

Solid Free-Form Fabrication of Tissue-Engineering Scaffolds with a Poly(lactic-co-glycolic acid) Grafted Hyaluronic Acid Conjugate Encapsulating an Intact Bone Morphogenetic Protein-2/Poly(ethylene glycol) Complex

Jung Kyu Park, Jin-Hyung Shim, Kyung Shin Kang, Junseok Yeom, Ho Sang Jung, Jong Young Kim, Keum Hong Lee, Tae-Ho Kim, Shin-Yoon Kim, Dong-Woo Cho,* and Sei Kwang Hahn*

Despite wide applications of bone morphogenetic protein-2 (BMP-2), there are few methods to incorporate BMP-2 within polymeric scaffolds while maintaining biological activity. Solid free-form fabrication (SFF) of tissue-engineering scaffold is successfully carried out with poly(lactic-co-glycolic acid) grafted hyaluronic acid (HA-PLGA) encapsulating intact BMP-2/poly(ethylene glycol) (PEG) complex. HA-PLGA conjugate is synthesized in dimethyl sulfoxide (DMSO) by the conjugation reaction of adipic acid dihydrazide modified HA (HA-ADH) and PLGA activated with *N,N'*-dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide (NHS). BMP-2 is complexed with PEG, which is encapsulated within the PLGA domain of the HA-PLGA conjugate by SFF to prepare tissue-engineering scaffolds. In vitro release tests confirm the sustained release of intact BMP-2 from the scaffolds for up to a month. After confirmation of the enhanced osteoblast cell growth, and high gene-expression levels of alkaline phosphatase (ALP), osteocalcin (OC), and osterix (OSX) in the cells, the HA-PLGA/PEG/BMP-2 scaffolds are implanted into calvarial bone defects of Sprague Dawley (SD) rats. Microcomputed tomography (μ CT) and histological analyses with Masson's trichrome, and hematoxylin and eosin (H&E) staining reveal effective bone regeneration on the scaffolds of HA-PLGA/PEG/BMP-2 blends.

1. Introduction

Conventionally, tissue-engineering scaffolds have been fabricated by solvent casting/particulate leaching, phase separation, emulsion freeze-drying, gas foaming, and fiber bonding.^[1–6] Although these methods allow easy fabrication of three-dimensional (3D) scaffolds, it is difficult to control the pore size and porosity of scaffolds precisely. Solid free-form fabrication (SFF) has been successfully exploited to prepare 3D scaffolds with a porous and fully interconnected structure for tissue-engineering applications. The multihead deposition system (MHDS), one type of SFF technique, may be more advantageous than others in fabricating well-defined 3D scaffolds.^[7,8] The multihead dispensers used in MHDS enable the preparation of hybrid scaffolds with various compositions of biomaterials. However, a thermal process to fabricate polymeric scaffolds using MHDS cannot be exploited to encapsulate growth factors such as bone morphogenetic proteins (BMPs) within the scaffolds due to the thermal protein denaturation.

J. K. Park, J. Yeom, H. S. Jung, Prof. S. K. Hahn
Department of Materials Science and Engineering
Pohang University of Science and Technology (POSTECH)
San 31, Hyoja-dong, Nam-gu, Pohang, Kyungbuk 790-784, Korea
E-mail: skhanb@postech.ac.kr

J.-H. Shim, K. S. Kang, Prof. D.-W. Cho
Department of Mechanical Engineering
and/or Division of Integrative Biosciences and Biotechnology
POSTECH, Korea
E-mail: dwcho@postech.ac.kr

Prof. J. Y. Kim
Department of Mechanical Engineering
Andong National University
388 Songchun-dong, Andong, Kyungbuk 760-749, Korea

DOI: 10.1002/adfm.201100612

K. H. Lee, Prof. S.-Y. Kim
Kyungpook National University Hospital
Kyungpook National University
101 Dongin-dong 2 Ga, Jung-gu, Daegu, 700-422, Korea
Prof. T.-H. Kim
Department of Medicine
School of Medicine
Kyungpook National University
101 Dongin-dong 2 Ga, Jung-gu, Daegu, 700-422, Korea

BMPs are a group of secreted proteins belonging to the transforming growth factor- β (TGF- β) super-family.^[9] BMPs are osteoinductive and originally identified in demineralized bones.^[10] Among these proteins, BMP-2 has been particularly known to induce healing in segmental bone defects. Nowadays, BMP-2 is used for the clinical treatment of bone fractures and spinal fusion procedures.^[11,12] Because BMP-2 diffuses rapidly from the site of local administration, BMP-2 needs a biomaterial carrier to maximize its osteogenic effect. BMP-2 has been delivered with various carriers for ectopic and orthotopic bone regeneration, including inorganic materials,^[13,14] synthetic polymers,^[15,16] natural polymers,^[17–19] and composites of the three kinds of materials.^[20,21] The disadvantages associated with inorganic and synthetic carriers of BMP-2 include protein denaturation, poor biodegradability, and possible inflammatory responses.^[22] Accordingly, there have been extensive research efforts to develop biodegradable materials that can encapsulate intact BMP-2 for bone tissue-engineering applications such as bone repair and regeneration.

Herein, a new polymer-solution-dispensing MHDS was successfully developed to prepare well-defined 3D tissue-engineering scaffolds encapsulating BMP-2. The heated-air-blower/air-knife system played an important role in rapid evaporation of the remaining solvent in the polymer solution. It was reported that various biomolecules such as plasmid DNA, proteins, and carbohydrates could be solubilized in selected organic solvents in the presence of poly(ethylene glycol) (PEG).^[23,24] Proteins and PEG appeared to form stable nanosized complexes in polar organic solvents. On the basis of PEG-assisted protein stabilization in organic solvent, 3D biodegradable tissue-engineering scaffolds with poly(lactic-co-glycolic acid) grafted hyaluronic acid (HA-PLGA) encapsulating intact BMP-2/PEG complexes were fabricated by using SFF technology. After *in vitro* release tests of BMP-2, the scaffolds of HA-PLGA/PEG/BMP-2 blend systems were implanted into calvarial bone defects in Sprague Dawley (SD) rats, and assessed for bone regeneration by means of microcomputed tomography (μ CT) and histological analyses with Masson's trichrome, and hematoxylin and eosin (H&E) staining.

