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Synthesis and characterization of biphasic calcium phosphate ceramics using a sponge coating method

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Biphasic calcium phosphate (BCP) bone grafts were successfully synthesized using the 45 ppi polyurethane sponge coating method. XRD results revealed that the BCP scaffolds were mainly composed of hydroxyapatite and β-tricalcium phosphate (β-TCP). As the number of BCP coatings increased from 1 to 5, the pore size and the wall size decreased from 480 ± 93 μm to 306 ± 120 μm and increased from 104 ± 25 μm to 186 ± 40 μm, respectively. The BCP scaffolds coated once, twice, three times, four times and five times, exhibited average cell viability of 106%, 109%, 114%, 107%, and 93%. The BCP scaffolds showed no evidence of causing cell lysis or toxicity. In addition, the cell proliferation results suggested that L-929 cells adhered well to the BCP scaffolds and proliferated continuously with increasing time, indicating that the BCP powders are highly applicable to the synthetic bone grafts.

Key words: Hydroxyapatite (HP), β-tricalcium phosphate (β-TCP), Biphasic calcium phosphate (BCP), Scaffold, Cytotoxicity.

Introduction

Autografts are known to be the most ideal for direct extraction of bone from other parts of the patient and transplantation to the site of bone defect, but the patient should experience the pain caused by the surgery and the limited amount of bone [1-4]. Among allograft, xenograft, and synthetic bone graft, the synthetic bone grafting is the most widely used due to safety issues. Calcium phosphate (CP) ceramics have been investigated extensively for biomedical applications such as dental and orthopedic bone graft materials because of its chemical composition close to that of bone. A biphasic calcium phosphate (BCP) composed of 60 wt% hydroxyapatite (HA) and 40 wt% β-tricalcium phosphate (β-TCP) is reported to exhibit excellent bioactivity and a scaffold matrix for the implant due to the excellent reactivity of β-TCP and the stability of HA [4-6]. The synthetic bone graft materials should resorb completely by leaving space for new bone formation. Therefore, the BCP is currently used in orthopedics and dentistry because of biodegradable properties while retaining a robust strength.

In this study, biocompatible calcium-phosphate bone graft materials were prepared by a sponge method. Fully interconnected porous sponges made of polyurethane (PU) were used. The PU template (45 pores per inch, Alfome Co., Korea) was coated with BCP powders in a distilled water-based slurry. To accomplish a homogeneous and continuous scaffold, the viscosity of the slurry was precisely controlled throughout the entire procedures. The PU sponges were coated up to five times, followed by heat treatment and pulverization to obtain a powder. The cytotoxicity and cell proliferation of synthetic bone graft materials were also evaluated [7-10].

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Experimental

Scaffold synthesis

N,N dimethylformamide (DMF, Sigma Aldrich, USA), carboxymethylformamide (CMC, Sigma Aldrich, USA), polyvinyl alchol (PVA, Sigma Aldrich, USA), NaOH, biphasic calcium phosphate (BCP, 60:40, Ossgen, Korea), Darvan C-N (Vanderbilt Co., USA), surfynol DF-58 (Air Products, USA) were purchased and used as received without any further purification. Prior to the first slurry coating, the polyurethane sponge (45 ppi, $25 \times 30 \times 14.5$ mm^3, Alfome Co., Korea) was immersed in 0.5 N NaOH for 40 min to enhance the adhesion between the slurry and the surface, followed by cleaning with distilled water. The slurries consisting of BCP, Darvan C-N (dispersant), surfynol DF-58 (antifoaming agent), DMF, and DI water were ball milled for 2 hrs 30 min. The cobinder (PVA + CMC) was added to the slurry and then paste mixed for 5 min. For the subsequent coatings, the slurry was prepared similarly. However, PVA was only

used as the cobinder without CMC. The mixture of slurry and cobinder was mixed for 5 min.

