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The effect of titanium with heparin/BMP-2 complex for improving osteoblast activity

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ABSTRACT

The objective of this study was to investigate the enhanced osteoblast activity of MG-63 cells cultured on titanium (Ti) with a heparin/BMP-2 (Hep/BMP-2) complex. The Ti substrates were initially modified by chemical grafting poly-L-lysine (PLL) using condensing agent, followed by immobilizing the heparin/BMP-2 complex to the PLL-grafted Ti substrate *via* electrostatic interactions. The surface modification of Ti substrates with PLL and/or Hep/BMP-2 complex were confirmed with scanning electron microscopy, contact angle measurements, and X-ray photoelectron spectroscopy. Immobilized BMP-2 was released from the Hep/BMP-2/Ti substrate in a sustained manner. *In vitro* studies revealed that osteoblasts grown on Hep/BMP-2/Ti substrate increased ALP activity, calcium deposition, ALP and osteocalcin levels as compared to those grown on pristine Ti or PLL-Ti. These results indicated that heparin/BMP-2 complex immobilized Ti substrate can be useful to effectively improve osteoblast activity.

1. Introduction

Commercially pure titanium (Ti) with excellent biocompatibility and bioactivity is widely used as an implant material in orthopedic and dental applications. Ti has low toxicity, favorable mechanical properties, and low corrosion rates compared to those of other metals (Buser, Belser, & Lang, 1998; Steinemann, 1998; Zhu et al., 2007). However, insufficient bioactivity of the Ti surface frequently caused the risk of implant loosening through time and low combination strength (Puleo & Nanci, 1999). Many researchers including orthopedic surgeons, dentists, and biomaterial experts have modified the Ti surface *via* hydroxyapatite coatings, immobilization of biomolecules and control of surface topography (Miron, Oates, Molenberg, Dard, & Hamilton, 2010; Piskounova, Forsgren, Brohede, Engqvist, & Strømme, 2009; Shi, Neoh, Kang, Poh, & Wang, 2009a; Shi, Neoh, Kang, Poh, & Wang, 2009b). For example, modifying the Ti surface with a peptide coating containing Arg-Gly-Asp (RGD) or lysine-arginine-serine-arginine (KRSR) to mediate the cellular response is an active area of research to enhance osseointegration and increase osteoblast adhesion (Balasundaram & Webster, 2007; Le Guillou-Buffello et al., 2008; Michael et al., 2009; Oya et al., 2009; Schuler et al., 2009). Therefore, the immobilization of bioactive proteins or peptides on the Ti surfaces is of tremendous interest owing to the enhancement of osteoblast activity and osteointegration after implant insertion.

In order to improve osteoblast activity and osteointegration after implant insertion, bone morphogenic proteins (BMPs), which play important roles in bone and cartilage regeneration, have been widely used. Among BMP family members, BMP-2 is a potent osteoinductive factor that plays key roles during bone formation (Karageorgiou et al., 2004; Liu, Enggist, Kuffer, Buser, & Hunziker, 2007). Previous studies have shown that BMP-2 stimulates differentiation of mesenchymal stem cells into osteogenic cells to repair facial skeleton defects, large bone defects, and fuse the spine (Boyne, 2001; Herford & Boyne, 2008; Maegawa et al., 2007). Also, BMP-2 is usually delivered by mixing it with collagen, demineralized bone matrix, or hydroxyapatite composites (Groeneveld & Burger, 2000; Katz, Nataraj, Jaw, Deigl, & Bursac, 2009; van den

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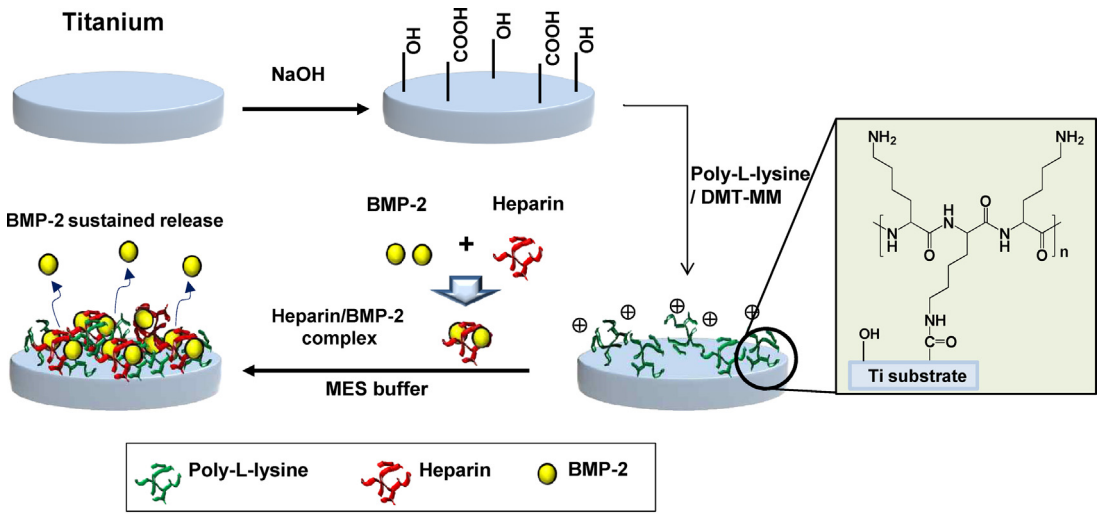

Fig. 1. Schematic diagram for the immobilization of heparin/bone morphogenic protein-2 (BMP-2) complex to poly-L-lysine (PLL) grafted Ti substrate.

Beucken et al., 2006). However, simple mixing or dip & 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 (Takahashi, Yamamoto, & Tabata, 2005). Thus, BMP-2 must be incorporated using biomaterials which can minimize the rapid diffusion or release from substrates and to improve its efficacy (Yang et al., 2012).