2. Results and Discussion

2.1. Synthesis and Characterization of HA-PLGA Conjugate

Biodegradable amphiphilic HA-PLGA conjugate was successfully synthesized for application in tissue-engineering scaffolds. HA was chemically modified by grafting adipic acid dihydrazide (ADH) onto the carboxyl group of HA in a mixed solvent of water and ethanol to prepare highly modified HA-ADH.^[25] The determination of peak assignment of HA-ADH in ^1H NMR spectra and the degree of HA modification were as described elsewhere.^[26] The degree of ADH modification increased by up to 85 mol% with increasing ethanol content in the mixed solvent. The addition of ethanol contributed to a high degree of ADH modification in HA-ADH. When the ADH content in HA-ADH was higher than ca. 80 mol%, HA-ADH became soluble in DMSO, which enabled the versatile chemical modification of HA in an organic solvent.^[25]

The carboxyl groups of PLGA were first activated with DCC and modified with NHS. HA-ADH in DMSO could be conjugated to the activated PLGA through the formation of an amide linkage between the amine groups of HA-ADH and the carboxyl terminal groups of PLGA. The resulting HA-PLGA conjugate was analyzed by using ^1H NMR spectroscopy. The peak assignment of PLGA and the degree of PLGA modification in HA-PLGA conjugate were determined as reported elsewhere.^[25] The degree of PLGA modification was quantitatively dependent on the amount of added PLGA. The ^1H NMR spectra showed the characteristic peaks of HA and PLGA, and confirmed the successful synthesis of HA-PLGA. The HA-PLGA conjugate with 30.5 mol% PLGA content was exploited for the preparation of barrier films and tissue-engineering scaffolds to encapsulate the BMP-2/PEG complex.

2.2. Solid Free-Form Fabrication of Tissue-Engineering Scaffolds

Tissue-engineering scaffolds encapsulating BMP-2/PEG complexes were prepared by means of SFF using solution-based MHDS. The heated air blower and suction systems were important for rapid evaporation of the remaining solvent, making 3D structure fabrication possible. After deposition of each layer, the scaffold was dried using heated air for 2 min. The temperature of extruded air passing through the air-knife system increased up to 37 °C, which appeared to be adequate to remove the remaining solvent without protein denaturation in the scaffolds (Figure 1). To fabricate 3D scaffolds with a controlled geometry and fully interconnected pores using solution-based MHDS, the viscosity of the feeding solution and the scan velocity of the nozzle should be optimized. If the solution viscosity is too low, it may be very difficult to fabricate well-defined structures and stack layers. In addition, if the solution viscosity is too high, the extrusion of polymer solution from a nozzle may be impossible. Two different organic solvents, dichloromethane and chloroform, were used for the MHDS. Since HA-PLGA conjugate dissolved more readily in dichloromethane than in chloroform, the feeding solution was prepared by dissolving HA-PLGA conjugate and BMP-2/PEG complex in dichloromethane. After solvent evaporation, the HA-PLGA/PEG/BMP-2 blend was redissolved in chloroform as its lower vapor pressure than dichloromethane enables a rapid solvent removal. Figure 2 shows the morphology of 3D tissue-engineering scaffolds of HA-PLGA conjugate encapsulating intact BMP-2/PEG complex fabricated by SFF technology. The scanning electron microscopy (SEM) image shown in Figure 2b reveals that the mean line width and pore size of the scaffolds were $250 \pm 20 \mu\text{m}$ and $300 \pm 20 \mu\text{m}$, respectively. A pore size in the range between $200\text{--}400 \mu\text{m}$ was reported to be optimal for effective bone regeneration.^[15,27,28] The lattice type of each layer was staggered and stacked up using layer-by-layer processes. We observed a stable nanosized complex of protein and PEG in the cross-sectional SEM image of the scaffold (Figure 2c). As reported elsewhere,^[23] protein can make a stable nanosized complex with PEG in polar organic solvents. HA brushes partitioned away from the BMP-2/PEG complexes, which matched well with the phase separation between PEG and HA domains in the aqueous solution.^[29] Interestingly, protein was reported