The polyurethane sponges were coated with the slurry. The slurry-soaked sponges were centrifuged for 1.5 min at 500 rpm to remove the remnant slurry in the sponge. The sponges were dried for 1 day and annealed for 6 hrs 40 min at 800 °C with a heating rate of 2 °C/min. Then, the samples were sintered for 3 hrs 50 min at 1100°C with a heating rate of 10°C/min. The sintered samples were coated again by using the adjusted slurry and the disposable pipette. The as-coated samples were centrifuged for 1 min at 500 rpm. The samples were coated up to five times. The samples were then heat-treated under the same conditions.

Characterization

The solution viscosity was measured at room temperature using a viscometer (DV 1M, Brookfield, USA) with spindle NO. SC4-31 at 5 rpm. The morphologies of the PU sponge (45 ppi) and the BCP coated scaffolds were examined using SEM (S-3000H, Hitachi, Japan) [8-10]. All specimens were coated with Au/Pd to ensure higher conductivity.

The crystalline phase of the scaffolds was analyzed by using an XRD (Mac Science, KFX-987228-SE, Japan). Differential scanning calorimetry (DSC) and thermogravimetric (TG, Netzsch STAS 409C/31F, Germany) studies were also performed as the temperature rose from room temperature to 90 °C at a heating rate of 10 °C/min. The crystalline phase of the scaffolds was analyzed by using an XRD (Mac Science, KFX-987228-SE, Japan). Values in the text were expressed as the means ± standard deviation, and $p < 0.05$ was considered statistically significant.

Cytotoxicity

The extract test method was conducted on the BCP bone grafts to evaluate the potential of cytotoxicity on the base of the International Organization for Standardization (ISO 10993-5) [10]. The BCP grafts were extracted aseptically in single strength Minimum Essential Medium (1X MEM, Dulbecco's Modified Eagles's Medium (Gibco) with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomysin) with serum. The ratio of the BCP grafts to extraction vehicle was 0.2 g/mL. The 96-well plate was incubated at a temperature of 37 °C in a 5% CO_2 atmosphere. The test extracts were maintained in an incubator for 24 hrs. The test extracts were placed onto three separate confluent monolayers of L-929 (NCTC Clone 929, ATCC, USA) mouse fibroblast cells propagated in 5% CO_2. For this test, confluent monolayer cells were trypsinized and seeded in 10 cm^2 wells (35 mm dishes) with a micropipette. Simultaneously, triplicates of reagent control, negative control (high density polyethylene film, RM-C), and positive control (polyurethane film, RM-A) were placed onto the confluent L-929 monolayers. All monolayers were incubated for 48 hrs at 37 °C in the presence of 5% CO_2. After incubation, the morphological change of the cell was examined to assess the biological reaction by using the inverted microscope (TS100-F, Nikon, Japan) and the iMark microplate absorbance spectrophotometer (Bio-Rad, USA) [9, 10]. Water-soluble tetrazolium salts (WSTs) are a series of other water-soluble dyes for MTT assays, which can provide different absorption spectra of the formed formazans. EZ-cytox yields a water-soluble formazan, which can be read directly. The absorbance of the colored solution is quantified by measuring at a wavelength of 415 nm with the microplate absorbance spectrophotometer [8-10]. The value of untreated cell (control sample, only cultured with culture medium) was set as 100% and those of the treated cells were expressed as the percentage of the control sample.

Cell proliferation

Cell counting kit-8 (CCK-8, Dojindo Molecular Technologies, Inc., Japan) was used for the assay of cell proliferation. CCK-8, being nonradioactive, allows sensitive colorimetric assays for the determination of the number of viable cells in cell proliferation [10]. WST is reduced by dehydrogenases in cells to give an orange colored product (formazan), which is soluble in the tissue culture medium. The amount of the formazan dye generated by dehydrogenases in cells is directly proportional to the number of living cells. The 96-well plate containing 100 mL of cell suspension (5×10^3 cells/well) was incubated for 24 h at a temperature of 37 °C in a 5% CO_2 atmosphere. The test extracts (10 μL) were added to the plate and maintained for an appropriate length of time (6, 12, 24, 48 hr) in an incubator. After adding 10 μL of CCK-8 solution to each well of the plate, the plate was incubated for 2 hrs. Then, the absorbance of the colored solution is quantified by measuring at a wavelength of 450 nm with the microplate absorbance spectrophotometer.