In our previous reports, we have used heparin to control BMP-2 delivery in a sustained manner. Due to its strong negative charge, heparin can bind to various growth factors such as basic fibroblast growth factor (bFGF), vascular endothelial growth factor, and BMP-2 (Sasisekharan, Ernst, & Venkataraman, 1997). Heparin grafted on biomaterials has also been used to immobilize growth factors through the growth factor binding domain (Nillesen et al., 2007; Steffens et al., 2004; Wissink, Beernink, Poot, et al., 2000; Wissink, Beernink, Scharenborg, et al., 2000). Therefore, biomaterials containing heparin can control the release of growth factors (Ishibe et al., 2009; Perets et al., 2003).

In this study, we investigated whether heparin/BMP-2 complex immobilized Ti substrates can promote osteoblast activity. In order to immobilize heparin/BMP-2 complex, Ti substrates were treated with alkali solution, and followed by chemically conjugating the positively charged Poly-L-lysine (PLL) to the surface of Ti substrates. Then, the heparin/BMP-2 complex was immobilized onto the surface of PLL-grafted Ti substrates using a layer-by-layer technique by which to directly immobilize oppositely charged nanoparticles and polymers (Fig. 1). In addition, we evaluated osteoblast activity such as cell proliferation, alkaline phosphatase (ALP) activity, the amount of calcium deposited, and ALP and osteocalcin gene expression *in vitro*.

2. Materials and methods

2.1. Materials

Ti discs (diameter × height: 1.2 cm × 0.3 cm) were purchased from Gai-Metal Co. Ltd. (Seoul, Korea). Poly-L-lysine (PLL, Mw = 30,000), 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM) and 2-(N-morpholino)ethanesulfonic acid (MES) were supplied by Sigma–Aldrich (St. Louis, MO, USA). Heparin sodium (molecular weight: 12,000–15,000 g/mol) was purchased from Acrose Organics (NJ, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), phosphate-buffered saline (PBS), and penicillin-streptomycin were purchased from Gibco BRL (Rockville, MD, USA). BMP-2 and an enzyme-linked immunosorbent assay (ELISA) kit were supplied by R&D Systems Inc. (Minneapolis, MN, USA). All other chemicals were of the purest analytical grade available.

2.2. PLL grafted Ti substrates

To graft the PLL onto the surface of pristine Ti substrates, the Ti substrates were treated with a 1 N NaOH solution and chemically modified with PLL in the presence of DMT-MM which is efficient condensing agent for chemical conjugation of carboxyl and amine group (Kunishima et al., 1999). In brief, pristine Ti substrates were cleaned with absolute ethanol using a bath-type ultrasonicator for 1 h. Then, the Ti substrates were soaked in 1 N NaOH solution at 60 °C for 24 h, washed with distilled water at 60 °C, and dried at 50 °C. PLL was chemically grafted to the surface of alkali-treated Ti substrates. Alkali-treated Ti substrates were immersed in 0.1 M MES buffer (pH 5.6) containing PLL (2 mg/mL) and DMT-MM (3 mg). The reaction was carried out for 24 h at room temperature (RT), washed with distilled water, and dried at RT. The PLL-grafted Ti substrates were designated as PLL/Ti.

2.3. Heparin/BMP-2 complex immobilized PLL/Ti substrates

The heparin/BMP-2 complex was immobilized on PLL/Ti substrates. Briefly, 10 or 50 ng/mL of BMP-2 was added to heparin (1 mg/mL) dissolved in 0.1 M MES buffer (pH 5.6) and simply mixed for 30 min. Then, the PLL/Ti substrates were immersed in the heparin/BMP-2 complex solution and allowed to immobilize heparin/BMP-2 complex onto the surface of PLL/Ti *via* electrostatic interactions. After 24 h, heparin/BMP-2 complex immobilized PLL/Ti substrates were carefully washed with distilled water and dried. The heparin/BMP-2 (10 ng) complex or heparin/BMP-2 (50 ng) complex immobilized PLL/Ti were designated as Hep/BMP-2 (10 ng)/Ti or Hep/BMP-2 (50 ng)/Ti, respectively.

2.4. Scanning electron microscopy (SEM)

The surface morphology of the pristine Ti and surface-modified Ti substrates (PLL/Ti, Hep/BMP-2 (10 ng)/Ti, and Hep/BMP-2 (50 ng)/Ti) were observed with a variable-pressure field emission scanning election microscope (FE-SEM; SUPRA-55VP, Carl Zeiss, Germany) at the Korea Basic Science Institute Chuncheon Center. Ti discs were coated with gold using a sputter-coater (Eiko IB, Japan).

The SEM was operated at 5 kV to observe the Ti disk surface morphology.

2.5. X-ray photoelectron spectroscopy (XPS)

The chemical composition of the pristine Ti and surface-modified Ti substrates was determined by a XPS K-Alpha spectrometer (Thermo Electron, Rockford, IL, USA) using a Al Kα source at 1486.6 eV. XPS spectra were generally recorded from C, O, N, and 2p Ti for 1s electrons. The C1 hydrocarbon peak at 284.84 eV was used as a reference for all binding energies.

2.6. Contact angle

To evaluate the general wettability of the surface, the contact angle was measured by the sessile drop method using a video contact angle instrument (Phoenix 150, SEO, Seoul, Korea) at RT. In brief, Ti or a surface-modified Ti substrate was placed on the stage, and then a water drop (3 μL) was placed onto a stage containing the Ti or surface-modified Ti substrates using a syringe and a stainless-steel needle. The contact angle was calculated as the angle between the Ti surface and the tangent of the liquid on the Ti surface using the AutoFAST algorithm and image analysis software.