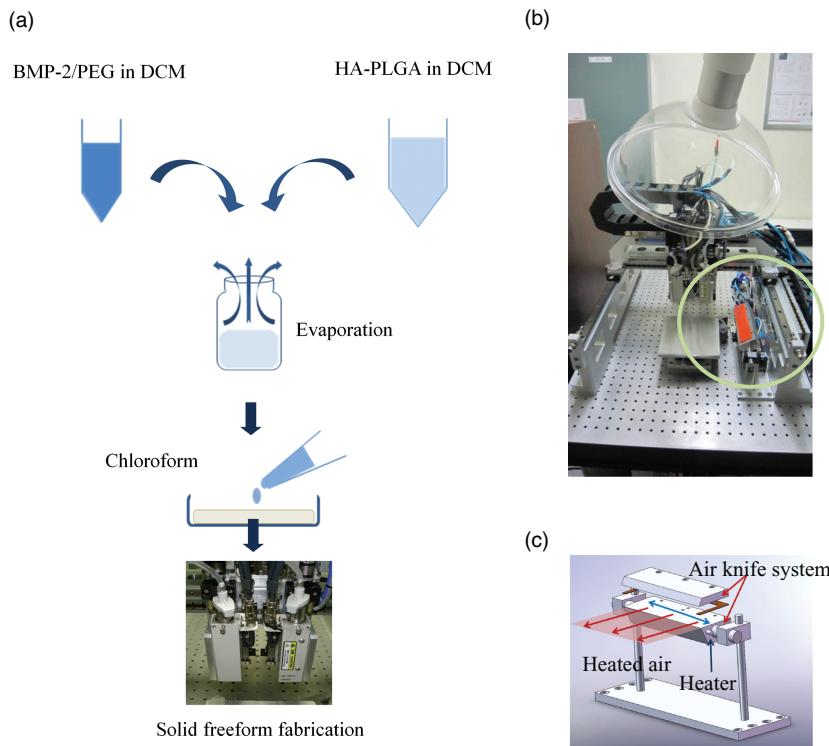


Figure 1. a) Schematic illustration of the preparation of feeding solution for SFF of tissue-engineering scaffolds using an MHDS, b) photograph of the solution-based MHDS with heated air blower/air knife (green circle) and suction systems, and c) schematic representation for the heated-air-blower/air-knife system in the MHDS.

to be placed in the PEG domain of the PEG/HA two phase systems.^[29] Although both HA and PEG are hydrophilic, the chemical structures of HA and PEG are quite different, which results in the phase separation. Dextran and PEG also showed similar phase separation behavior.^[30] The morphological

characteristics of HA-PLGA/PEG/BMP-2 scaffolds are schematically represented in Figure 2d. The amounts of BMP-2 and bovine serum albumin (BSA) encapsulated in the scaffolds were 20 ng mg⁻¹ and 20 µg mg⁻¹, respectively.

2.3. In Vitro Release of Proteins From HA-PLGA/PEG/Protein Scaffolds

In vitro release tests of BSA as a model protein and BMP-2 were carried out from the tissue-engineering scaffolds. As shown in Figure 3, the cumulative release profiles of BSA and BMP-2 from HA-PLGA/PEG/protein scaffolds were similar, with continuous release of proteins for up to a month. Interestingly, almost all the loaded proteins were released from the scaffolds, possibly due to the presence of PEG in HA-PLGA. PLGA has been widely used for controlled delivery of proteins in the form of microparticles.^[31] The conventional water-in-oil-in-water (W/O/W) method for the preparation of microparticles was reported to result in protein denaturation through exposure of the protein to the water–oil interface. To reduce protein denaturation during the formulation, nanosized complexes of proteins and PEG were dissolved in organic solvent, and directly spray-dried

to obtain PLGA microparticles encapsulating proteins.^[23] The release profile of BSA and BMP-2 from the scaffolds by SFF was well matched with that of BSA from PLGA microparticles in the literature.^[23] The immunological activity of released BMP-2 was confirmed by enzyme-linked immunosorption assay (ELISA).

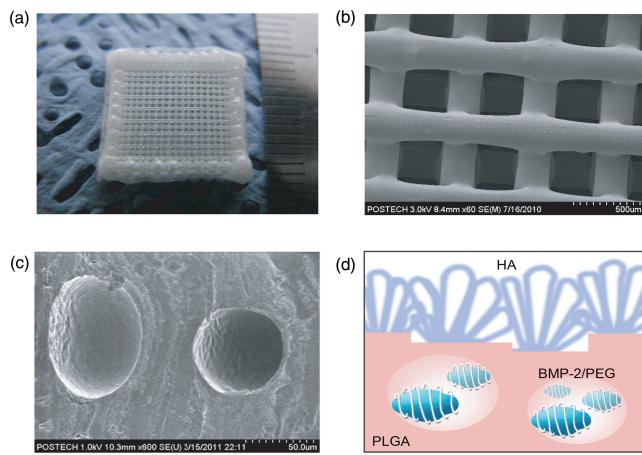


Figure 2. a) Photograph and b) SEM image of SFF HA-PLGA/PEG/BMP-2 scaffold at a magnification of $\times 60$. c) SEM image of the cross-section of the scaffold showing a pair of stable nanosized complexes of BMP-2 and PEG ($\times 300$). d) Schematic representation of HA-PLGA conjugate encapsulating BMP-2/PEG complex.

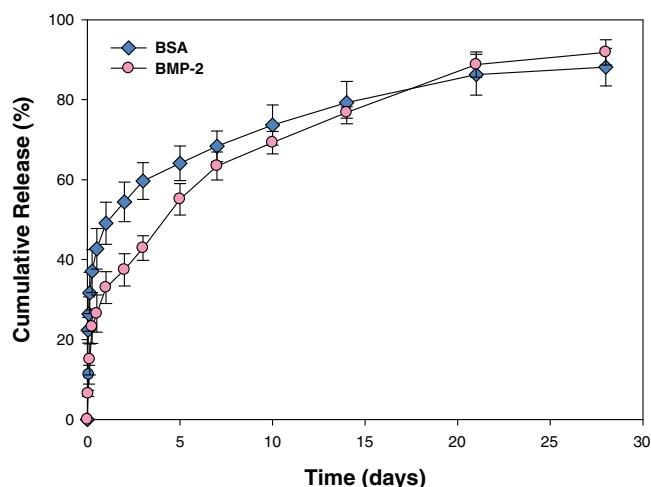


Figure 3. In vitro release profiles of BSA and BMP-2 from the SFF HA-PLGA/PEG/protein scaffolds.

