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The effect of macro/micro combination pore structure of biphasic calcium phosphate scaffold on bioactivity

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ABSTRACT

Cellular responses such as adhesion, spreading, growth and proliferation can be altered by macro and micro pores as well as surface patterns on its surface. In this study, the scaffold was prepared with interconnected macro pores by the polymeric sponge method and with interconnected micro pores by BCP slurry coating with different particle sizes on sintered porous body and necking sintering process. Two particle sizes for micro pores, 200–400 nm and 600–800 nm, were coated on a pre-sintered porous body. A combined macro and micro pore structure of the BCP scaffold can improve osteoblast cellular activity. *In vitro* study of MG63 cells suggest that the modified scaffolds improved cell response such as cell spreading, proliferation and differentiation. The scaffold that has a uniform distribution of micro pores ranging 1~5 μm in diameter on its surface yields the highest rate of cellular response.

1. Introduction

Biocompatible matrices have several key roles, which including facilitating cellular migration or invasion into the implanted materials, guiding wound healing and inducing tissue responses to the material. It has been demonstrated that interconnected macro pores (diameter > 100 μm) allows blood vessel in-growth to the pores and provides a scaffold for nutrients to cells and bone cell colonization [1], whereas micro pores (diameter < 10 μm) enable body fluid circulation [2]. It is also well established that synthetic scaffold architectures must include interconnected macro pores in the order of 100 μm or greater to allow bone in-growth [3], in accordance with the classical studies of Klawitter and Hulbert [1]. Kühne et al. found that coralline HA with an average 500 μm pore diameter showed good bone infiltration in a rabbit model. HA with an average 200 μm pore diameter showed poor bone infiltration [4], varying with and depending on the *in vivo* environment. An ideal pore size of bone substitutes would be that of cancellous bone ranging 500–600 μm, mimicking osteon-evacuated cancellous bone [5].

Cellular behavior such as adhesion, spreading, growth and proliferation is influenced by the roughness or micro pores on the surface of a scaffold. Osathanon et. al. reported that cell spreading was greater on rough surfaces than smooth ones and that cell proliferation were increased when cells were cultured on rough surfaces [6]. Sammons et. al. found that a rough surface of porous microstructures may enhance the rate of cell spreading, though differentiation and calcification occurring on surfaces of both rough and smooth microstructures [7]. Vandrovcová et. al. suggested that nanostructured surfaces preferentially adsorb extracellular matrix proteins, especially vitronectin, and this protein is preferentially recognized by osteoblasts over other cell types, which may improve cellular adhesion and spreading [8].

Most studies focus on macro porosity or micro roughness itself. However, the effects of macro and micro porous architecture, especially interconnected micro pores on cellular response is not yet fully understood.

Of various techniques for scaffold preparation, the polymeric sponge method has the benefit of producing a positive replica of the reticular foam, that is, an interconnected macro porous scaffold mimicking cancellous bone [9]. Though micro pores can be generally obtained by eliminating or calcining volatile organic components such as binders or PMMA particles in slurry [10], discrete micro pores usually appear in the body after sintering. Interconnected micro pores

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can be conveniently fabricated by necking the nano particles on sintering, where the pore size may be controlled by utilizing nano particles ranging from 200 to 800 nm.

Biphasic calcium phosphate (BCP) scaffolds composed with hydroxyapatite (HA) and tricalcium phosphate (β-TCP) are being used for bone regeneration in the field of dentistry and orthopedics. The solubility of HA in neutral aqueous media being very low, HA resorbs slowly *in vivo*, usually by osteoclastic resorption. In contrast, β-TCP dissolves much faster than HA *in vitro* and *in vivo*. Consequently, the dissolution of BCP can be accelerated unlike HA and be hindered as compared with β-TCP by controlling the ratio of HA and β-TCP and synchronizing the degradation rate with that of new bone formation [11,12].

The current study focuses on creation of different micro pore surfaces as experimental groups and with a smooth surface as a control to investigate cellular response. Human preosteoblastic MG63 cells were used to evaluate the effect of macro and micro pores on osteoblastic cellular response in terms of adhesion, spreading, proliferation, and differentiation. X-ray powder diffraction (XRD), Scanning Electron Microscope (SEM), Micro-Computed Tomography (Micro-CT) and water contact angle (WCA) measurement was employed to characterize the prepared BCP scaffold. SEM, MTT assay, ALP activity, and fluorescent microscope measurement were performed to evaluate cellular responses.

2. Materials and methods

2.1. Synthesis of BCP nano powders

Two sizes of BCP nano powders, 200–400 nm (BCP2040) and 600–800 nm (BCP6080), were prepared by the reaction of calcium hydroxide (98%, Duksan Pure Chemicals Co., Korea) and phosphoric acid (98%, Duksan Pure Chemicals Co., Korea), respectively. Ca/P ratio was set to 1.60 for 60% HA and 40% β-TCP and phosphoric acid was added dropwise into the aqueous calcium hydroxide solution. The pH of the solution was changed from high alkaline to neutral region after reaction. The solution was aged for 48 h in room temperature and filtered through a 1 μm pore size paper filter. The filter cake was dried at 150 °C in a dryer oven for 5 h and crushed down to fine powder. The powder was classified under 250 μm for BCP2040 and 500 μm for BCP6080 using plastic sieves. The BCP2040 powder was calcined at 950 ℃ for 30 min in a furnace. The BCP6080 powder was calcined at 1000 ℃ for 1 h and at 1100 ℃ for 30 min. The powders were milled using conventional ball milling with a zirconia ball media in ethanol for 6 h. The milled slurry was dried by a vacuum evaporator and the dried powders were gently grinded with mortar and pestle. The particle size and shape were examined by SEM. The powder composition was confirmed by XRD (Figs. 1, 2).

