# Poly(dopamine)-Assisted Immobilization of Arg-Gly-Asp Peptides, Hydroxyapatite, and Bone Morphogenic Protein-2 on Titanium to Improve the Osteogenesis of Bone Marrow Stem Cells

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**S** *Supporting Information*

**ABSTRACT:** Osteointegration of titanium implants in bone defects is clinically important for long-term performance of orthopaedic implants. In this work, we developed a facile and effective "one-pot" deposition method based on dopamine polymerization for the development of cell-adhesive, osteoconductive, and osteoinductive titanium implants. Arg-Gly-Asp (RGD)-conjugated polymers, hydroxyapatite (HAp) nanoparticles, and bone morphogenic protein-2 (BMP-2) were mixed with an alkaline dopamine solution, and then, titanium substrates were immersed in the mixture for an hour. During poly(dopamine) coating, the three types of bioactive substances were immobilized on the titanium surfaces. Our results indicate that RGD conjugation enhanced the adhesion of human bone marrow stem cell line, while HAp incorporation facilitated cellular osteodifferentiation. The immobilization of BMP-2 induced the osteogenesis of the stem cells, indicated by reverse-transcriptase polymerase chain reaction (RT-PCR) analysis. The mineralization on the deposited substrates was also enhanced greatly. This functionalized layer on titanium substrate promoted mesenchymal stem cell to osteoblast and improved osteogenic differentiation and mineralization. In conclusion, the surface modification method shows a great potential for enhancement of osteointegration of orthopaedic and dental implants.

**KEYWORDS:** osteoinduction, titanium implants, osteointegration, BMP-2, dopamine, bone marrow stem cells
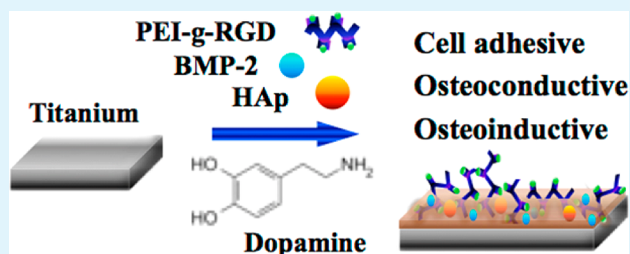
## 1. INTRODUCTION

Titanium alloys are being widely applied to orthopaedic and dental implants due to high mechanical strength, corrosion resistance, and biocompatibility. Integration of titanium implants with surrounding osseous tissues after being placed in bone defects in a clinical process is important for long-term performance of orthopaedic implants.[1] Appropriate surface modification of orthopaedic and dental implants for minimizing the time required for osteointegration could substantially improve patients' quality of life.

Surface modification of titanium implants for improving cell-affinity, osteoconductive, and/or osteoinductive properties is a common strategy for satisfactory osteointegration of orthopaedic or dental implants.[2,3] For example, peptides that mediate cell adhesion, such as Arg-Gly-Asp (RGD), enhance not only the adhesion but also mineralization of osteoblasts or mesenchymal stem cells (MSCs).[4,5] However, RGD peptides do not guarantee the osteoconduction and/or osteoinduction of osteoblasts or mesenchymal stem cells on the surface.[6,7] On the other hand, hydroxyapatite (HAp), a calcium phosphate-based ceramic, owing to similarity in chemistry and structure to the mineral components of natural bone,[8] is frequently used to improve osteointegration of titanium implants.[9,10] However, osteoconductive HAp possesses limited osteoinductive ability.[11] On the other hand, a family of bone morphogenetic proteins (BMPs) induces osteodifferentiation and bone formation.[12] Among the BMP family, bone morphogenic protein-2 (BMP-2), a proven strong osteoinductive factor, stimulates the differentiation of osteoprogenitor cells into mature osteoblasts.[13] Significant research efforts are being directed towards the surface conjugation of one or more of the above bioactive molecules to enhance osteointegration of titanium implants.