Results and Discussion

The viscosity of the slurry was examined at 25 °C using a viscometer with spindle NO. SC4-31 at 5 rpm. The viscosities of the first and the remaining slurries were 5800 cP and 25 cP, respectively. The dramatic reduction in viscosity of the slurries was due to the absence of CMC binder. Low viscosity slurries were suitable for subsequent sponge coatings without blocking sponge crevices. Although the 1st BCP coated scaffolds were so fragile that careful handling was required, the polyurethane sponge was burn out completely after heat treatment, as depicted in Fig. 1. The BCP synthetic bone grafts were easily crushed and powdered by rubbing the scaffolds with fingers.

The morphologies of the PU sponge (45 pores per inch, 45 ppi) and the BCP coated scaffolds were

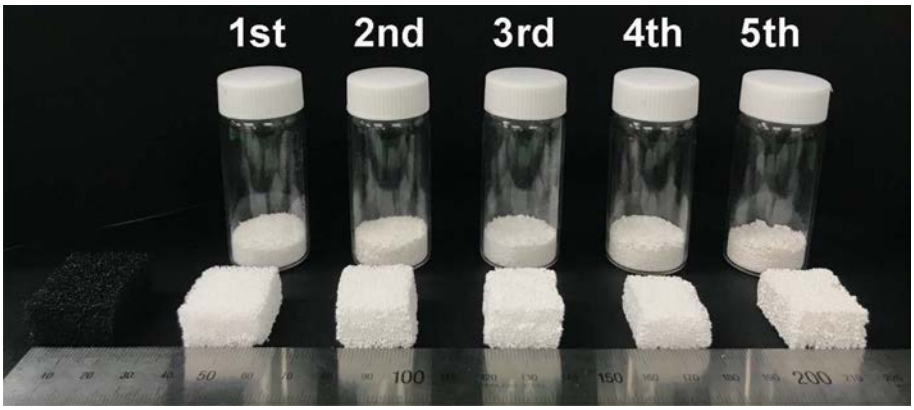

Fig. 1. Optical images of the PU sponge (45 ppi) and the BCP coated scaffolds. Note that the BCP scaffolds were annealed at 800 °C and then sintered at 1100 °C.