2.2. Macro porous with smooth surface scaffold preparation

Polyurethane (PU) sponge was etched in 2 wt% of NaOH solution for 10 min and dried slowly at 60 ℃ after washing with distilled water. Polyvinyl alcohol (PVA) binder was prepared by dissolving 3 wt% of PVA powder in distilled water at 150 °C. 0.2 wt% of polyacrylic ammonium salt as a dispersant was added and stirred to the PVA solution. BCP2040 powder was mixed and kneaded with PVA solution with a ratio of 1.5:1 in weight. The PU sponge was immersed into the BCP2040 paste and rolled with a rod, followed by repeated compression/release. BCP2040-coated PU sponge was dried at room temperature for 12 h and sintered at 1200 °C for 3 h, the heat-treatment condition being 3 °C/min to 280 °C, 2 °C/min from 280 °C to 400 °C (the volatile region of PU sponge), 3 °C/min to 600 °C (held for 1 h at 600 °C), and 5 °C/min to 1200 °C (Fig. 1).

2.3. Micro porous coating on fabricated scaffold

BCP2040 and BCP6080 slurry for second coating on fabricated macro porous body was prepared by dispersing each powder in PVA solution with a ratio of 1:2, 1:3, and 1:6 in weight, respectively. The sintered specimens were immersed in slurry and blown out by dried air, eliminating excessive slurry from the macro porous body. The scaffolds were dried at room temperature for 6 h and sintered at 1200 °C for 2 h at a heat rate of 5 °C/min (Fig. 1).

2.4. Measurement of macro and micro pore size

Macro pore size of the sintered scaffold and micro pore size on each strut surface coated with BCP2040 and BCP6080 was examined using field-emission scanning electron microscopy (FE-SEM, S-4200, HITACHI, Japan). The macro and micro pore size range was directly determined by measuring the diameter of pores on SEM picture compared with scale bar and magnification (Figs. 3, 4).

2.5. Water contact angle measurement

BCP discs coated with BCP2040 and BCP6080 were prepared to measure the water contact angles (WCA) on the flat surface. WCA was measured in triplicate by the static sessile drop method using a surface goniometer (OCA 15 plus, Data-Physics Instrument GmbH, Filderstadt, Germany), to examine hydrophilic or hydrophobic behavior of the surface (Fig. 5).

2.6. Seeding preosteoblasts onto the scaffolds for 3D culture

Each scaffold (Φ 10×3 mm) was sterilized by gamma radiation with a dose of 26 kGy. Human preosteoblastic MG63 cells (RIKEN CELL BANK, Japan) were cultured using the alpha minimum essential medium (α-MEM; WelGENE, Daegu, Korea) supplemented with 10% fetal bovine serum (FBS; Gibco Invitrogen, Grand Island, NY) and 100 units/mL penicillin (Gibco Invitrogen, Grand Island, NY). Cells were cultured at 37 °C in a 5% CO_2 humidified atmosphere incubator. In this study, the cells were seeded onto the BCP scaffolds in via 3D cell culture. The cells were seeded at a density of 3×10^4 cells/mL cell suspension onto the scaffolds, then cultured for up to 7 days for the proliferation test and 14 days for the differentiation test in the aforementioned medium at 37 °C with 5% CO_2. The medium was replenished every 2 or 3 days.

2.7. Cellular responses

Fluorescence microscopy was performed to observe morphological changes by actin formation. Staining of F-actin, which is known to be the major constituent of microfilaments, was performed using fluorescein isothiocyanate-labeled phalloidin. On the days of experiment, the scaffolds were washed with PBS and the cells were fixed with 4.0% paraformaldehyde. After washing, 0.1% Triton X-100 was added for cell permeabilization, and the blocking was performed using 1.0% bovine serum albumin (BSA) solution. After BSA solution removal, 50 μg/mL phalloidin solution in PBS was used to stain F-actin, and 4′,6-diamidino-2-phenylindole (DAPI) in antifade reagent was used to co-staining the cell nuclei while maintaining resistance to photobleaching. After a day, the observation of stained cells were observed using a fluorescence microscope.

The Cell Counting Kit-8 (CCK-8; Dojindo, Tokyo, Japan) was used to determine the number of viable cells. In this assay, 2-(2-methoxy-4-nitrophenyl)–3-(4-nitrophenyl)–5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8) is reduced by dehydrogenases in the cells to provide an orange-colored product (formazan) that is soluble in the tissue culture medium. The amount of the formazan dye generated by the dehydrogenases in the cells is directly proportional to the