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*Research Article*

# Plasma Treated High-Density Polyethylene (HDPE) Medpor Implant Immobilized with rhBMP-2 for Improving the Bone Regeneration

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We investigate the bone generation capacity of recombinant human bone morphogenetic protein-2 (rhBMP-2) immobilized Medpor surface through acrylic acid plasma-polymerization. Plasma-polymerization was carried out at a 20 W at an acrylic acid flow rate of 7 sccm for 5 min. The plasma-polymerized Medpor surface showed hydrophilic properties and possessed a high density of carboxyl groups. The rhBMP-2 was immobilized with covalently attached carboxyl groups using 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide and N-hydroxysuccinimide. Carboxyl groups and rhBMP-2 immobilization on the Medpor surface were identified by Fourier transform infrared spectroscopy. The activity of Medpor with rhBMP-2 immobilized was examined using an alkaline phosphatase assay on MC3T3-E1 cultured Medpor. These results showed that the rhBMP-2 immobilized Medpor increased the level of MC3T3-E1 cell differentiation. These results demonstrated that plasma surface modification has the potential to immobilize rhBMP-2 on polymer implant such as Medpor and can be used for the binding of bioactive nanomolecules in bone tissue engineering.

## 1. Introduction

Polymers are widely used in medical applications, for example, as vascular grafts [1], orthopedic bearings [2], screws [3], suture anchors [4], bone cement [5], soft-tissue reconstruction [6], or drug delivery systems [7]. Among the polymers, high-density polyethylene (HDPE) is used for containers, water pipelines, and industrial applications, but also as invasive medical device material because of its inert properties. Thus, Medpor HDPE facial implants are widely used in orbital reconstruction and augmentation of other defects of the facial skeleton [8–11]. In addition, porous structure of Medpor is rapidly infiltrated by host tissue. However, HDPE surface shows hydrophobic property due to the absence of polar functional groups in PE molecular chains, which limit their potential application in biomedical field [12, 13].

Recombinant human bone formation protein-2 (rhBMP-2) is a class of locally signaling molecules that promote bone formation by both osteoconduction and osteoinduction [14]. Recently, this promising protein has been immobilized onto various functionalized substrates, such as titanium and its alloys, polymers, and bioceramics [15–18]. The rhBMP-2, as a growth factor bound to biodegradable polycaprolactone 3D scaffolds, stimulates the differentiation of Mg-63 cells in vitro [19]. Most surfaces of the synthetic polymers underwent surface modification to induce a biological function at the interface owing to the poor hydrophobicity and lack of functional groups on its surface [20].

Plasma surface modification is a simple process with shorter treatment time than other surface modification methods including particularly wet surface modification methods, such as chemical polymerization with atom transfer radical polymerization and reversible addition-fragmentation chain transfer [21]. In addition, plasma-polymerization maintains more permanent hydrophilicity and functionality than a gas

and organic plasma treatment. Some studies have applied plasma-polymerization to bind the BMPs [22]. Plasma-polymerization has the potential to introduce a specific functionality to the surfaces of various substrates [23–25].

The objective of the present work was to deposit the polymer thin films on Medpor surface using an acrylic acid plasma-polymerization and investigate the bone regeneration of rhBMP-2 immobilized Medpor implant that was chosen as a bone substitute material for potential facial surgery use.

## 2. Materials and Method

*2.1. Materials.* The Medpor implants (Stryker Inc., USA) used as samples were cut into 5 mm × 5 mm. The rhBMP-2 was supplied by Cowellmedi (Korea). Acrylic acid (AA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), and N-hydroxysuccinimide (NHS) were obtained from Sigma-Aldrich.

*2.2. Acrylic Acid Plasma-Polymerization on Medpor Surface.* The equipment for plasma surface modification is reported elsewhere [16]. Plasma treatment of the Medpor was carried out using a radio frequency (RF, 13.56 MHz) capacitively coupled plasma system. The samples were placed on a stage 3 cm away from the top electrode in the vacuum chamber. The vapor of the acrylic acid monomer evaporated at room temperature (32°C in a water bath) from a container (10 mL in volume) was introduced to the vacuum chamber. The deposition conditions are as follows: RF discharge power = 20 W, monomer flow rate = 3 sccm, working pressure = 10 mTorr, and deposition time = 5 min.