2.7. In vitro bioactivity test

To evaluate the BMP-2 release profiles from Hep/BMP-2 (10 ng)/Ti and Hep/BMP-2 (50 ng)/Ti, each specimen was soaked in a 15 mL conical tube with 3 mL PBS. The tubes were incubated at 37 °C with gentle shaking at 100 rpm. At predetermined time intervals, the supernatant was collected and replaced with fresh PBS buffer. The amount of BMP-2 was determined using a commercially available ELISA kit according to the manufacturer's instructions. Sample absorbance was measured using a microplate reader at 495 nm.

2.8. Cell and culture condition

To evaluate the cellular response on the pristine Ti and surface-modified-Ti substrates, we analyzed cell proliferation, ALP activity, calcium content, and gene expression. MG 63 cells (human osteosarcoma cell line, Korea Cell Line Bank, KCLB No. 21427, Seoul, Korea) were cultured with DMEM supplemented with 10% FBS, 50 μg/mL ascorbic acid, 10 nM dexamethasone, and 10 mM β-glycerolphosphate in the presence of 100 U/mL penicillin and 100 μg/mL streptomycin in 75-T cell culture plates at 37 °C in a humidified atmosphere supplied with 5% CO_2. Cells at the fifth passage were used. The specimens were sterilized with 70% EtOH for 3 min and rinsed with PBS prior to use.

2.9. Cytotoxicity test and live/dead assay

A cytotoxicity test of the specimens was conducted using the DMEM extraction method under the ISO/EN 10993 Part 5 Guidelines to establish the possible toxic effects of the specimens. The specimens were immersed in DMEM medium and incubated for 24 h at 37 °C. After the incubation, extracted DMEM medium was added to MG 63 cells seeded at a density of 5×10^4 cells/wells in 96-well plates. The control contained DMEM medium without specimens. After 24 and 48 h incubations, cell viability and proliferation were measured with a Cell Counting Kit-8 (CCK-8, Dojindo, Gaithersburg, MD, USA) in accordance with the manufacturer's instructions. The DMEM medium was aspirated, the cells were washed with PBS, and the CCK-8 reagent was added and incubated for 1 h at 37 °C. Then, the absorbance of the solution was measured at 450 nm using a microplate reader. Cell viability on the surface of the pristine Ti or surface-modified Ti substrates was assessed by live/dead assay staining. Briefly, MG 63 cells were seeded at a density of 5×10^4 cells/mL on the Ti surface or the surface-modified Ti surface in 24-well plates. After a 24 h incubation, the cells/specimens were rinsed three times with PBS and then incubated with live/dead stain (2 μM calcein AM and 4 μM ethidium homodimer-1) for 30 min at RT. Viable (green) and dead cells (red) were counted using a confocal laser scanning microscope (CLSM, EZ-C1, Nikon, Tokyo, Japan).

2.10. Cell proliferation test

A CCK-8 kit was used to assess early cell proliferation in accordance with the manufacturer's instructions. Briefly, MG 63 cells were seeded and cultured for 7 days on the surface of specimens. At 1, 3, and 7 days of culture, the cells were rinsed with PBS, CCK-8 reagent was added, and the cells/specimens were incubated at 37 °C for 1 h. The optical density of cell proliferation was measured at 450 nm using a microplate reader.

2.11. Alkaline phosphatase (ALP) activity and calcium content

MG 63 cells were seeded on specimens at a density of 1×10^5 cells/mL to assess ALP activity and calcium content. ALP activity was measured spectrophotometrically using the conversion of *p*-nitrophenol from *p*-nitrophenyl phosphate. In brief, at 7, 14, and 21 days of culture, MG 63 cells were washed with PBS and 1× RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.25% deoxycholic acid, 1% NP-40, 1 mM EDTA, 1 mM PMSF, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 μg/mL pepstatin) was added to the cells. The cells were pulverized with a probe-type pulverizer (Vibra Cell™, Sonics & Materials Inc., Danbury, CT, USA) for 3 min at 4 °C. The cells lysates were centrifuged at 13,500 rpm and 4 °C for 1 min. The supernatant was collected and incubated with 100 μL p-nitrophenyl phosphate solution (Sigma) for 30 min at 37 °C. After the incubation, 1 N NaOH was added, and the reaction was stopped. The optical density was measured at 405 nm using a microplate reader.

To evaluate calcium deposition on the surface of the pristine Ti or surface-modified Ti substrates, the specimens were rinsed three times with PBS and homogenized with 1 mL of lysis buffer solution (0.1% Triton X-100) at 7, 14, and 21 days of culture. The cells/specimens were pulverized with a probe-type pulverizer for 3 min at 4 °C and centrifuged at 13,500 rpm and 4 °C for 1 min. The supernatant was used for the QuantiChrom™ Calcium Assay Kit (DICA-500), according to the manufacturer's instructions (BioAssay Systems, Hayward, CA, USA). The absorbance of the samples was read at 612 nm, 3 min after adding 200 μL of working reagents. Total calcium deposition was determined using standard solutions prepared in parallel.

2.12. Gene expression

Gene expression was analyzed using the real time polymerase chain reaction (RT-PCR) at 14 and 21 days after cell inoculation. Cells seeded on specimens were harvested and collected at predetermined time intervals using trypsin-EDTA. Total RNA was isolated with an RNeasy Plus Mini Kit (Qiagen, Valencia, CA, USA). Subsequently, cDNA was synthesized with 1 μg of total RNA using the *AccuPower* RT PreMix (Bioneer, Daejeon, Korea). RT-PCR reactions were conducted using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The primer sequences were used: ALP [(F) 5′-AAC CCA GAC ACA AGC ATT CC-3′, (R) 5′-GCC TTT GAG GTT TTT GGT CA-3′], Osteocalcin (OCN) [(F) 5′-TTG GTG CAC ACC TAG CAG AC-3′, (R) 5′-ACC TTA TTG CCC TCC TGC TT-3′], GAPDH [(F) 5′-ACT TTG TCA AGC TCA TTT CC-3′, (R) 5′-TGC AGC GAA CTT

