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Collagen immobilization of multi-layered BCP-ZrO_2 bone substitutes to enhance bone formation

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ABSTRACT

A porous microstructure of multi-layered BCP-ZrO_2 bone substitutes was fabricated using the sponge replica method in which the highly interconnected structure was immobilized with collagen via ethyl(dimethylaminopropyl)carbodiimide/*N*-hydroxysuccinimide crosslinking. Their struts are combined with a three-layered BCP/BCP-ZrO_2/ZrO_2 microstructure. Collagen fibers were firmly attached to the strut surface of the BCP-ZrO_2 scaffolds. With control of the three-layered microstructure and collagen immobilization, the compressive strength of the scaffolds increased significantly to 6.8 MPa compared to that of the monolithic BCP scaffolds (1.3 MPa). An in vitro study using MTT, confocal observation, and real-time polymer chain reaction analysis demonstrated that the proliferation and differentiation of the pre-osteoblast-like MC3T3-E1 cells was improved due to the collagen incorporation. Remarkable enhancement of bone regeneration was observed without any immunological reaction in the femurs of rabbits during 1 and 5 months of implantation. Furthermore, the interfaces between new bone and the scaffold struts bonded directly without any gaps.

1. Introduction

For clinical application, bone substitutes should meet requirements such as non-toxicity, non-immunogenicity, excellent biocompatibility, ease of accessibility and handling, and high mechanical strength [1]. Allografts and xenografts have been widely applied for bone regeneration within large defects [2–4]. Despite rules and regulations for tissue banks regarding processing and handling of hard human and animal tissues, some potential risks such as disease transmission and immunological disadvantage from the use of cadaver bones remain [5,6]. To overcome the drawbacks, many reports have detailed the development of polymer-based hydrogel for bone regeneration because they have shown excellent bone regeneration in the in vivo model [7–10]. However, it has been recognized that they cannot be widely applied for hard tissue regeneration, especially high load-bearing parts, due to their low mechanical strength. Thus, calcium/phosphate-based bioceramic scaffolds have recently been re-illuminated for clinical hard tissue regeneration [11–14]. To be ideal artificial bone substitutes, they should be chemically, biologically, and mechanically as close to natural bone as possible. Keeping this in mind, we hypothesized a novel design for collagen-immobilized porous multi-layered biphasic calcium phosphate (BCP)-ZrO_2 bone substitutes. Here, BCP, ZrO_2, and collagen were considered bone component, scaffolding reinforcement, and extracellular matrix (ECM), respectively. Although ZrO_2 is a typical bio-inert ceramic, it has some valuable properties such as non-toxicity, biocompatibility, and high strength. It has also been used in dental crowns and bridges as well as artificial hip joints [15–17].

To fabricate cancellous bone substitutes, the sponge replica method has been considered both convenient and easy [18]. This method generally yields a very open reticulated structure with high permeability. However, the main disadvantages of this method lay in the low mechanical properties of the end products since the structure is associated with hollow struts and a large number of flaws that result from burning out the polymer foam substrate [19]. In our earlier study, we reported that polycaprolactone infiltrated the struts of the sponge BCP scaffold and improved its strength, although the compressive strength did not increase remarkably [20]. Using the combination of ZrO_2 and BCP phases, we could remarkably improve the compressive strength [19]. In this case, the outer surface of the porous multi-layered scaffolds (BCP/BCP-ZrO_2/ZrO_2) was the BCP phase, which consists of hydroxyapatite (HAp) and tricalcium phosphate phases, possesses excellent

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properties, and serves as an efficient material for dental implants, bone replacements, and orthopedic surgery [21–23].

On the other hand, surface modifications of the scaffolds have been considered an important way to fabricate successful biocompatible materials in bone tissue engineering [24–26]. A key component of bone regeneration in tissue engineering is the scaffold that serves as a template for cell interactions and the formation of bone–ECM to provide structural support to the newly formed tissue. Some peptides and proteins, including bone morphogenetic proteins (BMPs) [27], fibronectin [25], and collagen [26,28], are used to improve scaffold bioactivity. Collagen type I, as a fibrous protein, is one of the major components of ECM and is secreted by osteoblasts. When collagen is adsorbed on the scaffolds, it plays a crucial role in bone cell attachment, proliferation, and differentiation. It also possesses several important properties such as excellent biological features and physicochemical characteristics for use in bone substitutes. In this study, the strut surfaces of multi-layered BCP-ZrO_2 scaffolds were modified with collagen using ethyl(dimethylaminopropyl)carbodiimide/*N*-hydroxysuccinimide (EDC/NHS) as a crosslinking agent, and we investigated the effects of collagen modification on the proliferation and differentiation of pre-osteoblast MC3T3-E1 cells as well as new bone formation in a rabbit model.

2. Materials and methods

2.1. Fabrication of porous multilayer BCP-ZrO_2 scaffolds

Porous BCP-ZrO_2 scaffolds were fabricated using the sponge replica method with ZrO_2 as a main frame [19]. To fabricate the multilayer structure, ZrO_2, BCP-ZrO_2 (volume fraction 50:50) and BCP slurries were prepared by mixing powder homogeneously in ethanol containing 5 wt% polyvinyl butyral. The sintered monolithic ZrO_2 scaffold was immersed in the BCP-ZrO_2 slurry and dried at 60 °C for 1 h. This step was repeated twice to obtain a uniform intermediate layer. After heat treatment at 1000 °C for 2 h to remove binder and at 1500 °C for 10 min for sintering, BCP-ZrO_2 and BCP slurry coating was conducted twice to obtain the outer layer. After the burning out and microwave sintering steps were completed, the multilayer structure was fabricated.