*2.3. rhBMP-2 Immobilization on Plasma-Polymerized Medpor Surface.* The rhBMP-2 powder was dissolved in sterilized water and adjusted to rhBMP-2 final concentrations of 1 $\mu$g/mL using PBS solution. The rhBMP-2 was immobilized onto the carboxylated Medpor surface by EDC-mediated reaction between the carboxyl groups of the Medpor surface and the primary amine groups of rhBMP-2. In brief, carboxylated Medpor was immersed in 10 mg EDC/6 mg NHS in 50 mM MES buffer (pH5.6) for 24 h at room temperature. After the reaction, the Medpor was thoroughly washed with distilled water and dried at room temperature. The Medpor was then immersed in 1 mL of rhBMP-2 solution for 24 h at room temperature. After immobilization, the samples were rinsed with MES buffer.

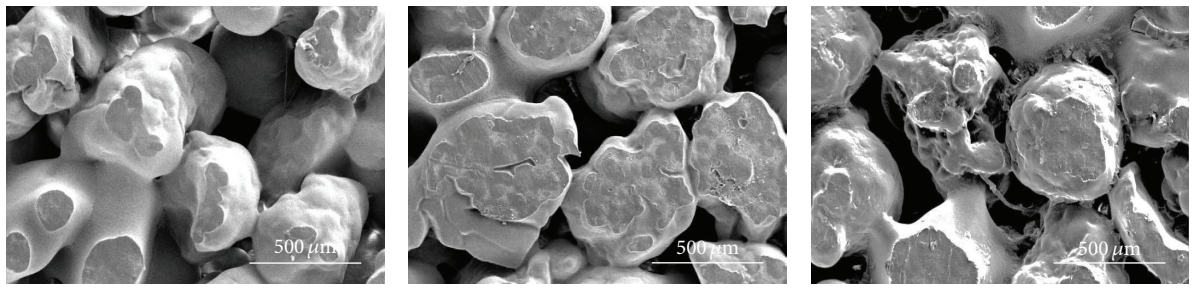
*2.4. Surface Characterization.* The hydrophilicity of the samples was examined by contact angle measurements (Surface Tech, Contact-Angle GS). Measurement temperature was set at 22°C, usually described as ambient temperature. Contact angle measurements were taken for each drop after 5 s deposition and the volume of the water droplet was 7 $\mu$L. Contact angle values presented in this study are always the mean of at least 5 individual measurements on each sample. The surface morphology was observed by scanning electron microscopy (SEM; SEC, SNE 3200 M). The substrates were coated with gold using a sputter-coater (MCM-100). The SEM was operated at 10 kV of acceleration voltage and SE detector mode. The chemical structure of the plasma-polymerized and rhBMP-2 immobilized Medpor surface was examined by attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR; PerkinElmer Spectrum 400).

*2.5. Cell Culture of MC3T3-E1 Cells and Cell Seeding into Medpor Specimens.* MC3T3-E1 (preosteoblastic cell line, ATCC CRL2593) cells were used to characterize the biocompatibility of the Medpor by analyzing cell proliferation, alkaline phosphatase activity, and live/dead assay. The cells were maintained in a humidified incubator at 37°C and 5% $CO_2$ atmosphere (Sanyo Electric). The cells were cultured in $\alpha$-modified Eagle's medium ($\alpha$-MEM; GIBCO) supplemented with 10% fetal bovine serum (PAA, Laboratories) and 1% solution of 100 U/mL penicillin and 100 $\mu$g/mL streptomycin (Lonza). Medium was changed every 2 days, and the cells were detached with 0.25% trypsin/EDTA (GIBCO) and passaged at 90% confluence. This study was used to passages 3 in MC3T3-E1 cells.

Before cell seeding, nonsterile Medpor specimens were sterilized by immersing for 30 min in 70% ethanol and rinsed twice with Dulbecco's Phosphate Buffered Saline (DPBS; Welgene). Sterilized specimens were placed in 48-well culture plate (SPL Inc.) and cell suspension was added to the top center of the Medpor specimens, avoiding tip/surface contact to reducing seeding efficiency. After 3 h, the specimens were transferred to another 48-well culture plate and each of the wells was filled with fresh media of 500 $\mu$g.