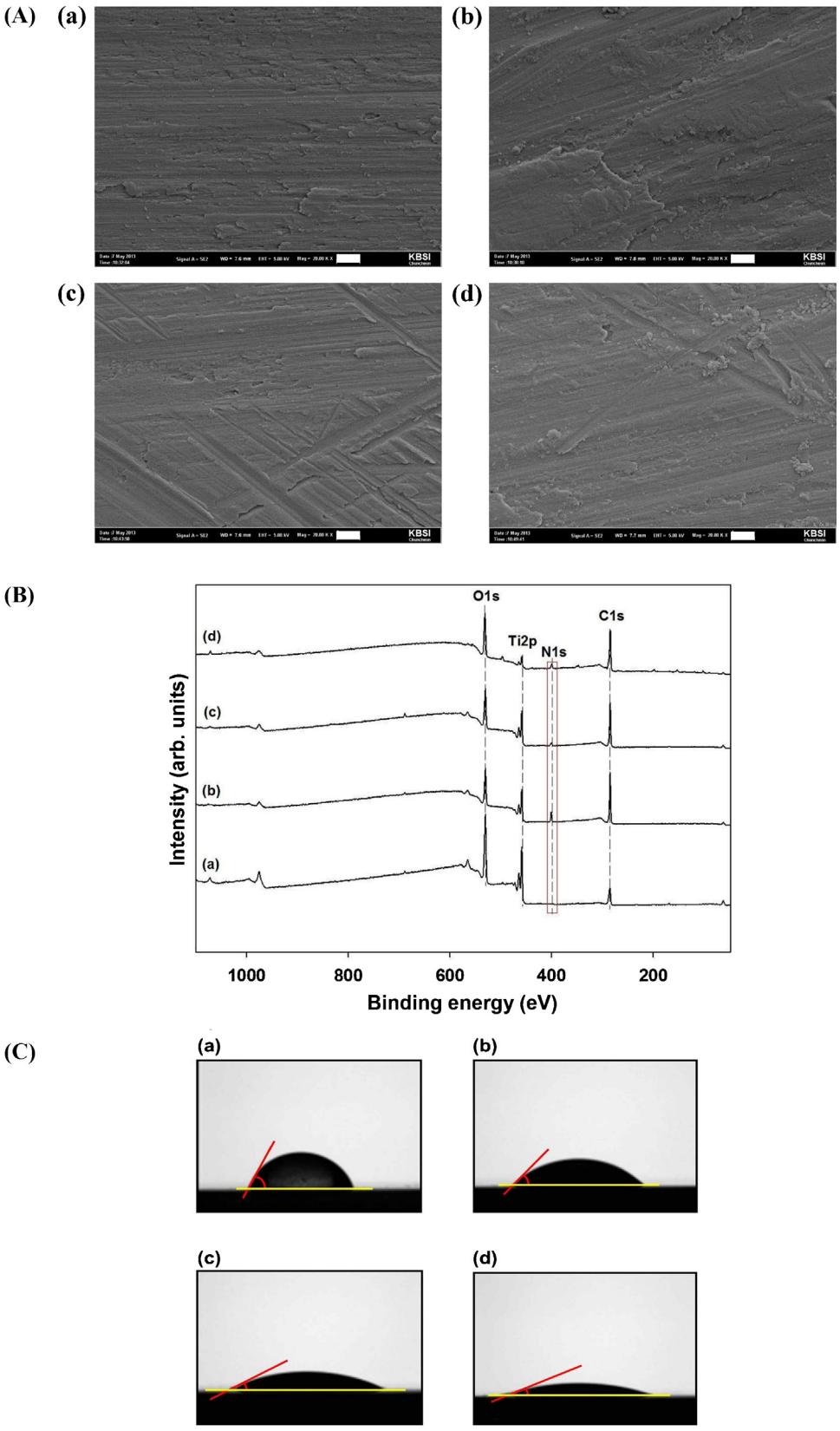

Fig. 2. (A) SEM images of the series of Ti substrates: (a) Pristine Ti, (b) PLL/Ti, (c) Heparin/BMP-2 (10 ng) complex-immobilized Ti (Hep/BMP-2 (10 ng)/Ti), and (d) Heparin/BMP-2 (50 ng) complex-immobilized Ti (Hep/BMP-2 (50 ng)/Ti). Scale bar: 1 μm. (B) XPS wide-scan spectra of various Ti substrates: (a) pristine Ti, (b) PLL/Ti, (c) Hep/BMP-2 (10 ng)/Ti, and (d) Hep/BMP-2 (50 ng)/Ti. (C) Contact angle of the series of Ti substrates: (a) pristine Ti, (b) PLL/Ti, (c) Hep/BMP-2 (10 ng)/Ti, and (d) Hep/BMP-2 (50 ng)/Ti.

Table 1
Elemental composition at the surfaces of pristine Ti and functionalized Ti with poly-L-lysine (PLL), heparin/BMP-2 (10 ng), and heparin/BMP-2 (50 ng) as determined by XPS.

Substrate	C%	N%	Ti%
Pristine Ti	22.86 ± 0.20	3.81 ± 0.12	73.32 ± 0.31
PLL-Ti	61.08 ± 0.79	19.53 ± 0.47	19.37 ± 0.38
Hep/BMP-2 (10 ng)/Ti	67.14 ± 0.93	8.26 ± 0.20	24.58 ± 0.76
Hep/BMP-2 (50 ng)/Ti	60.34 ± 0.39	13.75 ± 0.12	25.90 ± 0.39

TAT TGA TG-3′]. A DyNAmo™ SYBR® Green qPCR Kit (Finnzymes, Espoo, Finland) was used and PCR amplification and detection were conducted in an iCycler (Bio-Rad, Hercules, CA, USA).