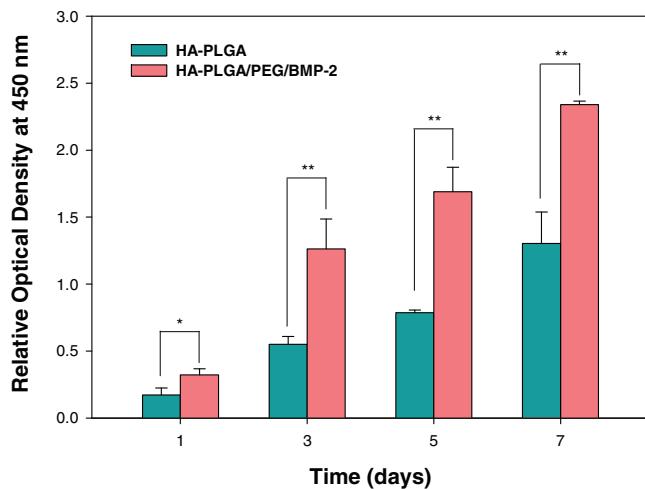


Figure 4. Relative optical density of MC3T3-E1 preosteoblasts cultured on HA-PLGA and HA-PLGA/PEG/BMP-2 scaffolds for up to 7 days (* $P < 0.05$, ** $P < 0.01$).

2.4. Effect of BMP-2 on the Growth of Osteoblasts

The effect of tissue-engineering scaffolds encapsulating BMP-2 on the growth of preosteoblasts was assessed by measuring the optical density (OD) of cells and the gene-expression levels of alkaline phosphatase (ALP), osteocalcin (OC), and osterix (OSX) in the cells. ALP is generally regarded as a biomarker of early osteogenesis,^[32] extracellular matrix mineralization can be characterized by OC expression, and OSX is known to regulate osteogenic differentiation.^[33] As shown in Figure 4, the relative OD increase of MC3T3-E1 preosteoblasts cultured on HA-PLGA/PEG/BMP-2 scaffold was always greater than that on HA-PLGA scaffold for up to seven days ($P < 0.05$ in 1 day, $P < 0.01$ in 3, 5, and 7 days). The results confirm the safety of fabricated scaffolds and the biological activity of BMP-2 released from the scaffold. The expression level of osteogenic mRNA was analyzed by real-time polymerase chain reaction (RT-PCR) over 14 days. Figure 5 shows the different gene expression levels of ALP, OC, and OSX of the HA-PLGA and HA-PLGA/PEG/BMP-2 scaffolds. ALP gene expression in the group of the HA-PLGA/PEG/BMP-2 scaffold was ca. 3.3 times higher than that in the HA-PLGA scaffold group in 14 days. HA-PLGA/PEG/BMP-2 scaffold also resulted in a 4.1-fold increase in the OC gene-expression level compared to the HA-PLGA scaffold. In addition, HA-PLGA/PEG/BMP-2 scaffold up-regulated OSX gene expression 2.3-fold over that of the HA-PLGA scaffold. From the results, it was concluded that all three target genes were more highly expressed when the cells were cultured on the HA-PLGA/PEG/BMP-2 scaffold than on the HA-PLGA scaffold. The results might be ascribed to the intact BMP-2 released from HA-PLGA/PEG/BMP-2 scaffold, which stimulated the differentiation of MC3T3-E1 preosteoblast cells.

2.5. In Vivo Bone Regeneration on HA-PLGA/PEG/BMP-2 Scaffold

The effect of HA-PLGA/PEG/BMP-2 scaffold on bone regeneration after implantation to the calvarial bone defects of SD

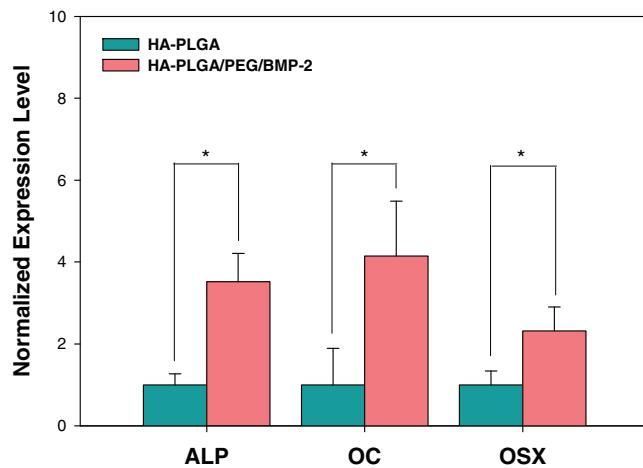


Figure 5. Normalized gene expression levels of ALP, OC, and OSX in MC3T3-E1 osteoblasts cultured on HA-PLGA and HA-PLGA/PEG/BMP-2 scaffolds for 2 weeks (* $P < 0.05$).

rats was assessed by using μ CT, and histological analyses with Masson's trichrome and H&E staining. Figure 6 shows μ CT images of regenerated bones on the control, HA-PLGA scaffold, and HA-PLGA/PEG/BMP-2 scaffold after implantation for four weeks. The regenerated bone volume (RBV) and the bone mineral density (BMD) were quantitatively analyzed using the 2.0⁺ ABA Microview software for the μ CT system. The RBV and BMD when using the HA-PLGA/PEG/BMP-2 scaffold were statistically higher than those from the control and HA-PLGA scaffolds (Figure 7). The RBV reflects the quantity of regenerated bone and the BMD reflects the quality of regenerated bone in the entire bone-defect region. The normal bone mineral density is known to be in the range of ca. 1,130–1,190 mg mL⁻¹.^[34] Effective bone regeneration by the HA-PLGA/PEG/BMP-2 scaffold was also confirmed by means of histological analyses with Masson's trichrome and H&E staining (Figure 8). In both the HA-PLGA and HA-PLGA/PEG/BMP-2 scaffolds, calcified bones were not observed near the scaffolds over four weeks. However, as shown in Figure 8e, there was plenty of osteoid formation. Osteoid is the organic portion of the bone matrix that forms before the maturation of bone tissue,^[35] and is composed of type 1 collagen, chondroitin sulfate, and osteocalcin. While bone regeneration was poor on the top of the HA-PLGA scaffold, relatively thick mineralized bone tissue was formed on the HA-PLGA/PEG/BMP-2 scaffold. In addition, bone also formed within the HA-PLGA/PEG/BMP-2 scaffold. Considering all these results, HA-PLGA/PEG/BMP-2 scaffolds with well-defined microstructures were thought to be effectively exploitable for various bone-tissue engineering applications.