*2.6. Proliferation of MC3T3-E1 Cells.* The proliferation rate of MC3T3-E1 cells cultured on Medpor specimens was determined by MTT (3-(4.5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide to a purple formazan product Sigma-Aldrich Co.) assay. In brief, $1 \times 10^5$ cells/mL were seeded on Medpor specimens and the cells were incubated for 1, 3, and 5 days. The MTT assay is described elsewhere [26].

*2.7. Differentiation of MC3T3-E1 Cells.* The cells were cultured at a density of $1 \times 10^5$ cells/mL and the medium was replaced with $\alpha$-MEM containing 10 mM $\beta$-glycerophosphate (Sigma) and 50 $\mu$g/mL ascorbic acid (JUNSEI). After 7 and 14 days, alkaline phosphatase (ALP) was assayed by measuring the release of p-nitrophenol (p-NP) from p-nitrophenyl phosphate (p-NPP). The Medpor specimens seeded with MC3T3-E1 cells were gently rinsed twice with DPBS (Welgene), lysed in 0.9% NaCl solution containing 0.2% Triton X-100 (Sigma) for 10 min, and sonicated using a Vibra cell instrument (SONICS) for 1 min at 65 W on ice. The lysate was centrifuged at 2,500 ×g for 10 min at 4°C and the clear supernatant was incubated with p-nitrophenyl phosphate solution for 30 min at 37°C. The reaction was stopped by adding 600 $\mu$L of 1.2 N NaOH. The ALP activity was determined by measuring the absorbance at 405 nm using ELISA reader (Thermal Fisher SCIENTIFIC) and normalized to the protein concentration. The protein concentration was determined by Bradford protein assay (Bio-Rad). The data was expressed as $\mu$mole p-NP/min/$\mu$g protein.




(a) (b) (c)

FIGURE 1: Surface morphology of (a) untreated, (b) acrylic acid plasma treated, and (c) rhBMP-2 immobilized Medpor.

*2.8. Live and Dead Cell Assay.* Cell viability/cytotoxicity was assessed by using molecular probes live/dead cell staining kit (Biovision). MC3T3-E1 cells were seeded at a density of $3 \times 10^5$ cells/mL on Medpor specimens in 48-well plates. After 3-day incubation, the culture media were removed from the wells and cells/specimens were rinsed three times with DPBS. Then staining solution (1 mM Live-Dye and 2.5 mg/mL of Propidium Iodide) of 0.25 mL per well was added and culture plates were returned to incubator for 20 min. Live cells (green) and dead cells (red) were imaged under a fluorescence microscopy (NIKON).

*2.9. Statistical Analysis.* The data were expressed as means standard deviations (SD) ($n = 3$). Statistical comparisons were performed by using Student's $t$-test. The statistical significance was considered at $^{*}P < 0.05$ and $^{**}P < 0.01$.

## 3. Results and Discussion

*3.1. Medpor Surface Analysis after Plasma Treatment and rhBMP-2 Immobilization.* Plasma-polymerization depends upon the power of the glow discharge, monomer pressure, and deposition time. In this work we have set up plasma process conditions such as RF discharge power and monomer pressure to optimize the rhBMP-2 immobilization process on Medpor surface.

Figure 1 shows the SEM image of pristine Medpor, Medpor treated AA plasma-polymerization, and rhBMP-2 immobilized Medpor surface. Regardless of plasma treatment, porous structure of Medpor surface was observed on all samples, and pore size was approximately 200–300 $\mu$m. We also observed formation of polymeric thin films of Medpor surface after the AA plasma-polymerization (Figure 1(b)). Some authors report that porous Si sample gives the evidence of the presence of a polymer layer onto the pSi surface, due to the PPAA coating. In addition, owing to polymer growth, free pores mouth results more narrowed as for longer deposition time [27].