2.13. Statistical analysis

Data are presented as means ± standard deviations ($n = 5$). Statistical comparisons were carried out *via* one-way analysis of variance using Systat software (Chicago, IL, USA). Differences were considered statistically significant at $^{*}P < 0.05$ and $^{**}P < 0.001$.

3. Results

3.1. Surface characterization of pristine Ti and surface-modified Ti

Fig. 2A shows the Ti surface morphology with or without surface modifications. No significant differences on the Ti surfaces were observed after immobilization of PLL, Hep/BMP-2 (10 ng) and Hep/BMP-2 (50 ng) relative to pristine Ti. We performed XPS to assess the surface chemical composition of Ti substrates. Fig. 2B and Table 1 show the XPS wide-scan spectra of the pristine Ti, PLL/Ti, Hep/BMP-2 (10 ng)/Ti, and Hep/BMP-2 (50 ng)/Ti, respectively. C1s, Ti2p, and O1s were used as the predominant components. Carbon was used as the internal reference. As shown in Table 1, successful grafting of PLL onto the Ti surface was confirmed by showing an increase in the C and N content. After immobilization of Hep/BMP complex, C1s signals were increased, while N1s signals were decreased in comparison with those on PLL/Ti. In particular, increase of N1s signal of Hep/BMP-2 (50 ng)/Ti substrate was observed compared to that of Hep/BMP-2 (10 ng) Ti due to the increase of immobilized BMP-2. The contact angle was measured to confirm the surface modification of pristine Ti substrate with PLL polymer and Hep/BMP-2 complex. As shown in Fig. 2 C, contact angle was decreased after surface modification of Ti with PLL and Hep/BMP-2 complex compared to that of pristine Ti. The contact angles on pristine Ti, PLL/Ti, Hep/BMP(10 ng)/Ti, and Hep/BMP(50 ng)/Ti were 75°, 45°, 25° and 18°, respectively.

3.2. In vitro BMP-2 release

The *in vitro* release of BMP-2 from the Hep/BMP-2 (10 ng)/Ti and Hep/BMP-2 (50 ng)/Ti was compared using an ELISA (Fig. 3). At first day, the released amounts of BMP-2 from Hep/BMP-2 (10 ng)/Ti and Hep/BMP-2 (50 ng)/Ti were 2.2 ± 0.1 and 8.1 ± 0.2 ng, respectively. At 28 days, 6.5 ± 0.6 and 20.8 ± 3.0 ng of the BMP-2 was released from Hep/BMP-2 (10 ng)/Ti and Hep/BMP-2 (50 ng)/Ti, respectively. This suggested that the BMP-2-loaded Ti exhibited a sustained release profile for different BMP-2 concentrations.

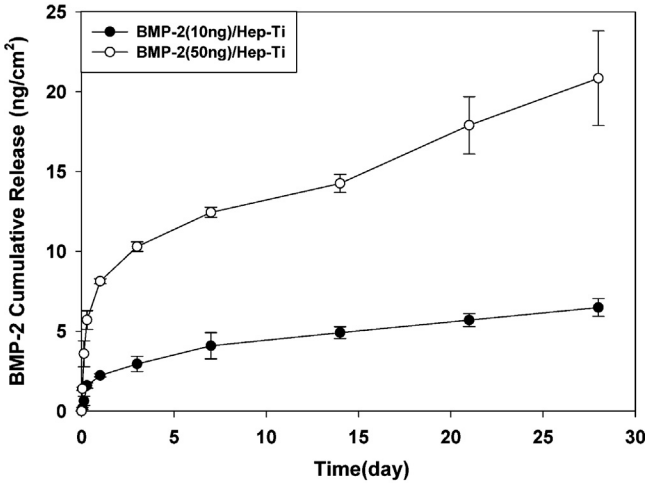

Fig. 3. *In vitro* release of BMP-2 from Hep/BMP-2 (10 ng)/Ti, and Hep/BMP-2 (50 ng)/Ti. The error bars represent mean ± SD ($n = 5$).

3.3. Cell viability

3.3.1. Cytotoxicity test and live/dead assay

No cytotoxic effects were observed on any of the Ti substrates compared to that in the control group for different culture periods up to 48 h (Fig. 4A). The cytotoxicity of pristine Ti and the surface-modified Ti substrates was also examined by fluorescence staining with a live/dead assay. As shown in Fig. 4B, almost MG 63 cells were alive after a 24 h exposure to the pristine Ti and surface-modified Ti substrates.

3.3.2. Cell proliferation

Fig. 4C shows MG 63 cell density on the surface of pristine Ti and the surface-modified Ti substrates. Cell growth was observed with increasing incubation period up to 7 days. Cell density on PLL/Ti, Hep/BMP-2 (10 ng)/Ti, and Hep/BMP-2 (50 ng)/Ti was not significantly different compared to that on pristine Ti during 7 days of culture.

3.4. Osteogenic differentiation

3.4.1. ALP activity

ALP is widely used as an early differentiation marker of osteoblasts and was measured on the pristine Ti and surface-modified Ti substrates at 7, 14, and 21 days. As shown in Fig. 5A, ALP activity of MG 63 cells grown on Hep/BMP-2 (10 ng)/Ti and Hep/BMP-2 (50 ng)/Ti for 21 days increased significantly compared to that on pristine Ti ($^{**}P < 0.001$). ALP activities of MG 63 cells on between pristine Ti and PLL/Ti were similar during the 21 day culture period. However, ALP activity for Hep/BMP-2/Ti group was significantly enhanced. A significant difference in ALP activity was also observed between Hep/BMP-2 (10 ng)/Ti and Hep/BMP-2 (50 ng)/Ti over the 21 days ($^{**}P < 0.001$). ALP activities of MG 63 cells grown on all substrates were increased for culture periods up to 14 days and decreased thereafter.