3. Conclusions

Tissue-engineering scaffolds of HA-PLGA conjugates encapsulating BMP-2/PEG complex were successfully developed by SFF using solution-based MHDS with a line-width of $250 \pm 20 \mu\text{m}$ and a pore size of $300 \pm 20 \mu\text{m}$. According to in vitro release tests, BMP-2 was controllably released from the scaffold for up

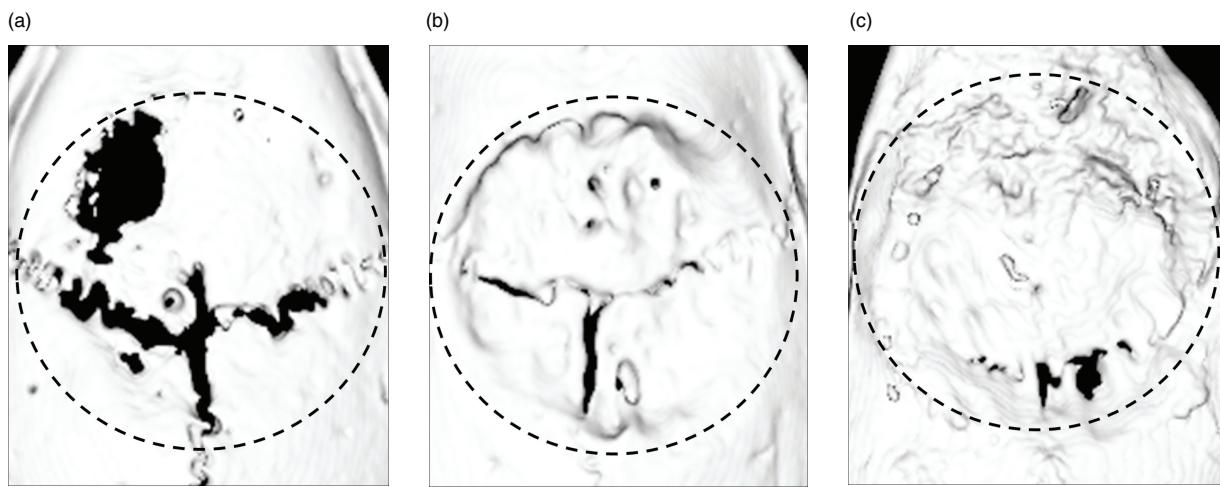


Figure 6. μ CT images of regenerated bones on a) the control, b) HA-PLGA scaffold, and c) HA-PLGA/PEG/BMP-2 scaffold after implantation for 4 weeks. Dashed circular lines represent the bone-defect area.

to a month, while maintaining the immunological activity, which might be ascribed to the PEG being complexed with BMP-2. In vitro assessment of optical cell density, and the gene-expression levels of OSX, OC, and ALP in MC3T3-E1 preosteoblast cells

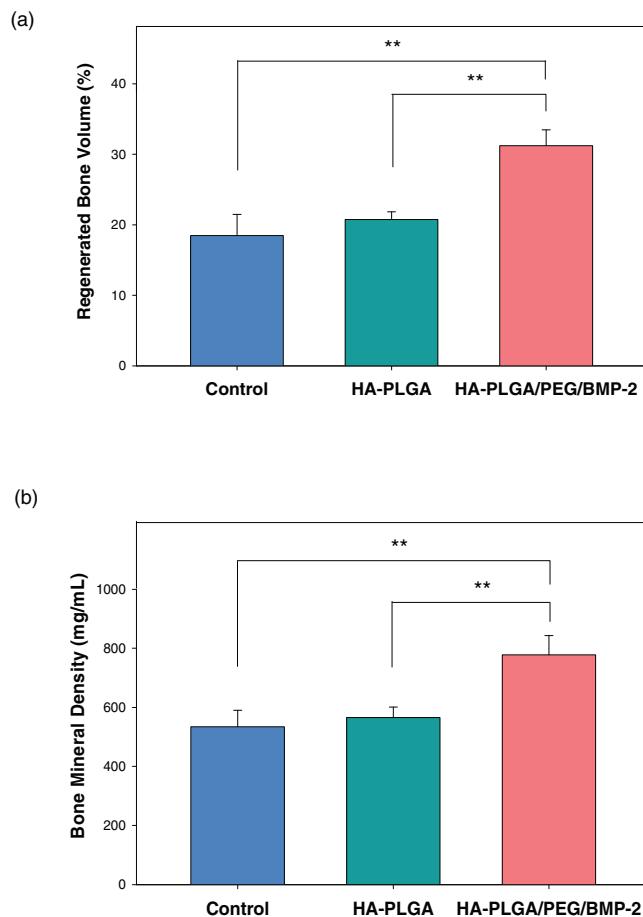


Figure 7. a) RBV (%) and b) BMD of the control, HA-PLGA scaffold and HA-PLGA/PEG/BMP-2 scaffold by μ CT analysis (** P < 0.01).

