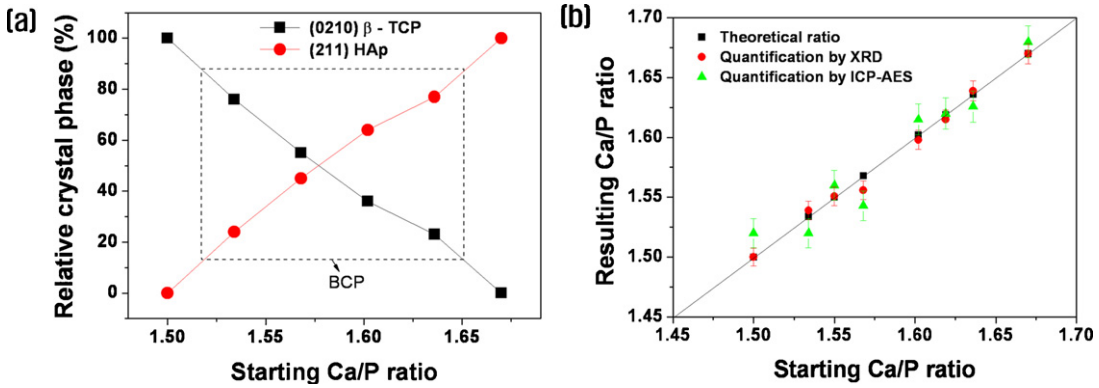

Fig. 10. XRD patterns of as-calcined powders; (a) relative crystal phase with different input Ca/P ratio, (b) correlation of the Ca/P ratio determined by chemical analyses and XRD results.

box in Fig. 10(a) shows the formation and stability domain of biphasic material within the annealing temperature range considered. Fig. 1 shows the transformation process of an intermediate composition (input Ca/P molar ratio of 1.602), in which the biphasic material evolves towards a higher HAp content. With increased annealing temperature, the initial biphasic material obtained after annealing at 700 °C turns into a β-TCP single phase at an annealing temperature of 1000 °C. In this temperature range, at an annealing temperature of 900 °C, the obtained biphasic materials have a similar composition ratio of HAp/β-TCP (62:38) to what would be expected.

FTIR characterization shows the progressive disappearance of the stretching mode and vibrational mode of the hydroxyl group and the substitution of the characteristic bands of the PO_4^{3-} of hydroxyapatite for those attributable to β-TCP when decreasing the input Ca/P molar ratio [10]. We have studied these samples in more detail, as depicted in Fig. 10(b), in order to determine the feasibility of this synthetic method, i.e., the agreement of the initial Ca/P molar ratios in the precursor solutions with those deduced from the quantity of HAp and β-TCP in these biphasic materials and their correspondence with those obtained by ICP. As regards the as-prepared biphasic materials, this method shows a reasonable accordance between the input Ca/P molar ratio in the solution, expressed as the theoretical quantity of HAp and β-TCP in the mixture, and that obtained by this method as shown in Fig. 10(a).

As for bioactivity, precipitates started to be formed at individual BCP granulates after immersing in HBSS for 1 week. These results are consistent with previous experiments [17] in which observation with TEM reveals the formation of a nanocrystalline apatite phase onto BCP scaffolds after 45 days in SBF. However, our present experiments show that the incubation time for producing newly formed apatite onto the specimen is faster than the previous reports. This could be explained by the fact that the formation of apatite on a specimen during immersion in SBF solution would be dependent on the specific surface area of specimen. On the basis of these results, the BCP granulates could be applied as a replacement for BCP scaffolds for quick response healing, as BCP scaffolds have a greater retention time for producing the apatite phase in implantation operations.

As for biocompatibility, MTT assays indicate that BCP granulates have no cytotoxic effects on MG-63 cells, and have good biocompatibility. More specifically, BCP granulates were implanted as bone substitute materials to reproduce bone structure in defect areas created in the skull bone of mice. During *in vivo* test, the implant place showed a smooth surface and no inflammatory response. This indicates that BCP granulates could be suitable substrates for Fcell attachment and proliferation as bone substitute materials. After 8 weeks *in vivo*, a significant increase in newly formed bone was revealed on the area implanted by BCP granulates (62% HAp and 38% β-TCP), which indicates powerful bone regeneration ability. In summary, implantation experiments in mouse skulls in the present study have revealed that BCP granulate is more effective for new bone generation than collagen implant materials.

5. Conclusion

This study demonstrates that the co-precipitation method is an effective technique for preparing BCPs whose content in β-TCP and HAp can be precisely determined from the input Ca/P molar ratio in the precursor solutions. After annealing at 900 °C for 24 h, HAp and β-TCP were obtained as a single phase at 1.67 and 1.5 of input Ca/P molar ratios, respectively. Between these two extremes, a whole range of BCPs were synthesized with an accurate control of starting reactants. BCP granulates with specific content of 62% HAp and 38% β-TCP were successfully prepared by crushing the scaffolds fabricated using the freeze-drying method. After immersion in HBSS for 1 week, precipitation started at individual small particles on the BCP granulates. With increases in the soaking time, the particles gradually grew together and formed a dense layer on the specimen surface. From the *in vitro* cell culture study, it was observed that BCP granulates have no cytotoxic effects on MG-63 cells and show good biocompatibility. Moreover, *in vivo* implantation experiments in mouse skulls revealed that BCP granulates provide a strong positive effect on mice, showing bone regeneration *in vivo*. Furthermore, BCP granulates have been shown to be more effective for new bone generation than collagen implant materials.

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