After AA plasma-polymerization on the Medpor samples, the wettability of polymer thin films was characterized by contact angle analysis. Figure 2 shows the contact angles of the AA thin film on pristine and plasma treated samples. The high water contact angle on the pristine Medpor was attributed to the hydrophobic property of the Medpor

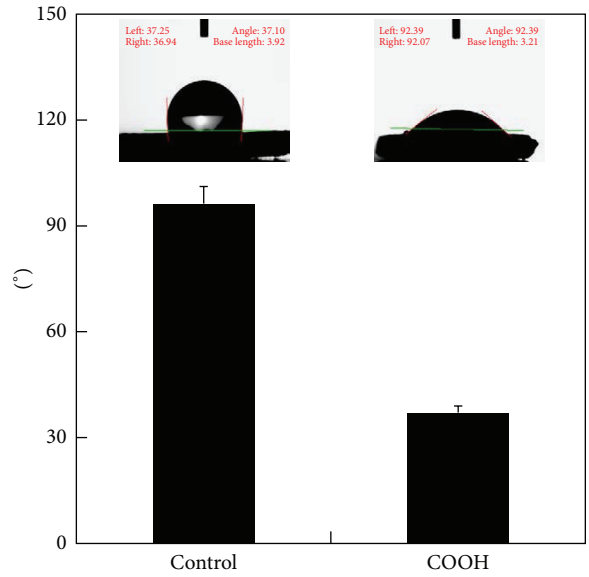



FIGURE 2: Contact angles of untreated and AA plasma treated Medpor surface.

surface. After AA plasma-polymerization the contact angles of Medpor surface decrease to values about 37.1°. These hydrophilic surfaces could be obtained as a consequence of the polar groups of AA thin films. The hydrophilic surface plays an important role in the cell-biomaterial interface [28]. However, moderate hydrophobic surfaces have good cell attachment [29]. It is well known that hydrophobic surfaces favor the adsorption of proteins from aqueous solution thermodynamically but may induce strongly irreversible adsorption and denature the protein's native conformation and bioactivity. On the other hand, a highly hydrophilic surface may expel any protein molecules and inhibit protein adsorption [30].

HDPE is made of the elements carbon (C) and hydrogen (H), which forms chains of repeating $-CH_2$-units [31]. Figure 3 shows the ATR-FTIR spectra of the HDPE (Medpor), HDPE modified by AA plasma, and rhBMP-2 immobilized HDPE. Stretching vibrations of C–H bonds of HDPE were observed through the presence of the two significant bands at 2847 and 2919 $cm^{-1}$ and bending vibrations as two bands at 1470 and 1472 $cm^{-1}$ (Figure 3, HDPE). After AA

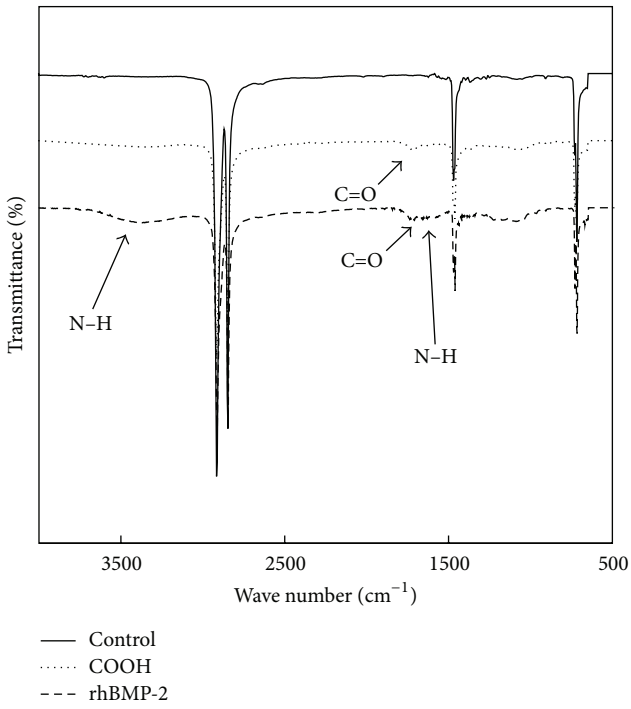



Figure 3: ATR-FTIR spectra of untreated, plasma treated, and rhBMP-2 immobilized Medpor surface.