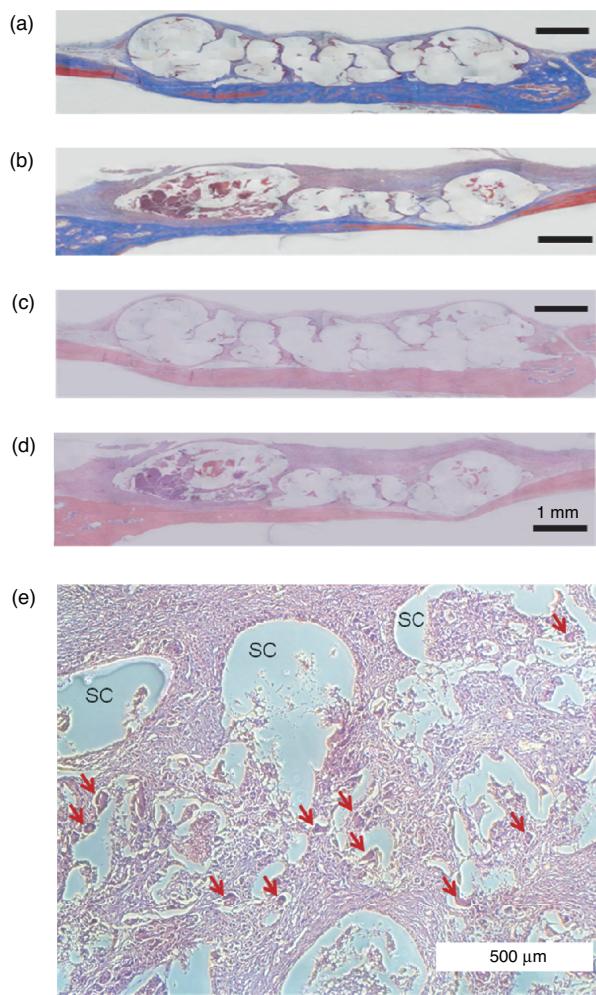


Figure 8. Histological analysis of regenerated bones with a,b) Masson's trichrome, and c,d) H&E staining 4 weeks after implantation of a,c) HA-PLGA scaffold and b,d) HA-PLGA/PEG/BMP-2 scaffold to the calvarial bone defects in SD rats. e) Magnified image of (d) showing osteoid formation with arrows (SC: scaffold).

confirmed the more efficient cell growth on HA-PLGA/PEG/BMP-2 scaffold than on HA-PLGA scaffold. Furthermore, after implantation into the calvarial bone defects in SD rats for four weeks, μCT and histological analyses with Masson's trichrome and H&E staining revealed active bone regeneration on the HA-PLGA/PEG/BMP-2 scaffold. The BMP-2 released from the scaffold was thought to contribute to enhanced bone regeneration. The solution-based SFF using MHDS might be exploited as a novel approach to fabricate various tissue-engineering scaffolds.

Experimental Section

Materials: HA with a molecular weight (MW) of 20,000 was purchased from Lifecore Co. (Chaska, MN). ADH and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC hydrochloride) were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). PEG with a MW of 4,000 was purchased from Daejung Chemicals and Metals Co. (Incheon, Korea). PLGA with a MW of 66,000–107,000, NHS, DCC, phosphate-buffered saline (PBS) tablet, BSA, β-glycerophosphate, and ascorbic acid were purchased from Sigma-Aldrich (Milwaukee, WI). BMP-2 and BMP-2 ELISA kit were purchased from Peprotech (Rocky Hill, NJ). Ethanol, HCl, sodium hydroxide, acetonitrile, dimethyl sulfoxide (DMSO), dichloromethane, and chloroform were obtained from Junsei Chemicals (Tokyo, Japan). Trizol and SuperScript synthesis systems were purchased from Invitrogen (Carlsbad, CA), and SYBR Green PCR premix Ex Taq from Takara Co. (Ohtsu, Japan). MC3T3-E1 cells were purchased from Riken Cell Bank (Tsukuba, Japan). All reagents were used without further purification.

Synthesis of HA-PLGA Conjugate: HA-PLGA conjugate was synthesized in DMSO by the conjugation of highly modified HA-ADH with PLGA activated with NHS and DCC. To increase the degree of ADH modification in HA-ADH, the protocol for HA-ADH synthesis by Luo et al.^[26] was slightly modified as follows: HA (100 mg) was dissolved in 20 mL of water to prepare an HA solution of 5 mg mL⁻¹. A 40 times molar excess of solid ADH (1.736 g) was added to the solution and dissolved completely by mixing for 10 min. The pH of the mixed solution was adjusted to 4.8 by the addition of 1.0 N HCl. Then, ethanol (20 mL, 50 vol%) was added and mixed for 30 min. After that, four time molar excess of solid EDC (0.191 g) was added. The pH of the mixed solution was maintained at a value of 4.8 by the addition of 1.0 N HCl. The reaction was stopped after 2 h by raising the pH of the reaction solution to 7.0 with 1.0 N NaOH. The reaction solution was poured into a prewashed dialysis membrane tube (MW cutoff of 7,000) and dialyzed against a large excess amount of 100 mM NaCl solution, followed by dialysis against 25 vol% ethanol and pure water. The resulting solution was finally lyophilized for 3 days. The purity of HA-ADH was determined by GPC (Waters, Milford, MA) and the degree of ADH modification was analyzed by using ¹H NMR spectroscopy (DPX300, Bruker, Germany).^[26] PLGA (4g, MW = 66k–107k) was dissolved in DMSO (10 mL) and activated by the addition of DCC (62 mg) and NHS (35 mg). Then, the prepared HA-ADH (35 mg, MW = 20,000, 83 mol% ADH) was dissolved in 5 mL DMSO and mixed with the activated PLGA solution for 12 h. The resulting HA-PLGA conjugate was recovered by dialysis against an excess amount of water, freeze-dried for 3 days, and analyzed by using ¹H NMR spectroscopy to determine the degree of PLGA modification in the HA-PLGA conjugate.^[25]

Solid Free-Form Fabrication of Tissue-Engineering Scaffolds: Tissue-engineering scaffolds were prepared by SFF using MHDS with a feed solution prepared by mixing HA-PLGA conjugate with BSA as a model protein or BMP-2/PEG complex in dichloromethane. In detail, BSA (10 mg) or BMP-2 (10 µg) was blended with PEG (50 mg) in 2 mL dichloromethane, which was mixed with HA-PLGA conjugate (450 mg) in 2 mL of dichloromethane using a vortex mixer (Vortex Genie 2; Scientific Industries Inc., Bohemia, NY). Then, the dichloromethane

was evaporated in a fume hood for a day to obtain a HA-PLGA/PEG/BSA or HA-PLGA/PEG/BMP-2 blend. The homogeneously mixed blend was dissolved again in chloroform to prepare a highly viscous solution at a concentration of 100 wt% for scaffold fabrication via MHDS. The mixed solution was moved into a syringe and dispensed by applying a pneumatic pressure of 650 kPa. In addition, the air-knife system for heated air blowing and the solvent suction system were specially designed and installed in the MHDS for the polymer-solution-based SFF. The pneumatic pressure supplied to the air blower was 250 kPa and the temperature of the heater was 330°C. After freeze-drying and gold-coating with a sputter coater for 60 s, the morphology of the prepared scaffolds was analyzed with an SEM (JSM-5300, JEOL, Japan) operated at 15 kV.