2.2. Immobilization of collagen on the multilayer BCP-ZrO_2 scaffold

The EDC, NHS, and 2-(*N*-morpho)ethanesulfonic acid [29] were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The BCP-ZrO_2 scaffolds were incubated with 1% (w/v%) bovine serum albumin (BSA) overnight, rinsed twice with distilled water, and then incubated in EDC/NHS crosslinker solution at a 5:2 molar ratio in MES (0.1 M) at 4 °C for 1 h. The scaffolds were incubated overnight in collagen solutions (5 mg/mL; Sigma). Glycine was added to stop the reaction.

2.3. Surface characterization

The microstructures of the non-collagen immobilized (BCP-ZrO_2) and collagen immobilized (Col-BCP-ZrO_2) scaffolds were characterized using a scanning electron microscope (SEM; JSM-6701F; JEOL, Japan) with platinum (Cressington 108 Auto) coating the sample holder. The samples were pulverized and subjected to Fourier transform infrared (FT-IR) imaging and microspectroscopy. Functional groups present in the scaffolds were analyzed using an FT-IR spectroscopic analysis Nicolet spectrometer system (Nicolet iS10; Thermo Scientific) and the spectrum was analyzed using accompanying Omnic Version 7.3 software over a range of 500–4000 cm^{-1} at a resolution of 8 cm^{-1}.

X-ray photoelectron spectroscopy (XPS; PHI 5400; Perkin-Elmer) spectra were obtained using a passing energy of 70 eV. High-resolution spectra were obtained using a passing energy of 20 eV. All binding energies were referenced to C 1s (carbon) peaks at 281.0 eV.

The porosity and pore size distribution of the samples was determined using a mercury intrusion porosity meter (PoreMaster-60; Quantachrome Porosity Meter, USA). Spongy bone scaffolds (9 mm high × 4 mm long × 4 mm wide) were sufficiently dried and preweighed at room temperature prior to testing. The samples were placed in the quartz penetrometers and weighed subsequently before being placed inside the machine for mercury intrusion. Low pressure was regulated at 60 psia and used to force the mercury inside the scaffold porous network. The porosity values and pore size distribution was automatically calculated by the included PoreMaster Data Reduction software.

2.4. Mechanical characterization

Compressive strength was determined using a universal testing machine (R&B UNITECH-T, Korea). The samples were prepared with dimensions of 5 mm wide × 7 mm long × 7 mm high. The measurements were taken using a 100 kN load cell. Load deformation data were recorded at a deforming speed of 1 mm/s.

2.5. In vitro study

2.5.1. Cell culture

Mouse pre-osteoblast MC3T3-E1 cells (ATCC-CRL-2593; USA) were used to study the cell behavior on porous BCP-ZrO_2 and Col-BCP-ZrO_2 surfaces. MC3T3-E1 were cultured in α-MEM (HyClone, Logan, UT, USA) and supplemented with 10% fetal bovine serum (FBS; Grand Island, NY) and 1% penicillin/streptomycin (Bio-Whittaker). The MC3T3-E1 cells were maintained and suspended in a humidified incubator at 37 °C in a 5% CO_2 atmosphere (ASTEC, Japan).

2.5.2. Cell viability and proliferation

Cell viability was done by seeding the non-modified and collagen-modified scaffolds with MC3T3-E1 pre-osteoblast-like cells (10^5 cells/mL). The optical density (OD) corresponds to the number of viable cells. The cell viability at 1, 3, and 7 days on the scaffolds was quantified by the addition of 100 μL of the MTT solution (5 mg/mL in phosphate buffered saline) to each well of the 24-well tissue culture plate. After 4-h incubation, the OD values of the solution were measured using an enzyme-linked immunosorbent assay (ELISA) reader (EL 312 Biokinetics reader; Bio-Tek Instruments) at a wavelength of 595 nm. For observation of the cell morphology on the scaffolds by confocal microscope, after 1, 3 and 7 days of incubation, the cells were immunostained using fluorescein isothiocyanate-conjugated phalloidin (25 μg/mL; Sigma) for 2 h at room temperature. The nuclei were counterstained with 4′,6′-diamidino-2-phenylindole. Finally, the scaffolds were mounted on glass slides and visualized under confocal fluorescent microscopy (FV10i-W).

2.5.3. Osteogenic differentiation

The osteogenic differentiation of MC3T3-E1 cells was analyzed by quantitative real-time polymerase chain reaction (RT-PCR) after 7 and 14 days of incubation. As a control we used cells grown only on media in the wells of tissue culture plate (TCP) without any samples. Cells were harvested, washed by centrifugation, and propagated in the control wells of TCPs with osteogenesis media. Total RNA from the samples was isolated using an RNeasy FFPE Kit (Qiagen, Valencia, CA, USA) and treated on-column with DNase I to eliminate contaminating DNA. The RNA was stored at −80 °C until