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Fabrication of a BMP-2-immobilized porous microsphere modified by heparin for bone tissue engineering

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ABSTRACT

The purpose of this study was to fabricate BMP-2-immobilized porous poly(lactide-co-glycolide) (PLGA) microspheres (PMS) modified with heparin for bone regeneration. A fluidic device was used to fabricate PMS and the fabricated PMS was modified with heparin-dopamine (Hep-DOPA). Bone morphogenic protein-2 (BMP-2) was immobilized on the heparinized PMS (Hep-PMS) *via* electrostatic interactions. Both PMS and modified PMS were characterized using scanning electron microscopy (SEM) and X-ray photoelectron spectroscopy (XPS). MG-63 cell activity on PMS and modified PMS were assessed *via* alkaline phosphatase (ALP) activity, calcium deposition, and osteocalcin and osteopontin mRNA expression. Immobilized Hep-DOPA and BMP-2 on PMS were demonstrated by XPS analysis. BMP-2-immobilized Hep-PMS provided significantly higher ALP activity, calcium deposition, and osteocalcin and osteopontin mRNA expression compared to PMS alone. These results suggest that BMP-2-immobilized Hep-PMS effectively improves MG-63 cell activity. In conclusion, BMP-2-immobilized Hep-PMS can be used to effectively regenerate bone defects.

1. Introduction

The research field and development of tissue engineering and regenerative medicine has progressed at a very rapid rate [1]. Tissue engineering has emerged as an attractive approach involving the combining of cells, scaffolds, and bioactive agents to fabricate functional new tissue to replace damaged tissue [2,3]. Recently, well-designed and porous three-dimensional (3D) biodegradable scaffold are of great importance in various tissue engineering applications because they guide tissue formation *in vitro* and *in vivo*. The porous 3D scaffold systems provide large surface area for cell attachment and proliferation. Also, they can be used as building blocks to construct a large scaffold as cell delivery carriers to reconstruct damaged tissue or organ [4–7].

When designing and developing these 3D scaffolds or injectable scaffolds, important factors such as osteoinductivity, osteoconductivity, and osteogenicity must be considered. In the case of 3D scaffolds, it is possible to regenerate and/or repair bones *via* an open surgical procedure, but these 3D scaffolds are not a reasonable method when compared to noninvasive or minimally invasive surgical modalities. Unlike 3D scaffolds, noninvasive or minimally invasive surgery using injectable scaffolds has been performed for the last 10 years, specifically on irregularly shaped bone defects [8]. Using injectable scaffolds for bone defects does offer some advantages, such as increased ease of the operation using 10- to 16-gauge needle syringes, shorter operative times, minimal scarring, and increased patient satisfaction. Moreover, as an injectable material, the microspheres with interconnective pores between the surface and interior are a good delivery vehicle not only for cells, but for the sustained release of various drugs or proteins as well [9–11]. Accordingly, microspheres have been indicated as a potential material for clinical applications requiring carriers for drugs, proteins and cells [12,13].

Bone morphogenic proteins (BMPs) have been widely used because they play important roles in bone and cartilage regeneration. Amongst other BMP, BMP-2 is a potent osteoinductive molecule that plays key roles during bone formation [14,15]. For example, BMP-2 significantly induces osteogenic differentiation

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from mesenchymal stem cells (MSCs) as well as enhancing bone formation [16,17]. Additionally, BMP-2 stimulates the gene expression of osteocalcin (OCN), osteopontin (OPN), bone sialoprotein, and alkaline phosphatase (ALP) during osteoblast differentiation *in vitro* [18,19]. Notably, BMP-2 has been used as an alternative to autogenous bone grafts in dental and orthopedic fields, and in 2004, BMP-2-mixed collagen sponge (INFUSE Bone Graft; Medtronic, Minneapolis, MN, USA) was approved by the U.S. Food and Drug Administration (FDA) for the treatment of interbody spine fusion, fresh tibial fractures, and oral maxillofacial bone grafting procedures. More recently, BMP-2-mixed β-tricalcium phosphate (β-TCP)/hydroxyapatite (HAp) (CowellBMP; Cowellmedi Co., Ltd., Seoul, Korea) was approved by the Korea Food and Drug Administration (KFDA) for the use of bone grafts in oral bone defects such as severely resorbed alveolar ridge augmentations, tooth extraction sockets, alveolar bone loss, and maxillary sinus bone loss. In these approaches, BMP-2 is delivered by simply mixing or dip & dry methods with scaffolds or biomaterials [20,21]. However, simple mixing or dip and dry methods make it difficult to maintain the BMP-2 concentration at defect sites because of the short biological half-life and rapid diffusion by body fluid [21,22]. To overcome these drawbacks, BMP-2 must be incorporated or immobilized using materials to facilitate its sustained release and improve its efficacy.

Poly(lactic-co-glycolic acid((PLGA) is an FDA approved polymer and attractive for tissue engineering applications due to its biocompatibility and biodegradability. PLGA scaffolds have been used for engineering a wide range of tissues such as adipocytes, tissue engineering, and muscle tissue [23–25]. However, unmodified PLGA scaffolds could not control the sustained release of bioactive molecules such as growth factors. To address this issue, we functionalized the surface of scaffolds by using heparin molecules [16,17,19]. Due to strong negative charge and high binding affinity with various growth factors such as basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and BMP-2 [10,16,17,19,26], heparin-immobilized scaffolds can control the release of growth factors and improve growth factor efficacy [27,28].