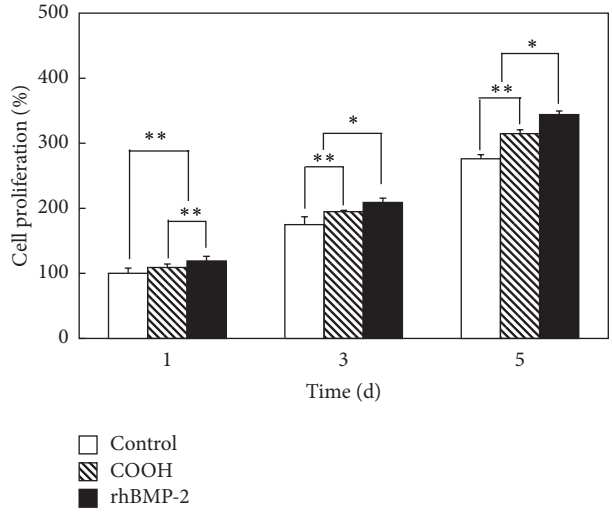



Figure 4: Cell proliferation of MC3T3-El on the different Medpor surface for 1, 3, and 5 days ($^{*}P < 0.05$, $^{**}P < 0.01$).

plasma treatment new peak appeared at 1718 cm$^{-1}$ which was assigned to the carboxyl group in the AA thin film. The most sensitive spectral region to the protein secondary structural components is the amide Ib and (1700–1600 cm$^{-1}$), which is due almost entirely to the C=O stretch vibrations of the peptide linkages [32]. The peak at approximately 1640 cm$^{-1}$ can be assigned to the bending modes of N–H bonds [33]. It was assumed that the HDPE surface was almost completely immobilized with rhBMP-2 molecules via covalent bonding between $-NH_2$ and –COOH.

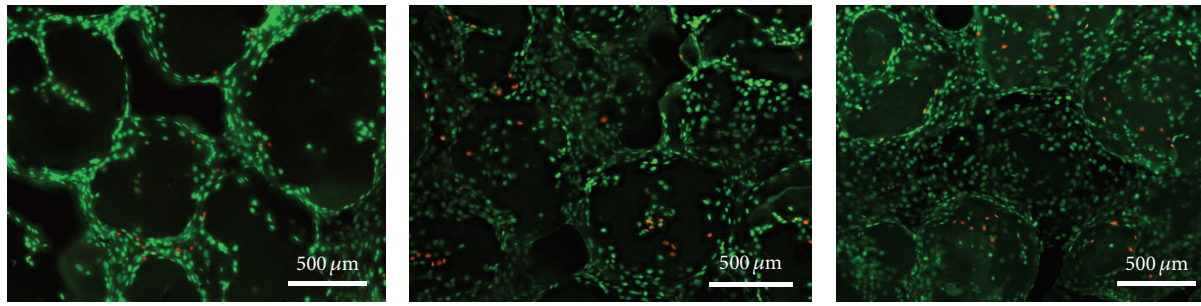
*3.2. Effect of the Surface Functionalization for MC3T3-E1 Cells Biological Response.* The proliferation of MC3T3-E1 cells was examined by MTT assay. Figure 4 shows the percent of cell proliferation after a culture period of 1, 3, and 5 days on the different samples. The pristine Medpor was used as control group in this study. The plasma treatment and rhBMP-2 had little effect on cell proliferation at initial stage (1 day). However, as the cell culture time increased, cell proliferation exhibited significant differences between experimental and control groups. It means that the chemical functional groups such as –COOH and $-NH_2$ groups on the functionalized Medpor surface could control cell behavior including adhesion and proliferation. Haddow et al. [34] and Daw et al. [35] found that substrates with –COOH groups on the surface encouraged the attachment and growth of human keratinocyte cells and ROS17/2.8 osteoblast-like cells.

The cell viability of pristine Medpor, plasma treated Medpor, and the rhBMP-2 immobilized Medpor was also examined by fluorescence staining with a live/dead assay. As shown in Figure 5, almost MC3T3-E1 cells were alive (green) after 3 days of culture on all the samples. The plasma treated and rhBMP-2 immobilized Medpor surface shows higher cell density than pristine Medpor. Dead cells were present in low numbers as detected by the low bright red fluorescence.