3.4.2. Calcium deposition

Calcium deposition is a marker of late osteoblast differentiation. As shown in Fig. 5B, the amount of calcium deposited increased on all substrates over time. In particular, the amount of calcium deposition by osteoblasts grown on Hep/BMP/Ti substrates was much higher than by those grown on pristine Ti at 7, 14, and 21 days of culture ($^{**}P < 0.001$). Also, the level of calcium deposition was significantly enhanced with increasing BMP-2 concentration ($^{*}P < 0.05$ or $^{**}P < 0.001$) for culture periods up to 21 days. In contrast, no significant difference of calcium deposition was observed between PLL/Ti and pristine Ti.

3.4.3. Gene expression

Osteoblastic gene expressions for ALP and OCN were evaluated by real time-PCR, and total mRNA was isolated from MG 63 cells

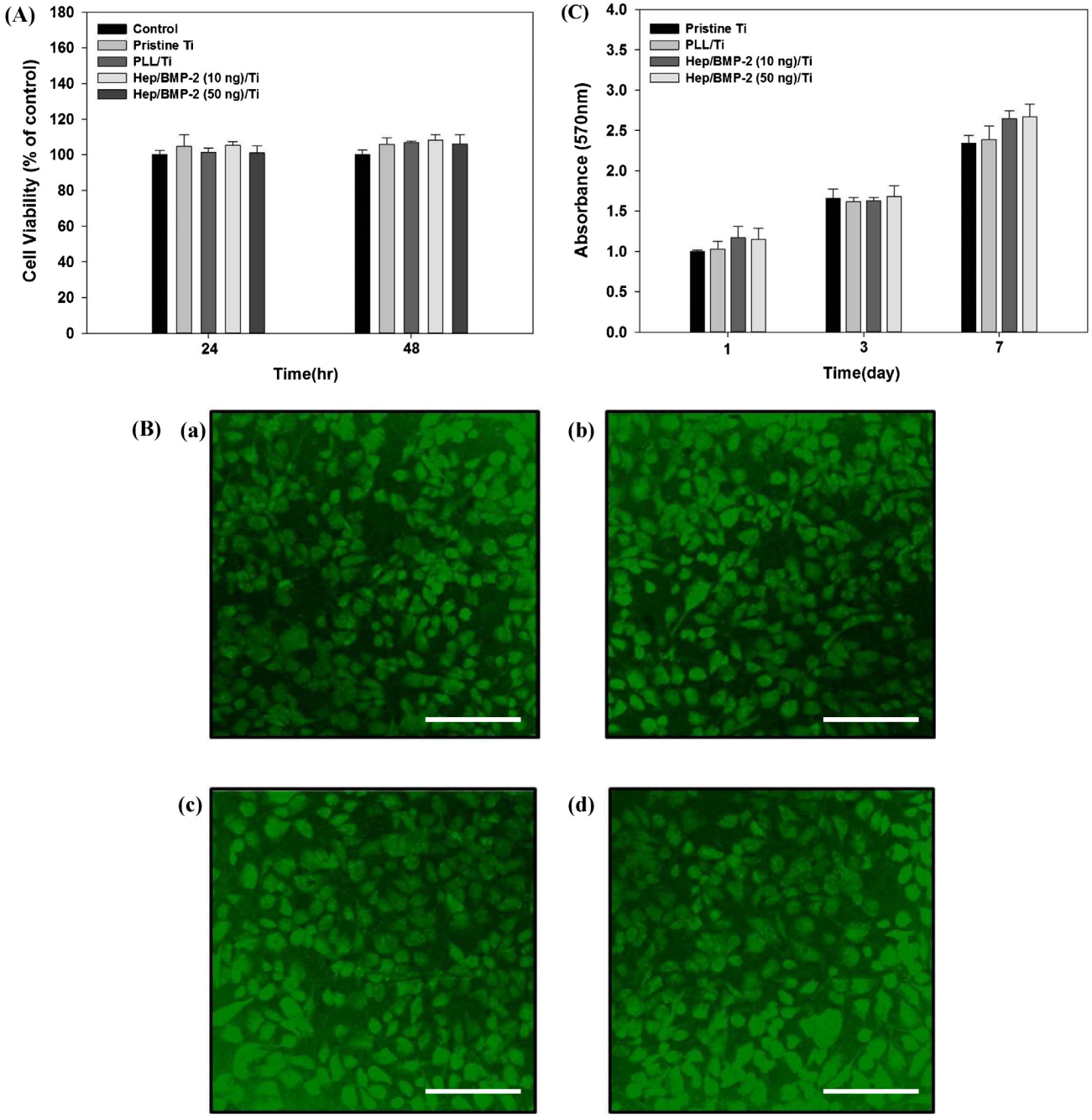

Fig. 4. (A) Cytotoxicity test of pristine Ti, PLL/Ti, Hep/BMP-2 (10 ng)/Ti, and Hep/BMP-2 (50 ng)/Ti against osteoblasts (MG-63 cells). (B) Live/dead assay of various Ti substrates: (a) pristine Ti, (b) PLL/Ti, (c) Hep/BMP-2 (10 ng)/Ti, and (d) Hep/BMP-2 (50 ng)/Ti. Scale bar: 100 μm. (C) Cell proliferation of osteoblast-like cells (MG-63 cells) grown on pristine Ti, PLL/Ti, Hep/BMP-2 (10 ng)/Ti, and Hep/BMP-2 (50 ng)/Ti after 1, 3, and 7 day incubation. The error bars represent mean ± SD ($n = 5$). These experiments were repeated thrice.

on days 14 and 21 of culture because ALP is an early marker and OCN is a late marker of osteoblastic differentiation. As shown in Fig. 5C-a, the ALP gene expression level in MG 63 cells grown on BMP-2-containing Ti substrates was significantly higher than that in MG 63 cells grown on pristine Ti at 14 and 21 days of culture ($^{**}P < 0.001$). Also, ALP gene expression level for Hep/BMP-2 (50 ng)/Ti was higher than that for Hep/BMP-2 (10 ng)/Ti at 14 and 21 days ($^{**}P < 0.001$ or $^{*}P < 0.05$). As expected, OCN gene expression for BMP-2-containing Ti substrates was much higher than those for pristine Ti at 14 and 21 days of culture (Fig. 5C-b) ($^{**}P < 0.001$). However, ALP and OCN gene expression for between PLL/Ti and pristine Ti were not significantly different (Fig. 5C-a and -b).

