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Incorporation of BMP-2 nanoparticles on the surface of a 3D-printed hydroxyapatite scaffold using an ε-polycaprolactone polymer emulsion coating method for bone tissue engineering

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ABSTRACT

Hydroxyapatite (HAp)-based three-dimensional (3D) scaffolding is an excellent method for the fabrication of complex-shaped scaffolds to reconstruct bone defects. This study aimed at improving the osteoinductivity and compressive strength of the HAp-based 3D scaffold for bone regeneration. Bone morphogenetic protein-2–loaded nanoparticles (BMP-2/NPs) were prepared by a double emulsion-solvent evaporation method and incorporated onto the surface of 3D scaffolds using ε-polycaprolactone (PCL) and NPs emulsion solution. The surface morphology of the scaffold was characterized using scanning electron microscopy and its biocompatibility and osteogenic effects evaluated *in vitro* using human mesenchymal stem cells. The *in vivo* bone regeneration efficiency was determined using a rabbit calvarial bone defect model. We obtained 3D HAp scaffolds with NPs using PCL coating process. BMP-2/NPs were uniformly distributed on the scaffold surface and BMP-2 was gradually released. Furthermore, PCL coating improved the compressive strength of the scaffold. The cell proliferation, adhesion, and osteogenic differentiation properties were improved with PCL_BMP-2/NPs coated scaffold. *In vivo* experiments showed that the formation of new bone was significantly higher in the PCL_BMP-2/NPs group than in the uncoated scaffold-implanted group. The coating method using PCL and NPs emulsion solutions was useful not only to incorporate BMP-2/NPs onto the surface of the scaffold, but also to improve the compressive strength, which enhanced bone regeneration.

1. Introduction

Artificial bone replacement techniques using a three-dimensional (3D) scaffold display high clinical potential to resolve bone defects [1]. In recent years, 3D printing techniques have been used for the generation of 3D scaffolds and may serve as a useful tool, as these techniques allow control over the pore size, porosity, and outer shape of the scaffold [2]. Calcium phosphate materials are commonly used in bone tissue engineering as a part of 3D printing system for the preparation of bone tissue scaffolds [3]. Of these materials, hydroxyapatite (HAp, $Ca_5(PO_4)_3OH$) is frequently used as a scaffold material [4], as it is the main component of bones. Although several studies have reported the preparation of 3D scaffolds with HAp powder, it is still difficult to find an effective binder for HAp powder [5,6]. Gypsum can be transformed into HAp by a hydrothermal reaction in ammonium phosphate solution [11] and the 3D scaffolds fabricated with calcium sulfate powder can be successfully transformed into HAp, with their shape unchanged by the hydrothermal reaction [12]. These findings suggest that the HAp scaffold could be directly 3D printed using calcium sulfate powder.

The brittleness and low strength of 3D-printed HAp scaffolds need further attention. Therefore, to improve the mechanical strength of HAp scaffold, sintering [7] and polymer infiltration [8] methods have been used. In the sintering method, optimal sintering temperature and time condition is affected by grain size, density, and crystallite size, and leads to improved mechanical strength. However, sometimes the grain size obtained by sintering is too large, which causes unexpected results, such as the interruption of the degradation rate by osteoclastic bone resorption [9]. On the contrary, polymer coating increases the mechanical strength of a brittle scaffold associated with polymer infiltration into the spaces between the particles [8]. In addition, this approach

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has the advantage that polymer phase can act as carrier material for functional or bioactive biomolecules [10]. Generally, in polymeric infiltration methods, synthetic polymers, such as poly (lactic-co-glycolic acid) (PLGA) or polycaprolactone (PCL), have been widely used to improve the compressive strength of the scaffold. Especially, PCL polymer has been used to improve the compressive strength of HAp scaffolds because the fracture energy of PCL is higher than that of other polymers [11].

Although HAp is considered an acceptable material for bone tissue, its osteoinductive properties are insufficient to permit healing of extensive bone defects [12]. To overcome these disadvantages, several bioactive molecules, including growth factors, have been used in bone tissue engineering [13]. Bone morphogenetic protein-2 (BMP-2) is the gold standard growth factor used to enhance bone regeneration and has been successfully applied in several studies [14]. However, the clinical application of BMP-2 has been limited by the necessity for an initial high dose of BMP-2 and maintenance of a therapeutic concentration, owing to its short half-life *in vivo*. Poly(lactic-co-glycolic acid) (PLGA) is a biocompatible, biodegradable polymer [15] and has attracted attention as a biocompatible polymer for nanoparticles (NPs) [16]. Although drug-encapsulated PLGA particles are easily fabricated using a double emulsion-solvent evaporation method [17], it is important to achieve homogeneous and stable localization of polymeric NPs on the scaffold surface.

Here, we fabricated BMP-2–loaded NPs (BMP-2/NPs) with the double emulsion-solvent evaporation method. To incorporate BMP-2/NPs on the surface of scaffold, 3D HAp scaffold was coated with ε-polycaprolactone (PCL) and NP composite emulsion. The fabricated scaffold was evaluated for its mechanical properties and *in vitro* biological behavior. In addition, the bone-healing capacity of the scaffold was examined in a rabbit calvarial defect model *in vivo*.