The biological activity of rhBMP-2 bound to Medpor surface was evaluated by measuring ALP activity of MC3T3-E1 cells cultured on the Medpor surface. The ALP activity of MC3T3-E1 cells cultured on various Medpor surfaces for 7 and 14 days was shown in Figure 6. The ALP activity tended to increase with increasing culture period. During the experiment period, the ALP activity of the rhBMP-2 immobilized Medpor was higher than that of the untreated Medpor. In addition, AA plasma treated Medpor showed better ALP activity than pristine Medpor. Bone morphogenetic protein (BMP) is well known as a growth factor that plays a crucial role in bone formation and repair [36]. BMP regulate cell growth and differentiation of a variety of cell types including osteoblasts and chondrocytes [36, 37]. Recently, to improve polylactone-type biodegradable polymer scaffolds, a number of strategies including physisorption, ionic interaction, and blending have been designed to immobilize BMP-2 on the polylactone-type scaffolds [38, 39]. In this respect, the surface chemistry and topography are the two major elements found to affect protein adsorption to the biomaterial surface [40]. Based on these results, we proved that the rhBMP-2 of Medpor implant using plasma-polymerization has a positive effect on the adhesion, proliferation, and differentiation of MC3T3-E1 cells.

## 4. Conclusion

Acrylic acid plasma treatment can offer suitable hydrophilicity and functional groups on the surface of Medpor implants. Due to the functionalized surface rhBMP-2 was successfully immobilized to the surface of Medpor implants. Furthermore, results of ATR-FTIR analysis confirmed the




(a) (b) (c)

Figure 5: Live/dead fluorescent stain images of MC3T3-El cells on (a) untreated, (b) AA plasma treated, and (c) rhBMP-2 immobilized Medpor surface for 3 days. Viable cells were stained green and dead cells stained red.

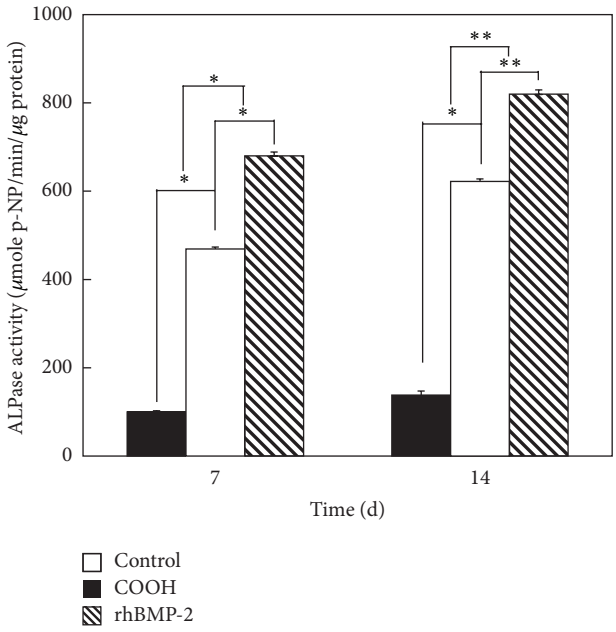



Figure 6: ALP activity of MC3T3-El cells on the different Medpor surface for 7 and 14 days ($^{*}P < 0.05$, $^{**}P < 0.01$).

immobilization of rhBMP-2 via polymer polymerization. The effect of immobilized rhBMP-2 on proliferation of the MC3T3-E1 cells was gradually increased in a time-dependent manner. In addition, the immobilized rhBMP-2 and plasma treatment stimulated the differentiation of MC3T3-E1 cells and accelerated the process of bone regeneration of MC3T3-E1 cells. In conclusion, the rhBMP-2 immobilized Medpor implants by the AA plasma treatment could be utilized as a promising bone substitute for clinical facial surgery. Finally, plasma surface modification techniques presented in this study can be used as a potential tool for functionalizing a biomaterial surface.

## Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

## Authors' Contribution

Jin-Su Lim and Min-Suk Kook contributed equally to this work.

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