A simple and convenient process for incorporation of cell-adhesive peptides, as well as HAp, and BMP-2 on titanium implants could prove beneficial for implant osteointegration by leveraging combining the best of each kind of surface modification. Several approaches have been applied to immobilization of RGD on titanium surfaces, such as layer-by-layer technique,[14] electro-deposition,[15] plasma treatment,[16] and chemical immobilization.[17] However, many of these methods possess disadvantages such as complex chemical reaction and/or a time-consuming process. Thermal plasma spraying is commonly applied to HAp deposition onto titanium implants.[18] However, this technology is not compatible to the methods for RGD conjugation. Several studies combine HAp and BMP-2 to achieve osteoconductive and osteoinductive
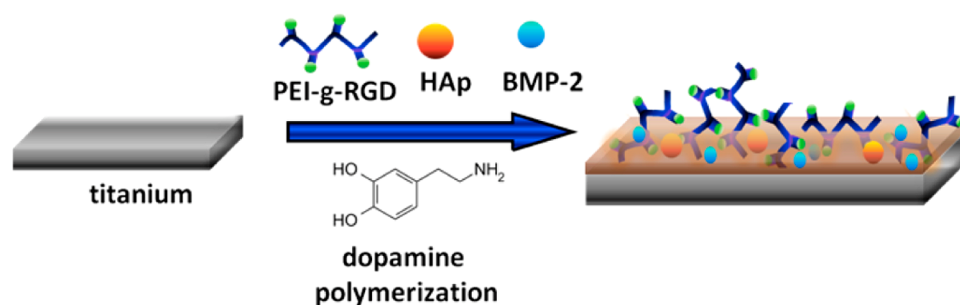
**Figure 1.** Schematic illustration of dopamine-assisted immobilization of PEI-g-RGD, hydroxyapatite (HAp) nanoparticles, and rhBMP-2 on a titanium substrate.

capacities of titanium implants by soaking HAp modified substrates in BMP-2 solution.[19,20] This strategy may cause weak bonding and mismatching bone healing time.[21] Therefore, an easy, quick, and effective method for coating all of these three categories of osteoactive molecules could prove beneficial in achieving more effective osteointegration of bone implants.

Inspired by mussels' adhesive mechanism, Messersmith's group recently created a versatile poly(dopamine) coating method by simply dipping a substrate into an alkaline dopamine solution.[22] In an alkaline environment, the catechol groups of dopamine oxidize to form a poly(dopamine) ad-layer on a wide range of organic and inorganic materials, such as polymers, metals, and metal oxides. Many biomacromolecules including proteins that contain amino or thiol groups can be further conjugated onto poly(dopamine) ad-layer via secondary reaction.[22−24] Poly(dopamine) coating has been proved to promote the adhesion of certain cell types to various surfaces due to an increase in immobilization of serum adhesive proteins.[25−27] We suggest that this simple technique is suitable for conjugation of different bioactive substances on titanium implants.

We previously demonstrated that HAp nanoparticles could be immobilized onto titanium substrates via dopamine polymerization to create an osteoconductive surface.[28] Furthermore, biomolecule-conjugated poly(ethyleneimine) is able to be immobilized on substrates to create a bioactive surface via poly(dopamine) deposition.[24,27] The objective of this work was to enhance cell affinity, osteoconductivity, and osteoinductivity of titanium surfaces via a "one-spot" poly-(dopamine) coating process. Poly(ethyleneimine)-graft-RGD to facilitate cell adhesion, HAp for osteoconduction, and BMP-2 for osteoinduction were codeposited on titanium substrates in a single step via dopamine polymerization, as shown in Figure 1. The adhesion, proliferation, and osteodifferentiation of human bone marrow stem cells were investigated to evaluate the efficacy of the surface modification.

## 2. EXPERIMENTAL SECTION

**2.1. Materials.** Reagents were received from Sigma-Aldrich (St. Louis, USA) unless specified otherwise. Cell culture medium consisted of Dulbecco's modified Eagle medium-low glucose (DMEM-LG) (Gibco, Grand Island, NY), 10% (v/v) fetal bovine serum (JRH Biosciences, Australia), 100 U/mL penicillin, and 10 $\mu$g/mL streptomycin. The culture medium supplemented with 1 mM sodium glycerophosphate, 0.1 $\mu$M dexamethasone, and 50 $\mu$g/mL L-ascorbate constitutes the osteogenic medium. RGD-containing peptides (Ac-GRGDSPGYG) were purchased from Kelowna Inc. (Taipei, Taiwan). The tyrosine (Y) residue incorporated in the peptide sequence was used for quantification of peptide conjugation in PEI, according to the absorbance at 275 nm (molar adsorption coefficient of tyrosine: 1420

$M^{-1}cm^{-1}$). Recombinant human morphogenetic protein-2 (rhBMP-2) was provided from Industrial Technology Research Institute (Hsinchu, Taiwan). The activity and purity of rhBMP-2 was demonstrated (Figure S1, Supporting Information).

Titanium discs (3 mm thick) were prepared by cutting cylindrical rods (6 mm in diameter and 8 cm in length) of Ti-6A1-4 V titanium alloy (grade V) by a discharge-cutting device. The titanium substrates were cleaned by soaking in 1 M HCl, followed by rinses with deionized water. After the titanium discs were ultrasonicated in 70 v/v% isopropyl alcohol, the samples were ready for poly(dopamine) coating.