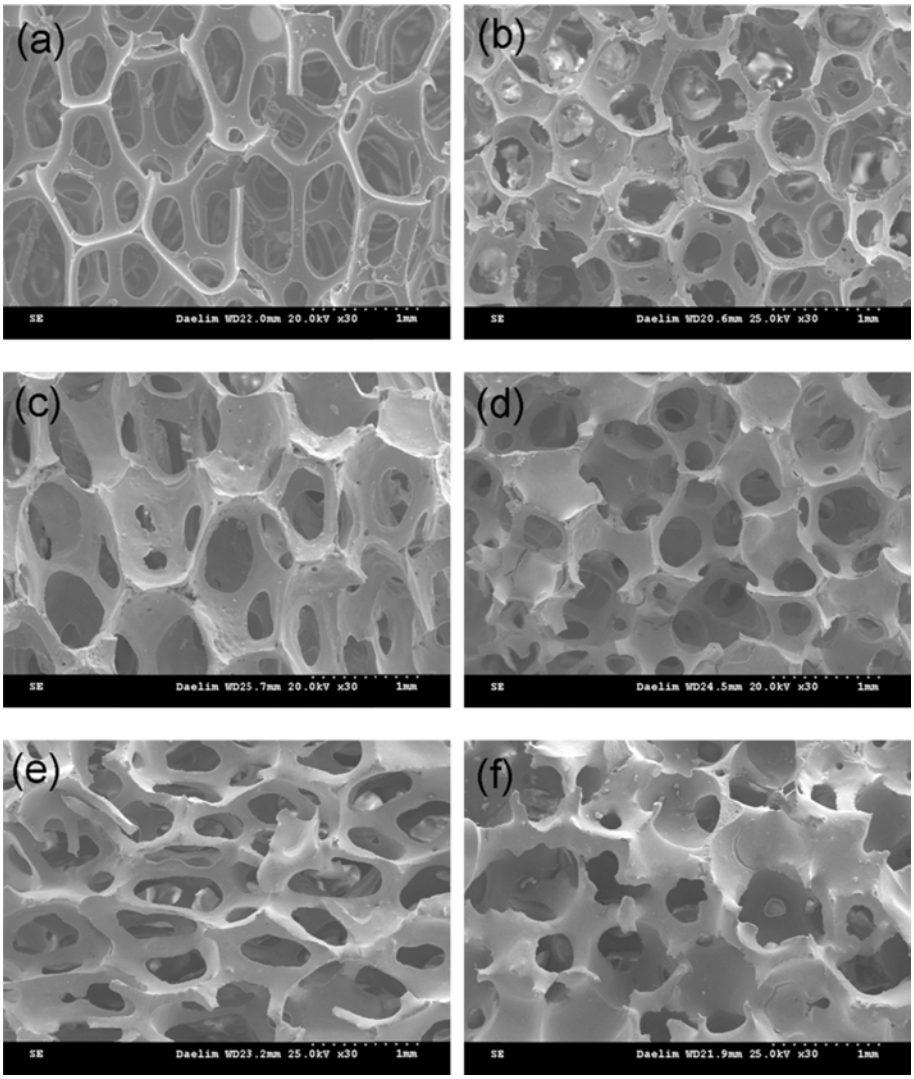

Fig. 2. SEM images of (a) PU sponge (45 ppi) and BCP scaffolds coated (b) once, (c) twice, (d) three times, (e) four times, and (f) five times. Note that the BCP scaffolds were annealed at 800 °C and then sintered at 1100 °C.

examined. Fully interconnected porous sponges made of polyurethane (PU) was clearly visible, as shown in Fig. 2(a). Oh *et al.* reported that the HA-based scaffolds with micro-channels and nano-pores were prepared by using a polyurethane template coating method to overcome some the limitations by addressing fluid absorbance and retention via capillary action [11, 12]. The scaffolds had 3 basic structures such as a porous

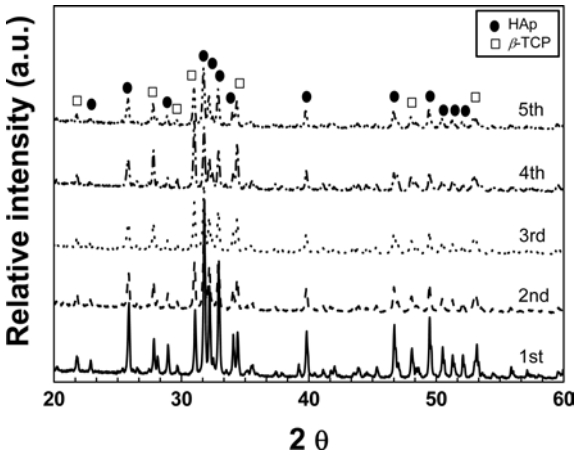

Fig. 3. XRD patterns of the BCP coated scaffolds. Note that the scaffolds were coated up to five times.

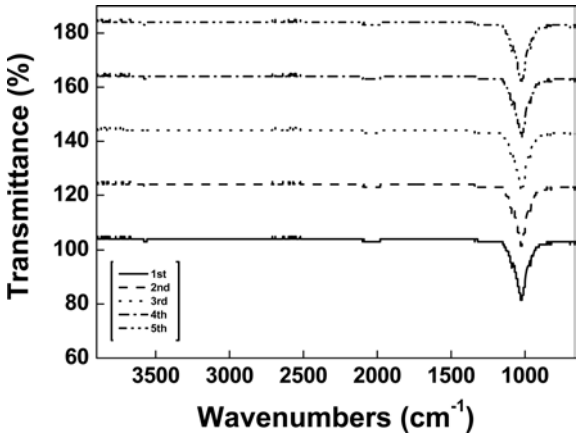

Fig. 4. FT-IR spectrum of the BCP coated scaffolds.