In Vitro Release Tests of BSA and BMP-2: The tissue-engineering scaffold of HA-PLGA/PEG/protein blend was inserted into a vial containing 10 mL PBS solution, which was placed in a shaking incubator at 37 °C. At the predetermined time intervals, the solution was sampled to determine the amount of released protein by HPLC for BSA and ELISA for BMP-2, respectively. For the ELISA of BMP-2, a standard curve was prepared in the range from 62.5 to 4,000 pg mL⁻¹. The same volume of fresh PBS solution was replenished at each sampling time. Triplicates were carried out for the in vitro release tests.

In Vitro Cell-Culture Tests on The Fabricated Scaffolds: MC3T3-E1 preosteoblasts were seeded onto both HA-PLGA scaffold and HA-PLGA/PEG/BMP-2 scaffold by pipetting 10 µL of the cell suspension containing 1 × 10⁵ cells.^[15] Before seeding cells, scaffolds were immersed in deionized water for 2 days, frozen at -70 °C overnight, and freeze-dried at -90 °C for 3 days to remove the remaining toxic solvent in the scaffold. After preincubation at 37 °C and 5% CO₂ for 1 h, the seeded cells were cultured in α-minimum essential medium (α-MEM) supplemented with 10 vol% fetal bovine serum (FBS) and 1 wt% penicillin at 37 °C in a 5% CO₂ incubator for 7 days. The culture media was refreshed every other day. The number of cells on the scaffolds was measured in 1, 3, 5, and 7 days using a cell-counting kit-8 (CCK-8, Dojindo, Tokyo, Japan).^[36] To induce the differentiation of preosteoblasts into osteoblasts, 10 mM β-glycerophosphate and 50 µg mL⁻¹ of ascorbic acid were added to the cell-culture medium. Total RNA was prepared from cultured cells using Trizol. The cDNAs were synthesized from 1 µg of RNA using SuperScript synthesis system. Quantitative RT-PCR was performed to present the relative expression from the threshold cycle (Ct) numbers for the different samples on the LightCycler 2.0 (Roche Diagnostics, Mannheim, Germany) using SYBR Green PCR premix Ex Taq. The amplification reaction was performed for 45 cycles with denaturation at 95 °C for 10 min, followed by annealing at 95 °C for 15 s, and extension and detection at 60 °C for 1 min. The expression level of each gene was normalized against that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primer sequences used for GAPDH, ALP, OC, and OSX were as follows: GAPDH (sense, 5'-GCACAGTCAGGCCGAGAAT-3'; antisense, 5'-GCCTTCTCATGGTGGTAA-3'), ALP (sense, 5'-GTGCCCTGACTGAGGCTGTC-3'; antisense, 5'-GGATCATCGTCTGCTCAC-3'), OC (sense, 5'-CCGGGAGCAGTGTGAGCTTA-3'); antisense, 5'-TAGATG CGTTGTAGGGCGTC-3'), and OSX (sense, 5'-CCCTTCTCAAGCACCAATGG-3'; antisense, 5'-AGGGTGGGTAGTCATTTCATAG-3').

In Vivo Bone Regeneration Tests on HA-PLGA/PEG/BMP-2 Scaffold: Two kinds of scaffolds, HA-PLGA and HA-PLGA/PEG/BMP-2 scaffolds (ϕ = 8 mm × 1.5 mm), were implanted to the calvarial bone defects with 1.5 mm thickness and a diameter of 8 mm in SD rats at a mean age of 6 weeks, and covered with HA-PLGA barrier film.^[25,37] HA-PLGA film was prepared as described elsewhere.^[25] After bone regeneration for 4 weeks, the bone-defect region was retrieved for further investigation by μCT (eExplore Locus SP, GE Healthcare), and histological analyses with Masson's trichrome and H&E staining. The bone specimens were placed in 10 vol% formalin and embedded in paraffin after decalcification with ethylenediamine tetraacetic acid (EDTA).

Statistical Analysis: The data are expressed as means ± SD from several separate experiments. Statistical analysis was carried out via

the one-way analysis of variance (ANOVA) tests using the software of MINITAB version 14.2. $P < 0.05$ was considered statistically significant.

Acknowledgements

J.K.P. and J.-H. S. contributed equally to this work. This study was supported by a grant of the Korea Health 21 R&D Project, Ministry of Health & Welfare, Republic of Korea (A084132). This research was also supported by the Converging Research Center Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2009-0081871), and by the National Research Foundation of Korea (NRF) grant funded by the Korean government (MEST) (No. 2010-0018294). Note: This article has been amended on August 9, 2011 to update Figure 1 and 6, which were corrupted in the version originally published online.