4. Discussion

The efficacy of Ti implant materials depends on the adhesion and proliferation of osteoblasts. However, Ti implants still have limitations, including a low success rate in patients with poor bone quality and those with poor healing/regeneration capability. To overcome these problems, many methods such as surface modification and immobilizations with biomolecules and bioactive proteins have been studied to improve osteoblast activity and enhance osteointegration (Das, Bose, & Bandyopadhyay, 2007; Lin, Li, Li, & Swain, 2010; Shi et al., 2009a, 2009b). Here, the aim of this study was to develop a functionalized Ti substrate to induce

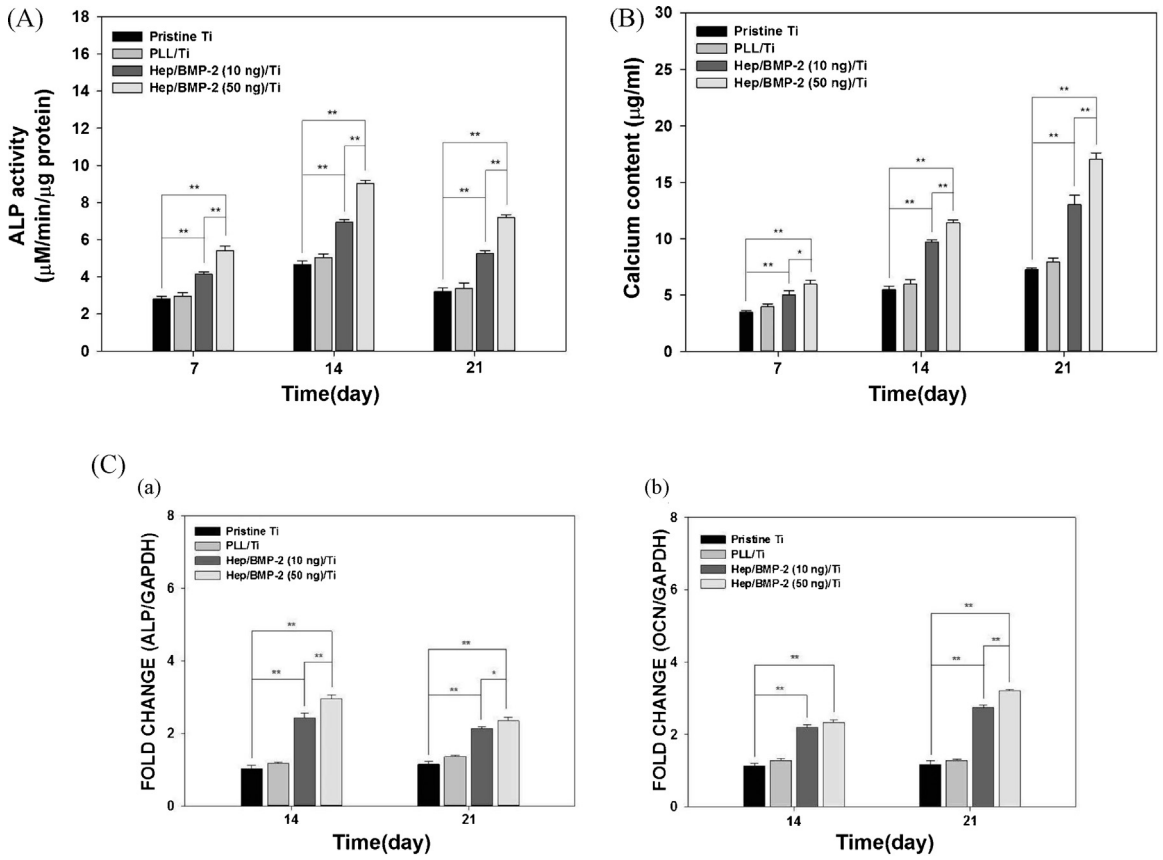

Fig. 5. (A) ALP activity of osteoblast-like cells (MG-63 cells) grown on pristine Ti, PLL/Ti, Hep/BMP-2 (10 ng)/Ti, and Hep/BMP-2 (50 ng)/Ti against osteoblasts (MG-63 cells) after 7, 14, and 21 day incubation. (B) Calcium deposition of osteoblast-like cells (MG-63 cells) grown on pristine Ti, PLL/Ti, Hep/BMP-2 (10 ng)/Ti, and Hep/BMP-2 (50 ng)/Ti against osteoblasts (MG-63 cells) after 7, 14, and 21 day incubation. (C) Real-time PCR analysis for (a) Alkaline phosphatase (ALP) and (b) osteocalcin (OCN) expressions of each groups cultured MG-63 cells after 14 and 21 day incubation. The error bars represent mean ± SD ($n = 5$). These experiments were repeated thrice (*$P < 0.05$ and **P 0.001).

osteoblast activity using immobilization of Hep/BMP-2 complex on Ti substrates.