trabecular network similar to that of human trabecular bones (300 ~ 400 μm), micro-sized channels (25 ~ 70 μm) within each trabecular septum, and the surface of each septum having nano-sized pores (100 ~ 400 nm) [11]. These scaffolds induced excellent cell attachment, proliferation and differentiation as well as in nutrient

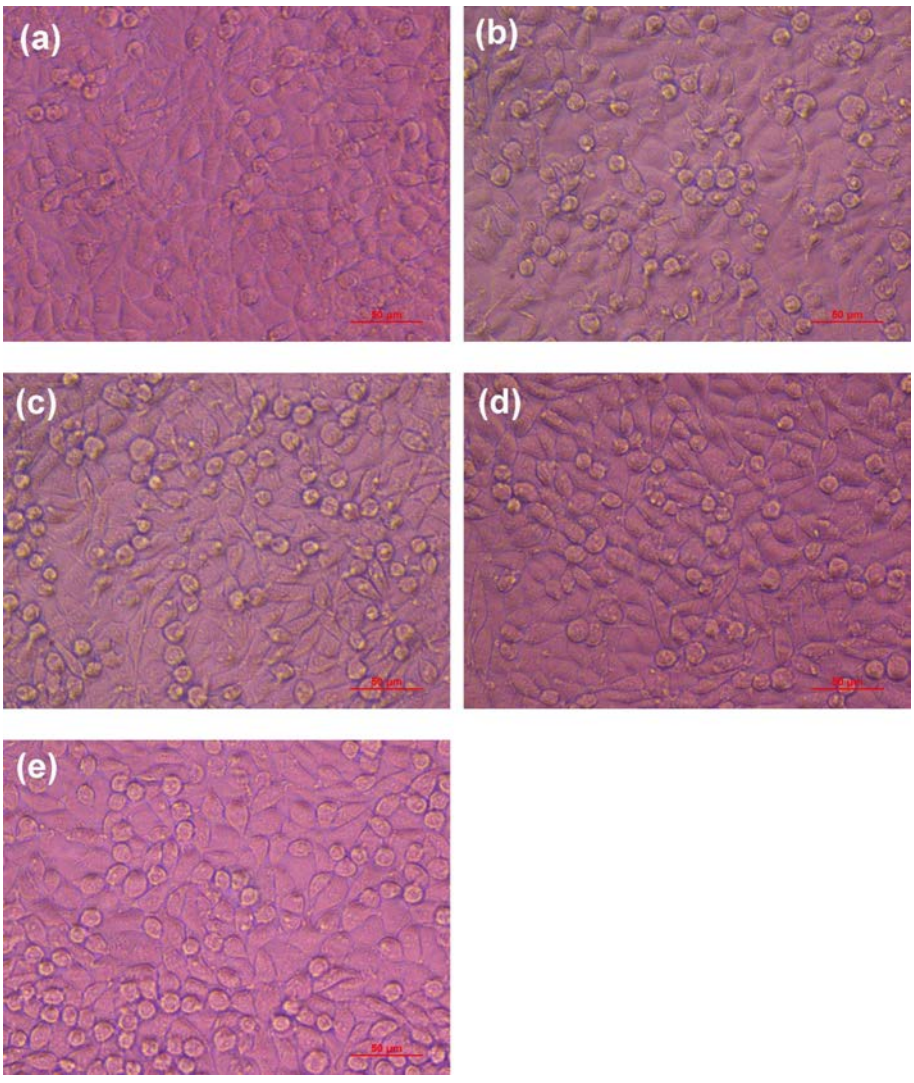

Fig. 5. Photographs of cell morphologies: the BCP scaffolds coated (a) once, (b) twice, (c) three times, (d) four times, and (d) five times from WST assay (EZ-cytox) after exposing with the scaffold suspensions for 48 hrs.