2. Materials and methods

2.1. Fabrication of 3D HAp scaffold

For the fabrication of 3D HAp scaffolds, calcium sulfate hemihydrate powder ($CaSO_4$-1/2 H_2O) was transformed into HAp by hydrothermal treatment, as previously reported [18]. Calcium sulfate hemihydrate powder (average particle size, 10–20 μm) and ZB 63, a water-based binder (Z corporation, UK), were used to fabricate the scaffolds. A porous structure was designed using 3D CAD program (SolidWorks, Concord, MA, USA). Calcium sulfate hemihydrate 3D scaffolds were printed using 3DP equipment (Z350, Z-Corporation). After building the specimen, calcium sulfate hemihydrate 3D scaffolds were transformed into HAp in a hydrothermal reaction [19].

2.2. Microstructural observation

To evaluate the morphological properties of 3D HAp scaffolds, specimens were sputter-coated with gold and their microstructural and surface morphology observed by scanning electron microscopy (SEM; EM-30, COXEM, Daejeon, Korea) under vacuum.

2.3. X-ray diffraction analysis

X-ray diffraction analysis (XRD) was performed with CuKα radiation at a scan rate of 0.02°/min (2θ) and 30 mÅ range of 20–60° to evaluate the transformation of calcium sulfate hemihydrate into HAp.

2.4. Preparation of BMP-2–loaded PLGA NPs

Recombinant human BMP-2 (rhBMP-2) was purchased from Genoss (Suwon, Korea). PLGA (50:50; MW 4000 g/mol) and poly(vinyl alcohol) (PVA; MW 30,000–70,000 g/mol) were used as NP materials. BMP-2–loaded NPs were prepared using a water/oil/water (W/O/W) double emulsion-solvent evaporation technique [17]. Briefly, 30 mg PLGA was dissolved in 1 mL dichloromethane (Sigma-Aldrich, St. Louis, MO, USA) and treated with 10 mg rhBMP-2 in 100 μL distilled water. The mixture was emulsified with a sonicator (Sonicator XL; Misonix, Farmingdale, NY, USA) for 30 s at 20 W in an ice bath. The primary emulsion solution was added to 12 mL of 1% PVA aqueous solution and a second emulsification step performed using a sonicator for 5 min at 20 W in an ice bath. To evaporate the organic solvent, the emulsion was stirred on a magnetic stir plate and BMP-2/NPs were recovered by centrifugation at 10,000 ×*g* for 20 min at 4 °C. NPs were washed twice with distilled water during centrifugation to remove any remaining PVA and untrapped protein, followed by their lyophilization.

2.5. Characterization of BMP-2–loaded PLGA NPs

The prepared BMP-2/NPs were assayed for particle size and zeta potential using a Zetasizer Nano ZS from Malvern Instruments (Malvern, UK). The freeze-dried sample (5 mg) was diluted in 10 mL distilled water and its particle size and zeta potential determined. In addition, the encapsulation efficiency (EE %) of BMP-2 was determined as previously described [20], with some modifications. Briefly, 10 mg lyophilized BMP-2/NPs were resuspended in 750 μL dimethyl sulfoxide for 1 h and treated with 0.5% sodium lauryl sulfate/0.2 N sodium hydroxide (NaOH) solution. The mixture was incubated for 1 h to completely dissolve NPs. After neutralization with hydrochloric acid (HCl), the total extracted BMP-2 concentration was measured with a bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA).

2.6. Incorporation of BMP-2/NPs on the surface of 3D scaffolds

PCL pellets (MW 10,000; Sigma-Aldrich) were dissolved in dichloromethane (Sigma-Aldrich) at a concentration of 5% (w/v). For the incorporation of BMP-2/NPs on the surface of 3D scaffolds, freeze-dried BMP-2/NPs (100 mg) were dispersed in 200 μL distilled water and treated with 2 mL PCL solution. The mixture was emulsified with homogenizer for 30 s to prepare PCL and NP emulsions. 3D HAp scaffolds were dipped into PCL_BMP-2/NPs emulsion solutions. To improve the coverage of PCL on the porous structure, the dipping process was performed in a vacuum. The excess solution was removed by centrifugation at 300 rpm for 30 s. PCL-coated scaffolds were dried for 5 days at room temperature to allow evaporation of excess of dichloromethane (Fig. 1).

2.7. Microstructural observation

To confirm the incorporation of BMP-2/NPs onto the surface of scaffolds by PCL coating, specimens were sputter-coated with gold and observed by SEM (EM-30).

2.8. Analysis of compressive strength and porosity

To measure the compressive strength and porosity, a porous square-shaped (10 × 10 × 10 mm) specimen was used in this study. The compressive strength of the specimen was evaluated using an Instron test machine (Model 4505, UK) by applying load *via* 1-kN load cell at a crosshead speed of 0.5 mm/min. The effect of PCL coating on scaffold porosity was evaluated using a mercury intrusion porosimeter (AutoPore IV9500; Oak Ridge, TN, USA). Briefly, scaffolds were sealed in a penetrometer, weighed, and subjected to analysis [21].

2.9. In vitro release study

To measure the *in vitro* BMP-2 release, the 3D scaffold were immersed in 5 mL phosphate-buffered saline (PBS) and incubated at 37 °C. At each time interval (0, 1, 2, 3, 7, 10, 15, 20, 25, and 30 days), the supernatant was collected and fresh PBS added for continuous