To realize sustained delivery of BMP-2 on the surface of the Ti substrate, Ti substrate was treated with 1 N NaOH and functional groups such as hydroxyl and carboxyl groups were introduced on the surface of the Ti substrate (Lu et al., 2007). Then, PLL was chemically grafted to carboxyl group of the Ti substrate in the presence of DMT-MM as condensing agent. To immobilize BMP-2 on PLL/Ti surface, strong negative charged heparin was simply mixed with BMP-2 which has the positive charges of the amino acid residues to make heparin/BMP-2 complex *via* electrostatic interactions (Zhao et al., 2006). Then, heparin/BMP-2 complex was subsequently immobilized on the PLL/Ti surface *via* electrostatic interactions. The successful immobilization of PLL and Hep/BMP-2 onto the pristine Ti substrate was confirmed with SEM, XPS and contact angles. In particular, successful anchoring of the heparin/BMP-2 complex on the Ti substrate resulted in both an increase in C content and a decrease in N content compared to those of the PLL grafted Ti substrate. Furthermore, the heparin/BMP-2 complex immobilized on the Ti surface showed a lower contact angle than that of pristine Ti and PLL-Ti, suggesting that Hep/BMP-2 complex with hydrophilic property was successfully immobilized on the surface of Ti substrate.

BMP-2 is one of the most popular osteoinductive growth factors for bone formation because it effectively enhances osteointegration and osteoblast activity. However, BMP-2 has a short half-life in the blood stream leading to decrease of its bioactivity (Takahashi et al., 2005). Thus, a large dose of BMP-2 is required for clinical treatment. However, such a large dose of BMP-2 can cause side effects including bone overgrowth and an immune response due to diffusion away from the defected site of bone regeneration (Yang et al., 2010). To overcome these problems, an effective BMP-2 delivery system with a sufficient time period of release and appropriate concentrations must be developed for bone regeneration. In this study, heparin was used to develop an implant material for enhanced osteoblast activity and a long-term delivery system. Through immobilization of Hep/BMP-2 complex onto PLL grafted Ti substrate, the release of BMP-2 from Ti substrate could be maintained for a long period. Previous studies reported that heparin is very useful for controlled delivery of growth factors with various bioactive implantable matrices (Perets et al., 2003; Steffens et al., 2004). For example, Chung, Kim, Yoon, and Park (2006) showed that loading bFGF onto heparin immobilized PLGA porous microspheres leads to increased angiogenic potential when compared to loading the same amount of bFGF onto non-heparinized PLGA porous microspheres. As another example, PLGA/heparin system loaded with BMP-2 also promotes enhanced bone formation (Jeon, Song, Kang, Putnam, & Kim, 2007; Kim et al., 2008). We also confirmed that all Ti substrates did not damage osteoblast cells. As we showed in the live/dead assay and proliferation study, all osteoblast grow healthily on Ti substrates and no dead osteoblasts were observed. Also, osteoblasts proliferated well on all Ti substrates in a time-dependent manner. These results indicated that all Ti substrates are safe and suitable materials for the growth of osteoblasts, as observed in previous studies (Kim et al., 2011). In other words, it implies that immobilization of Hep/BMP-2 complexes on PLL-Ti substrate does not damage osteoblasts.

ALP activity and calcium deposition are important markers for enhanced osseointegration. ALP activity is widely used as an early differentiation marker of osteoblast-cells (Shi et al., 2009a, 2009b; Turksen, Bhargava, Moe, & Aubin, 1992). We found no significant difference in ALP activity or calcium deposition between PLL grafted Ti and pristine Ti throughout 21 days of culture. In contrast, ALP activity of osteoblasts cultivated on the heparin/BMP-2 complex immobilized Ti was significantly higher than those on pristine Ti and PLL grafted Ti. However, osteoblast ALP activity on all substrates at 21 days was slightly lower than that on all substrates at 14 days. Because ALP usually peaks before mineralization actually begins, ALP activity showed slightly diminishing mineralization and slowly increasing calcium deposition on all substrates after 14 days of culture (Bancroft et al., 2002). Calcium deposition is a marker of late osteoblast-like cell differentiation (van den Beucken et al., 2006). The calcium amount deposited on the heparin/BMP-2 complex immobilized Ti was significantly higher than those on pristine Ti and PLL grafted Ti during the 21 day culture. Calcium deposition on PLL grafted Ti was similar to that on pristine Ti throughout the 21 day culture. These results indicate that the heparin/BMP-2 complex immobilized Ti substrate had a stimulating effect on matrix formation and enhanced osteoblast activity consistent with the ALP activity results.

Osteogenic differentiation of osteoblasts cultivated on the different substrates was investigated by determining ALP and OCN expression by real time-PCR analysis at 14 and 21 days of culture. ALP gene expression was consistent with the ALP activity results. OCN gene expression on the heparin/BMP-2 complex immobilized Ti substrates was significantly different compared to that on pristine Ti at 14 and 21 days of culture. A previous study reported that OCN is a late differentiation marker expressed at the matrix mineralization stage (Song, Jeon, Yang, Han, & Kim, 2007). Thus, BMP-2 effectively stimulated osteogenic markers such as ALP and OCN during osteogenic differentiation *in vitro*. The Heparin/BMP-2 complex immobilized Ti substrates released BMP-2 locally over long time periods at appropriate concentrations, and this system could contribute to reducing the BMP-2 dose for enhanced osteoblast activity. In addition, an *in vitro* study to compare small and large doses of BMP-2 and an animal study may be needed to determine enhanced osseointegration.

5. Conclusions

Ti substrates were successfully modified with PLL *via* chemical conjugation and Hep/BMP-2 complex was immobilized on the surface of PLL-Ti substrate *via* electrostatic interactions. The PLL grafted Ti substrate did not show different ALP activity, calcium deposition, or gene expression compared to those of the other substrates. The heparin/BMP-2 complex immobilized Ti substrate was effective for stimulating osteoblast activity. Furthermore, the heparin/BMP-2 (50 ng) system showed the significantly improved osteogenic markers such as ALP activity, calcium deposition, ALP and osteocalcin gene expression compared to that of the heparin/BMP-2 (10 ng) system. Thus, the heparin/BMP-2 complex immobilized Ti substrate is a valuable system to enhance osteoblast activity by delivering BMP-2 and can be further applied to orthopedic implant materials or dental applications.

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