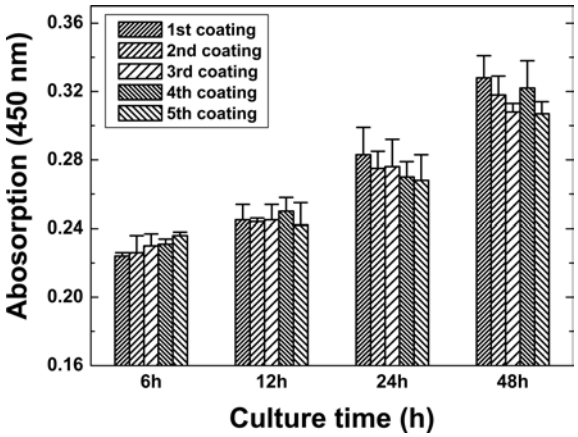

Fig. 6. Proliferation of L-929 cells on various BCP scaffolds.

flow and cell communication, which is crucial for proper bone healing [11, 12]. The pore and the wall size of the 45 ppi PU sponge were determined to be 580 ± 180 μm and 94 ± 15 μm, respectively. As the number of BCP coatings increased, the pore size decreased from 480 ± 93 mm (Fig. 2(b)) to 306 ± 120 μm (Fig. 2(f)), but the wall size increased from 104 ± 25 μm to 186 ± 40 μm. Although porous scaffolds reduced mechanical strength due to the presence of pores, a porous body improved cell affinity and drug delivery ability [8]. In particular, the presence of macro/micro-pores is determined to be very effective for bone ingrowth [8, 11, 12]. The chemical composition of the BCP coated scaffolds was almost the same regardless of the number of coatings, as displayed in Figs. 3 and 4. XRD results (Fig. 3) revealed that the BCP scaffolds were mainly composed of hydroxyapatite and β-TCP. No other peaks were visible. FT-IR analysis also exhibited a similar chemical composition of calcium phosphate for the BCP scaffolds coated various times, as depicted in Fig. 4.

A cytotoxicity test of the BCP scaffolds determines whether a product or compound will have a toxic effect on living cells [8-10]. The test extract with the BCP scaffolds coated various times showed no evidence of causing cell lysis or toxicity, as depicted in Fig. 5. The BCP scaffolds coated once, twice, three times, four times and five times, exhibited average cell viability of 106%, 109%, 114%, 107%, and 93% compared to the negative control, respectively, as measured at a wavelength of 415 nm by using the microplate absorbance spectrophotometer. The qualitative morphological grading of cytotoxicity of the BCP scaffolds was determined to be scale 0. As a result of cell counting kit (CCK)-8 cell proliferation experiment [10], L-929 cells adhered well to the BCP scaffolds and proliferated continuously with increasing time, as shown in Fig. 6. Therefore, it is conceivable that the BCP scaffolds are considered to be clinically safe and effective due to the absence of cytotoxicity and excellent cell proliferation under the condition of this study.

Conclusions

BCP bone grafts were successfully synthesized using the sponge coating method. FT-IR analysis also exhibited a similar chemical composition of calcium phosphate for the BCP scaffolds coated various times. XRD results revealed that the BCP scaffolds were mainly composed of hydroxyapatite and β-TCP. The pore and the wall size of the 45 ppi PU sponge were 580 ± 180 μm and 94 ± 15 μm, respectively. As the number of BCP coatings increased from 1 to 5, the pore size and the wall size decreased from 480 ± 93 μm to 306 ± 120 μm and increased from 104 ± 25 μm to 186 ± 40 μm, respectively. All the BCP scaffolds coated up to five times exhibited average cell viability of more than 93%. The cell proliferation results suggested that L-929 cells adhered well to the BCP scaffolds and proliferated continuously with increasing time, indicating that the BCP powders are highly adequate to the synthetic bone grafts.

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