In this study, we fabricated porous PLGA microspheres by a fluidic device method and then functionalized the surface of the porous PLGA microspheres with heparin-dopamine (Hep-DOPA). BMP-2 subsequently immobilized these functionalized porous microspheres *via* the electrostatic interactions between carboxyl and sulfate groups of heparin and a positively charged domain of BMP-2. In addition, we evaluated markers of osteoblast activity such as cell proliferation, alkaline phosphatase (ALP) activity, calcium deposition, and osteocalcin (OCN) and osteopontin (OPN) gene expression though sustained release of BMP-2 on BMP-2-immobilized porous microspheres *in vitro*.

2. Materials and methods

2.1. Materials

Poly D,L-lactide-co-glycolide (PLGA, 50:50, molecular weight: 30,000–60,000), poly vinyl alcohol (PVA, molecular weight: 13,000–23,000, 98% hydrolyzed), dichloromethane, gelatin from porcine skin, ascorbic acid, dexamethasone, β-glycerophosphate, and dopamine were from Sigma–Aldrich (St. Louis, MO, USA). *Escherichia coli*-derived recombinant human bone morphogenetic protein-2 (BMP-2) was donated by Cowellmedi (Busan, Korea). Heparin sodium (molecular weight: 12,000–15,000 g/mol) was from Acrose Organics (NJ, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS), and penicillin-streptomycin were from Gibco BRL (Rockville, MD, USA). Cell counting kit-8 (CCK-8) reagents were supplied from Dojindo, INC. (Kumamoto, Japan). BMP-2 ELISA kits were purchased from PeproTech Inc. (Rocky Hill, NJ, USA). All other chemicals were of the purest analytical grade available.

2.2. Fabrication of porous microsphere by heparin–dopamine (Hep-DOPA) and BMP-2

To immobilize heparin on the surface of PMS, heparin was chemically coupled with dopamine. Briefly, heparin (400 mg), EDAC (169.4 mg) and N-hydroxysuccinimide (102.2 mg) was reacted in 10 mL of 2-(N-morpholino) ethanesulfonic acid (MES) buffer (pH 4.5) for 10 min to activate carboxyl groups of heparin. Then, dopamine hydrochloride (90.8 mg) dissolved in 1 mL of MES buffer (pH 4.5) was added to the activated heparin, and allowed to react overnight. The mixture was dialyzed (MWCO = 2000, Spectrum®) against acidified distilled water (DW) for two days and lyophilized. To fabricate porous microspheres (PMS) immobilized with Hep-DOPA and BMP-2, a fluidic device method was used as previously described by Kim et al. [9]. PLGA (140 mg) was dissolved in 7 mL of dichloromethane solution. PVA (0.2 g) and gelatin (750 mg) were dissolved in 10 mL distilled water (DW). 500 μL PVA and 3 mL gelatin were added to a PLGA solution. The resulting solution was homogenized with a homogenizer (Ultra-Turrax T-25 Basic, IKA) at 13,500 rpm for 3 min to make water-in-oil (W/O) solutions. The W/O solution was introduced into the discontinuous phase. The continuous phase was PVA (1 wt%) solution. The discontinuous and continuous phases had flow rates of 0.05 mL/min and 2 mL/min, respectively. Harvested microspheres were immersed in warm DW at 45 °C under gentle stirring for 4 h to remove residual gelatin and were then washed with DW several times. PMS was lyophilized for 3 days. The Hep-DOPA and BMP-2 were then immobilized on PMS. To start, the Hep-DOPA was immobilized on PMS first. Briefly, 2 mg/mL Hep-DOPA was dissolved in 10 mM Tris buffer (pH 8.0). PMS was added to the Hep-DOPA solution and gently shaken overnight under dark conditions. Hep-DOPA immobilized on PMS is referred to as Hep-PMS in this study. Additionally, to immobilize the BMP-2 on PMS or Hep-PMS, microspheres were immersed a 0.1 M MES buffer (pH 5.6), followed by the addition of BMP-2 (500 ng/mL). After overnight incubation, BMP-2 in the supernatant was measured with an enzyme-linked immunosorbent assay (ELISA) kit. The amount of immobilized BMP-2 on microspheres was calculated as a difference in values of original BMP-2 solution (500 ng/mL) and in the supernatant solution. On the other hand, the collected microspheres (BMP-2 immobilized-PMS or -Hep-PMS) were rinsed with DW and then lyophilized for 3 days. BMP-2 immobilized on PMS or Hep-PMS were referred to as BMP-2/PMS or BMP-2/Hep-PMS, respectively, in this study (Fig. 1). Before using the PLGA microspheres for *in vitro* studies, the microspheres were sterilized with 70% Ethanol and UV light for 30 min.

2.3. Characterization of PMS and modified PMS

The morphologies of PMS, Hep-PMS, BMP-2/PMS, and BMP-2/Hep-PMS were evaluated using a scanning electron microscopy (SEM, S-2300, Hitachi, Tokyo, Japan). Specimens were coated with Pt using a sputter-coater (Eiko IB, Japan). The SEM was operated at a setting for 3 kV. The PMS, Hep-PMS, BMP-2/PMS, and BMP-2/Hep-PMS pore sizes were determined using the ImageJ program (Version 1.2). The average pore sizes of randomly selected porous microspheres ($n = 30$) for each group were determined by using the image analysis software "Image J" based on SEM images. X-ray photoelectron spectroscopy (XPS) on a K-alpha spectrometer (ESCALAB250 XPS System, Theta Probe AR-XPS System, Thermo Fisher Scientific, UK) with an Al Kα X-ray source (1486, 6 eV photons) at the Korean Basic Science Institute Busan Center was used