**2.2. Synthesis of Hydroxyapatite Nanoparticles.** Hydroxyapatite (HAp) nanoparticles with a Ca/P molar ratio of 1/50 were synthesized by coprecipitation of $Ca(CH_3COO)_2$ and $H_3PO_4$.[29] Briefly, 0.001 wt % poly(acrylic acid) (PAA) (MW 2000 Da) was added into alkalized $H_3PO_4$ solution (0.33 M, pH > 12). $Ca(CH_3COO)_2$ solution (0.5 M) was then dropped into the 60°C $H_3PO_4$ solution at a rate of 5 mL/min. HAp nanoparticles were collected by filtration. The crystallinity of HAp was confirmed by X-ray diffraction as shown in the previous study.[30] The needle-like morphology of HAp nanoparticles was visualized by transmission electron microscopy. The dimension of HAp nanoparticles was 155 nm in length and 7.1 aspect ratio (length/diameter) (Figure S2, Supporting Information).

**2.3. Synthesis of PEI-g-RGD.** PEI-g-RGD was prepared by conjugation of RGD-containing peptides onto poly(ethyleneimine) (PEI) via a carbodiimide reaction, and the detailed procedure was described in a previous study.[31] The graft ratio of RGD to PEI was estimated as 3.10 mol % with respect to the total moles of the primary amino groups of PEI.

**2.4. Dopamine-Assisted Immobilization of Biomolecules onto Titanium Substrates.** 3-Hydroxytyramine hydrochloride (dopamine hydrochloride, abbreviated as DA, cat. # H8502, Sigma-Aldrich) and PEI-g-RGD were dissolved in 10 mM Tris buffer (pH 8.5) to 4 mg/mL, while HAp was suspended in the same buffer to 4 mg/mL. rhBMP-2 was dissolved in 0.1 % acetic acid to 40 $\mu$g/mL. The four solutions were mixed to obtain the final concentrations as listed in Table 1. The titanium discs were covered by 100 $\mu$L of the mixture in 96-well polystyrene plates for 60 min of incubation.

**2.5. Surface Characterization.** Electron spectroscopy for chemical analysis (ESCA) was used to characterize the surface chemical compositions of substrates. ESCA spectra for the samples were recorded on a VG Microtech MT-500 Spectrometer (UK) with

**Table 1. Compositions of Poly(dopamine) Coatings**

| abbreviation for surface types | DA (mg/mL) | PEI-g-RGD (mg/mL) | HAp (mg/mL) | rhBMP-2 ($\mu$g/mL) |
|---|---|---|---|---|
| Ti-DA | 1 | − | − | − |
| Ti-DA/RGD | 1 | 0.1, 0.5, or 1 | | |
| Ti-DA/HAp | 1 | − | 1 | − |
| Ti-DA/RGD/HAp | 1 | 1 | 1 | − |
| Ti-DA/RGD/HAp/ rhBMP-2 | 1 | 1 | 1 | 1 or 10 |

**Table 2. Primary Sequences of GAPDH, RunX2, ALP, OPN, BSP, and OCN**

| gene | primer (F: forward; R: reverse) | product size (bp) | NCBI reference sequence number |
|---|---|---|---|
| GAPDH | F: GTTCCAATATGATTCCACCC | 400 | M33197 |
| | R: TGAGTCCTTCCACGATACC | | |
| RunX2 | F: GTTTGTTCTCTGACCGCCTC | 318 | L40992 |
| | R: CCAGTTCTGAAGCACCTGAAA | | |
| ALP | F: CCCAAAGGCTTCTTCTTG | 356 | NM_000478.4 |
| | R: CTGGTAGTTGTTGTGAGCAT | | |
| OPN | F: CTAGGCATCACCTGTGCCATACC | 371 | NM_000582.2 |
| | R: CAGTGACCAGTTCATCAGATTCATC | | |
| BSP | F: TCAGCATTTTGGGAATGGCC | 657 | NM_004967.3 |
| | R: GAGGTTGTTGTCTTCGAGGT | | |
| OCN | F: CATGAGAGCCCTCACA | 310 | NM_199173.4 |
| | R: AGAGCGACACCCTAGAC | | |

**Table 3. The Elemental Compositions of Unmodified and Modified Titanium Substrates, Analyzed by ESCA**

| element (atomic concentration in %) | Ti | C | O | N | Ca | P |
|---|---|---|---|---|---|---|
| unmodified Ti | 75.8 | | 24.2 | | | |
| Ti-DA | 0.6 | 78.4 | 15.5 | 5.5 | | |
| Ti-DA/RGD (1 mg/mL) | 0.4 | 77.0 | 15.0 | 7.6 | | |
| Ti-DA/HAp | 1.2 | 51.3 | 28.4 | 6.6 | 7.0 | 5.5 |
| Ti-DA/RGD/HAp | 0.2 | 43.7 | 31.1 | 8.2 | 10.0 | 6.9 |
| Ti-DA/RGD/HAp/BMP-2 (1 μg/mL) | 0.3 | 41.5 | 30.9 | 6.4 | 13.3 | 7.6 |
| Ti-DA/RGD/HAp/BMP-2 (10 μg/mL) | | 52.3 | 23.4 | 11.3 | 7.1 | 6.9 |

an Mg K$\alpha$ X-ray source radiation.[32] The atomic compositions of the surfaces were calculated from the high-resolution spectrum of each element. Atom force microscopy (AFM) with a tapping mode was used to explore the surface topography of the substrates (AFM, Digital Instruments, Nanoscope III, 125 μm AFM scanning head).