Received: March 18, 2011

Revised: April 21, 2011

Published online: July 4, 2011

- [1] S. C. Baker, G. Rohman, J. Southgate, N. R. Cameron, *Biomaterials* **2009**, *30*, 1321.
- [2] S. W. Choi, J. Xie, Y. Xia, *Adv. Mater.* **2009**, *21*, 2997.
- [3] J. Guan, J. J. Stankus, W. R. Wagner, *J. Control. Release* **2007**, *120*, 70.
- [4] H. Wang, Y. Li, Y. Zuo, J. Li, S. Ma, L. Cheng, *Biomaterials* **2007**, *28*, 3338.
- [5] A. T. Mehlhorn, J. Zwingmann, G. Finkenzeller, P. Niermeyer, M. Dauner, B. Stark, N. P. Südkamp, H. Schmal, *Tissue Eng. Part A* **2009**, *15*, 1159.
- [6] F. T. Moutos, L. E. Freed, F. Guilak, *Nat. Mater.* **2007**, *6*, 89.
- [7] S. J. Holister, *Nat. Mater.* **2005**, *4*, 518.
- [8] J. Y. Kim, J. J. Yoon, E. K. Park, D. S. Kim, S. Y. Kim, D. W. Cho, *Biofabrication* **2009**, *1*, 015002.
- [9] J. D. Kretlow, S. Young, L. Klouda, M. Wong, A. G. Mikos, *Adv. Mater.* **2009**, *21*, 3368.
- [10] M. R. Urist, H. Nogami, *Nature* **1970**, *225*, 1051.
- [11] S. D. Boden, T. A. Zdeblick, H. S. Sandhu, S. E. Heim, *Spine* **2000**, *25*, 376.
- [12] G. E. Riedel, A. Valentin-Opran, *Orthopedics* **1999**, *22*, 663.
- [13] P. Q. Ruhe, H. C. Kroese-Deutman, J. G. Wolke, P. H. Spauwen, J. A. Jansen, *Biomaterials* **2004**, *25*, 2123.
- [14] H. J. Seeherman, K. Azari, S. Bidic, L. Rogers, X. J. Li, J. O. Hollinger, J. M. Wozney, *J. Bone Joint Surg. Am.* **2006**, *88*, 1553.
- [15] J. W. Lee, K. S. Kang, S. H. Lee, J. Y. Kim, B. K. Lee, D. W. Cho, *Biomaterials* **2011**, *32*, 744.
- [16] H. Schliephake, H. A. Weich, C. Dullin, R. Gruber, S. Frahse, *Biomaterials* **2008**, *29*, 103.
- [17] J. Kim, I. S. Kim, T. H. Cho, K. B. Lee, S. J. Hwang, G. Tae, I. Noh, S. H. Lee, Y. Park, K. Sun, *Biomaterials* **2007**, *28*, 1830.
- [18] H. D. Kim, R. F. Valentini, *J. Biomed. Mater. Res.* **2002**, *59*, 573.
- [19] M. P. Ludolf, J. A. Hubbel, *Nat. Biotechnol.* **2005**, *23*, 47.
- [20] Y. Takahashi, M. Yamamoto, Y. Tabata, *Biomaterials* **2005**, *26*, 4856.
- [21] Y. C. Fu, H. Nie, M. L. Ho, C. K. Wang, C. H. Wang, *Biotechnol. Bioeng.* **2008**, *99*, 996.
- [22] H. Seeherman, J. Wozney, R. Li, *Spine* **2002**, *27*, S16–23.
- [23] H. Mok, T. G. Park, *Eur. J. Pharm. Biopharm.* **2008**, *70*, 137.
- [24] H. Mok, H. J. Kim, T. G. Park, *Int. J. Pharm.* **2008**, *356*, 306.
- [25] J. K. Park, J. Yeom, E. J. Oh, M. Reddy, J. Y. Kim, D. W. Cho, H. P. Lim, N. S. Kim, S. W. Park, H. I. Shin, D. J. Yang, K. B. Park, S. K. Hahn, *Acta Biomater.* **2009**, *5*, 3394.
- [26] Y. Luo, K. R. Kirker, G. D. Prestwich, *J. Control. Release* **2000**, *69*, 169.
- [27] K. Kim, A. Yeatts, D. Dean, J. P. Fisher, *Tissue Eng. Part B* **2010**, *16*, 523.
- [28] L. Shor, S. Güceri, R. Chang, J. Gordon, Q. Kang, L. Hartsock, Y. An, W. Sun, *Biofabrication* **2009**, *1*, 015003.
- [29] K. Moriyama, T. Ooya, N. Yui, *J. Control. Release* **1999**, *59*, 77.
- [30] O. Franssen, R. J. H. Stenekes, W. E. Hennink, *J. Control. Release* **1999**, *59*, 219.
- [31] N. Saito, T. Okada, H. Horiuchi, H. Ota, J. Takahashi, N. Murakami, M. Nawata, S. Kojima, K. Nozaki, K. Takaoka, *Bone* **2003**, *32*, 381.
- [32] Y. F. Chou, J. Y. Dunn, B. M. Wu, *J. Biomed. Mater. Res. B* **2005**, *75*, 81.
- [33] M. M. Ali, T. Yoshizawa, O. Ishibashi, A. Matsuda, M. Ikegami, J. Shimomura, H. Mera, K. Nakashima, H. Kawashima, *J. Cell Sci.* **2007**, *1*, 2565.
- [34] A. Koistinen, S. S. Santavirta, H. Kröger, R. Lappalainen, *Biomaterials* **2005**, *26*, 5687.
- [35] D. W. Lee, K. T. Koo, Y. J. Seol, Y. M. Lee, Y. Ku, I. C. Rhyu, C. P. Chung, T. I. Kim, *J. Periodontal Implant Sci.* **2010**, *40*, 132.
- [36] J. H. Shim, J. Y. Kim, J. K. Park, S. K. Hahn, J. W. Rhee, S. W. Kang, S. H. Lee, D. W. Cho, *J. Biomater. Sci. Polym. Ed.* **2010**, *21*, 1069.
- [37] J. K. Park, Y. J. Kim, J. Yeom, J. H. Jeon, G. C. Yi, J. H. Je, S. K. Hahn, *Adv. Mater.* **2010**, *22*, 4857.