Enzyme-linked immunosorbent assay (ELISA) was used to evaluate immobilization of rhBMP-2. The BMP-2/dopamine-modified titanium discs were first blocked by 2% bovine serum albumin (100 μL) at 37°C for 1 h, followed by rinses of PBST (PBS supplemented with 0.05% Tween 20). The blocked samples were then incubated with 100 μL of rabbit anti-human-BMP-2 polyclonal antibody (1:10 000, catalog No. APH960, AbD Serotec., USA) for 1 h and were rinsed with PBST. Next, 100 μL of horseradish peroxidase-conjugated anti-rabbit IgG (1:10 000, catalog No. ab6721, Abcam, USA) was added to each sample and incubated at 37°C for 1 h. The samples were then rinsed with PBST, followed by the addition of 40 mg/mL 1,3,3′,5,5′-tetramethylbenzidine dihydrochloride in dimethyl sulfoxide for a 10 min reaction on a shaker at room temperature. The reaction was stopped by adding 50 μL of 2N sulfuric acid. The OD of the substrate solution was read at 450 nm with a microtiter plate reader.

**2.6. Culture of hBMSC 3A6 Cells.** The isolation and maintenance of human bone marrow stem cell line (hBMSC 3A6 cells) followed a previously reported protocol,[33] which was approved by the institutional review board of Veteran's General Hospital-Taipei. Prior to cell experiments, the samples were sterilized by soaking in 70% ethanol, followed by rinses with sterilized PBS. 3A6 cells were seeded on the poly(dopamine)-modified samples at a density of $2 \times 10^4$ cells/cm$^2$ and cultured in a humidified incubator containing 5% CO$_2$ at 37°C. The cell culture medium was changed every 2 days.

After culture, the cells were lysed with 1% Triton X-100 in PBS. The cell numbers were determined by a lactate dehydrogenase assay, according to a previous procedure.[4] Intracellular alkaline phosphatase (ALP) activities were determined by determining enzymatic conversion of 4-nitrophenyl phosphate disodium salt to p-nitrophenol, as reported previously.[34]

**2.7. Reverse-Transcriptase Polymerase Chain Reaction (RT-PCR) for Analysis of the Expression of Osteogenic Genes.** The expression of several osteogenic genes, RunX2 (Runt-related transcription factor 2), ALP (alkaline phosphatase), OPN (osteopondin), BSP (bone sialoprotein), and OCN (osteocalcin), was analyzed by RT-PCR. The extraction of mRNA and the reverse-transcription into

complementary DNA (cDNA) were performed according to a previous procedure.[35] Briefly, RNA extraction was performed using RNeasy mini kit (Qiagen, USA) according to the manufacturer's instructions. Total RNA was reverse-transcribed using SuperScript III transcriptase according to the manufacturer's protocols (Invitrogen, USA). The PCR reactions were performed using Master Mix (Promega, USA). The PCR amplification cycles included denaturation for 30 s at 95°C, annealing for 90 s, and extension for 2 min at 72°C for 30 cycles. Primer sequences are listed in Table 2. PCR products were analyzed on 2% agarose and visualized under UV light after ethidium bromide staining. The amount of every PCR product was analyzed by using NIH Image J for quantification of the band intensities on the images of electrophoresis gels. The intensities of RunX2, ALP, OPN, BSP, and OCN were divided by the value of GAPDH of the same sample for normalization to cell numbers.

**2.8. Mineralization Culture.** After a one-day culture in the normal culture medium, the cells were cultured in the osteogenic medium with daily supplement of L-ascorbate (50 μg/mL). After the osteogenic culture, calcium deposited by the cells was extracted in 0.6 N HCl at 4°C overnight and then quantitated by a commercial calcium kit (Diagnostic Chemicals Limited, USA).[34]

**2.9. Statistical Analysis.** The data was reported as means ± standard deviation. The statistical analyses between different groups were determined using Student's t-test. Probabilities of $p \leq 0.05$ were considered as significant differences. All statistical analyses were performed using GraphPad Instat 3.0 (GraphPad Software, USA).

## 3. RESULTS AND DISCUSSION

**3.1. Surface Characterization of Poly(Dopamine)-Deposited Titanium.** The deposition of dopamine, PEI-g-RGD, HAp, and rhBMP-2 on the titanium substrates was verified by ESCA (Table 3). The oxygen atoms appearing on the unmodified titanium indicate that the surface is overlaid with a layer of titanium oxide. Poly(dopamine) deposition created the surface carbon and nitrogen contents to 78.4% and 5.5%, respectively. Incorporation of PEI-g-RGD further increased the nitrogen content to 7.6% (Ti-DA/RGD), which is contributed by the rich nitrogen atoms in PEI and the RGD peptides. On the other hand, the immobilization of HAp
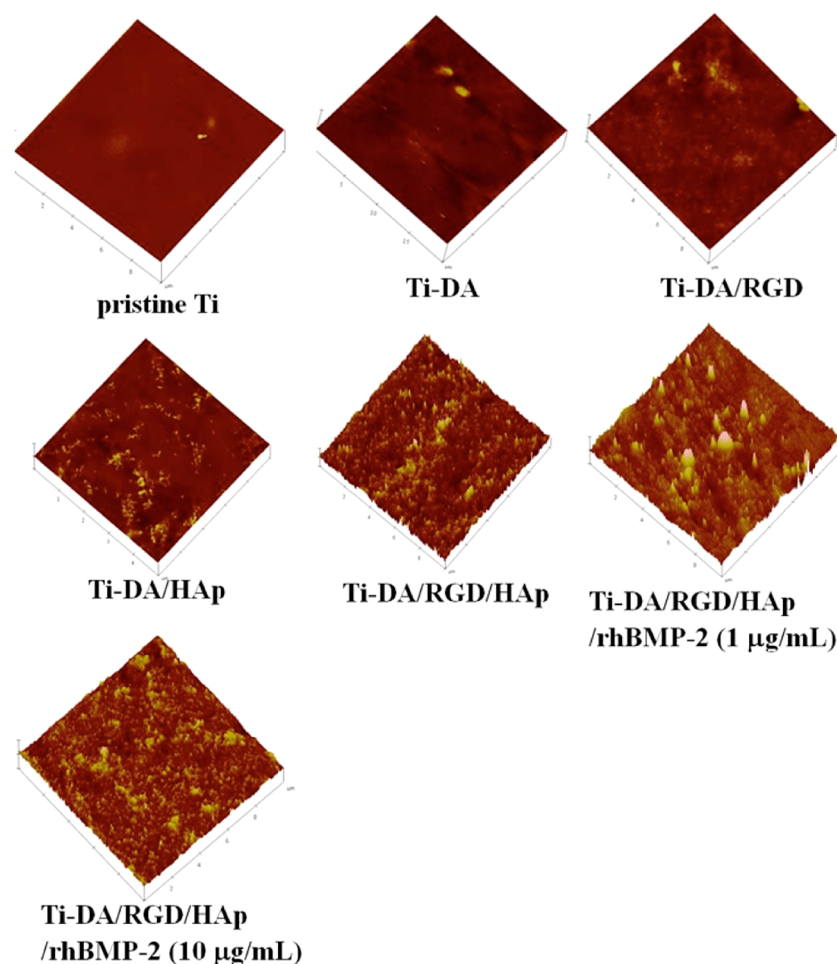
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**Figure 2.** AFM images of surface topography of different titanium substrates: pristine titanium (Ti), dopamine-deposited titanium (Ti-DA), dopamine/PEI-g-RGD-deposited titanium (Ti-DA/RGD), dopamine/HAp-deposited titanium (Ti-DA/HAp), dopamine/PEI-g-RGD/HAp-deposited titanium (Ti-DA/RGD/HAp), and dopamine/PEI-g-RGD/HAp/rhBMP-2 (1 or 10 μg/mL)-deposited titanium.

nanoparticles was revealed by the appearance of the calcium and phosphate elements on Ti-DA/HAp. Although incorporation of 1 μg/mL rhBMP-2 did not increase the surface nitrogen content, incorporation of 10 μg/mL rhBMP-2 increased the nitrogen element to 11.3%.

The topography of poly(dopamine)-modified titanium surfaces was analyzed using AFM (Figure 2, the corresponding enlarged AFM images are shown in Figure S3, Supporting Information). No significant topographic change was observed on Ti-DA and Ti-DA/RGD compared to the unmodified titanium. Tiny particulates appeared on the surfaces incorporated with HAp (Ti-DA/HAp and Ti-DA/RGD/HAp), indicating the deposition of HAp naoparticles. No further topographic change was found after incorporation of rhBMP-2.

**3.2. Culture of 3A6 Cells on the Titanium Deposited with Poly(dopamine)/PEI-g-RGD.** Improvement of cell adhesion and proliferation on bone implants is important for osteointegration. Poly(dopamine) deposition onto substrates has been demonstrated to enhance cell adhesion.[25,36,37] We previously demonstrated that the enhancement of cell adhesion on poly(dopamine)-deposited surfaces is ascribed to the increase in surface immobilized serum adhesive proteins.[25] A similar conclusion was also obtained in this study. After 1 and 7 days of culture, the cell numbers on poly(dopamine)-coated Ti surfaces were increased by 16.3% and 45.5%, respectively, with respect to the unmodified titanium ($p < 0.05$, Figure 3A).

Furthermore, after 7 days of osteogenic culture, the calcium content on the poly(dopamine)-deposited titanium was 31.8% more than the unmodified titanium surface ($p < 0.05$, Figure 3B).

Cell adhesive peptides, such as RGD, are known to bind cell membrane integrins and thus mediate cell adhesion. Surface immobilization of RGD has been shown as an effective way to improve the adhesion and mineralization of osteoblasts.[4,31,38−40] We previously conjugated bioactive molecules, such as galactose or PEG to PEI, and then deposited the grafted polymers on various surfaces via dopamine polymerization in order to enhance or inhibit cell attachment.[24,36] PEI provides multiple primary, secondary, and tertiary amines for covalent bonding with biomolecules via suitable reaction and incorporation with poly(dopamine). In this study, RGD peptides were grafted on PEI prior to codeposition with poly(dopamine) on titanium.

Incorporation of 0.1 or 0.5 mg/mL PEI-g-RGD only slightly increase cell attachment compared to Ti-DA. When PEI-g-RGD was increased to 1 mg/mL, cell attachment was increased to $4.59 \times 10^4$ cells/cm$^2$, approximately a 27% increase compared to Ti-DA ($p < 0.05$, Figure 3A). The trend continued through 7 days of culture. Furthermore, calcium deposition by 3A6 cells was increased with the incorporation of PEI-g-RGD in a concentration-dependent manner (Figure 3B). The amount of calcium deposition on Ti-DA/RGD (1 mg/
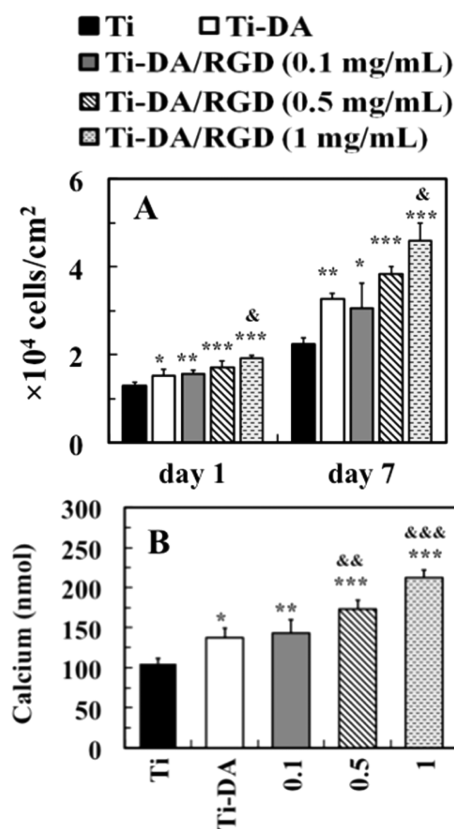
**Figure 3.** 3A6 cells were cultured in the osteogenic medium for 1 or 7 days on different substrates: titanium (Ti), dopamine-deposited titanium (Ti-DA), and dopamine/PEI-g-RGD (0.1, 0.5, or 1.0 mg/mL)-deposited titanium (Ti-DA/RGD). (A) The cell numbers and (B) calcium deposition were determined after cell culture. *, **, and *** represent $p < 0.05$, 0.01, and 0.001 vs. Ti, respectively. &, &&, and &&& represent $p < 0.05$, 0.01, and 0.001 vs. Ti-DA, respectively. Value = mean ± standard deviation; $n = 4$.
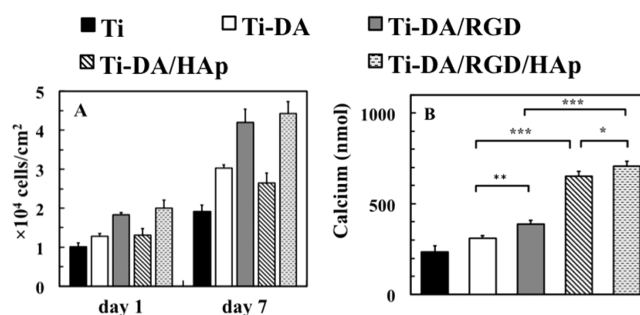


**Figure 4.** 3A6 cells were cultured in the osteogenic medium on different substrates: titanium (Ti), dopamine-deposited titanium (Ti-DA), dopamine/PEI-g-RGD-deposited titanium (Ti-DA/RGD), dopamine/HAp nanoparticle-deposited titanium (Ti-DA/HAp), and dopamine/PEI-g-RGD/HAp nanoparticle-deposited titanium (Ti-DA/RGD/HAp). (A) The cell numbers for 1 or 7 days of osteogenic culture and (B) calcium deposition after 14 days of osteogenic culture were determined. *, **, and *** represented $p < 0.05$, 0.01, and 0.001, respectively. Value = mean ± standard deviation; $n = 4$.

mL) was increased to 212 nmol, 1.54-fold of that on Ti-DA ($p < 0.001$). The increase in calcium deposition on Ti-DA/RGD should be due to the enhancement in cell population. Since 1 mg/mL RGD was most effective in the enhancement of cell attachment, proliferation, and calcium deposition among the three concentrations used in this study, this concentration was applied in the subsequent experiments.

**3.3. Culture of 3A6 Cells on Dopamine/PEI-g-RGD/HAp Deposited Titanium.** Previously, we showed that HAp nanoparticles were immobilized on titanium substrate via poly(dopamine) deposition to enhance the substrate's osteoconductivity.[28] The conjugation of HAp nanoparticles onto titanium was stable even under ultrasonication. We suggest that the immobilization of HAp nanoparticles on titanium surfaces is achieved by entrapment and/or conjugation with poly(dopamine). The conjugation of HAp nanoparticles greatly enhances the mineralization of osteoblasts on titanium substrates.

In this study, HAp nanoparticles were mixed with PEI-g-RGD for codeposition with poly(dopamine) on titanium substrates to investigate whether the osteogenic differentiation of 3A6 cells could be enhanced. Incorporation of RGD increased the cell attachment and proliferation. After 7 days of culture, the cell number on Ti-DA/RGD/HAp was about 4.42 × $10^4$ cells/cm², significantly higher than the value on Ti-DA/HAp (2.65 × $10^4$ cells/cm²) (Figure 4A, $p < 0.001$). On the

other hand, cell adhesion and proliferation was not affected by the incorporation of HAp nanoparticles. The cell numbers between Ti-DA and Ti-DA/HAp and between Ti-DA/RGD and Ti-DA/RGD/HAp were comparable after 1 or 7 days of culture. Therefore, the enhancement of cell adhesion and proliferation was mainly due to poly(dopamine) deposition and RGD incorporation but not HAp nanoparticles.

The osteodifferentiation of 3A6 cells was also analyzed by the expression of several osteogenic genes by RT-PCR. The electrophoretic images of the PCR products were shown in Figure S4, Supporting Information. After 4 days of osteogenic culture, only the substrates incorporated with HAp nanoparticles possessed elevated expression levels of RunX2, ALP, BSP, and OCN genes (Figure 5). The trend continued through 7 days of osteogenic culture. On the other hand, the effect of RGD on osteodifferentiation was not significant. No significant difference was found between Ti-DA/RGD/HAp and Ti-DA/HAp.

We further determined the calcium deposition, the final stage of osteogenesis. After 14 days of osteogenic culture, the mineralization of 3A6 cells was greatly enhanced by the incorporation of HAp nanoparticles. The amount of calcium deposition on Ti-DA/HAp was about 651 nmol, 2.1-fold of the value on Ti-DA ($p < 0.001$, Figure 4B). The calcium deposition on Ti-DA/RGD/HAp was further enhanced to 707 nmol, 1.82-fold of the value on Ti-DA/RGD. The enhanced calcium deposition indicates that HAp nanoparticles immobilized by dopamine polymerization possess osteoconductivity. On the other hand, RGD further enhanced calcium deposition, indicated by calcium deposition being higher on Ti-DA/RGD/HAp compared to Ti-DA/HAp ($p < 0.05$).

**3.4. Incorporation of rhBMP-2 for Enhancement of Osteoinduction.** Titanium substrates become more cell adhesive and osteoconductive via codeposition of RGD and HAp nanoparticles with poly(dopamine). However, the titanium surface still lacks osteoinductivity. In this study, rhBMP-2 was mixed in dopamine solution. We expect that rhBMP-2 would be incorporated in the poly(dopamine) films by entrapment or form covalent bonds with poly(dopamine).

Incorporation of rhBMP-2 was first evaluated using ELISA. Incorporation of 1 or 10 $\mu$g/mL rhBMP-2 increased the OD value to 0.34 or 1.29, respectively, greatly higher than the values
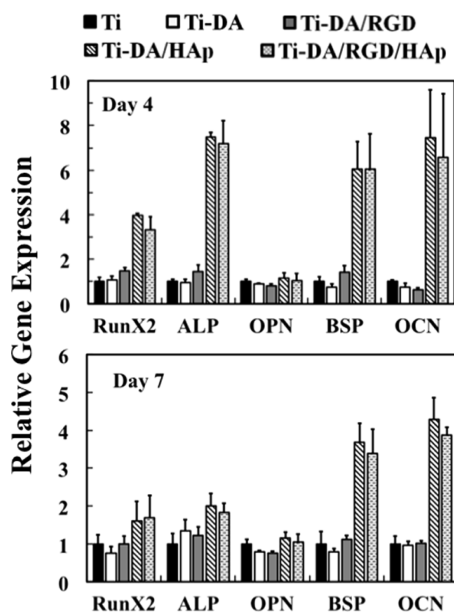
**Figure 5.** 3A6 cells were cultured in the osteogenic medium on different substrates: titanium (Ti), dopamine-deposited titanium (Ti-DA), dopamine/PEI-g-RGD-deposited titanium (Ti-DA/RGD), dopamine/HAp nanoparticle-deposited titanium (Ti-DA/HAp), and dopamine/PEI-g-RGD/HAp nanoparticle-deposited titanium (Ti-DA/RGD/HAp). After 4 or 7 days of osteogenic culture, the expression of several osteogenic genes such as RunX2 (Runt-related transcription factor 2), ALP (alkaline phosphatase), OPN (osteopondin), BSP (bone sialoprotein), and OCN (osteocalcin) was analyzed by RT-PCR. The intensity of each gene was normalized to the value of GADPH. Value = mean ± standard deviation; $n = 3$.

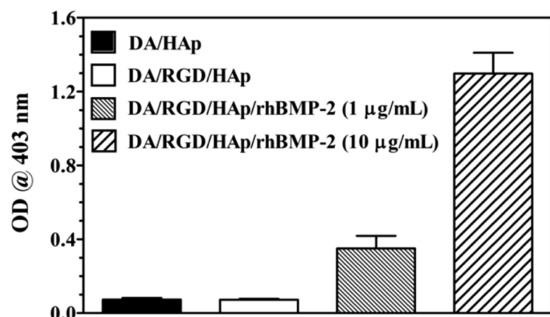on Ti-DA/HAp and Ti-DA/RGD/HAp (OD ∼ 0.08, $p <$ 0.001, Figure 6). The ELISA results indicate that rhBMP-2 is



**Figure 6.** Surface immobilization of rhBMP-2 was evaluated by ELISA. 1 or 10 $\mu$g/mL indicates the concentration of rhBMP-2. Value = mean ± standard deviation; $n = 4$.

exposed on the titanium surface. However, the ELISA assay cannot quantify the exact amount of incorporated rhBMP-2. Using radioactive proteins for quantification of surface-bound proteins is an accurate method. Unfortunately, we do not have the facility and isotope-labeled rhBMP-2. Therefore, we attempted to use gold nanoparticles (AuNPs) to mimic the incorporation of rhBMP-2. The preparation of AuNPs was detailed in the Supporting Information. The size of AuNPs was ∼10 nm, comparable to the size of rhBMP-2 (∼4 nm). AuNPs (1 or 10 $\mu$g/mL) were deposited with 1 mg/mL dopamine. The deposited AuNPs were dissolved in 20% (v/v) $HNO_3$ solution, and the amount of Au was then determined by an

inductively-coupled plasma for spectrometry. The results indicate that ∼28% and ∼63.5 % of AuNPs was immobilized along with poly(dopamine) from 1 and 10 $\mu$g/mL AuNPs, respectively (Table S1, Supporting Information). The amount of deposited AuNPs from 10 $\mu$g/mL was ∼22-fold of the value from 1 $\mu$g/mL. Both ELISA and AuNPs data suggest that rhBMP-2 is immobilized at a higher amount when a higher concentration is used. However, the exact amount of immobilized rhBMP-2 is still unknown.

The osteoinductive ability of the immobilized rhBMP-2 was then evaluated under osteogenic and non-osteogenic culture. For the osteogenic culture, the cells were cultured in the normal culture medium for 1 day, and then, the culture medium was replaced by the osteogenic medium for a further 7 days of culture. For the non-osteogenic culture, the culture medium was replaced by fresh normal culture medium after 2 days of seeding, followed by another 6 days of culture in normal culture medium. Therefore, the total culturing time was 8 days for both culture conditions. After 2 days of osteogenic culture, the cell number on Ti-DA/RGD/HAp was ∼$1.86 \times 10^4$ cells/$cm^2$, 1.7 times of the value on Ti-DA/HAp (Figure 7A). Incorporation of 1 $\mu$g/mL rhBMP-2 did not further increase cell attachment, while cell adhesion was increased to $2.43 \times 10^4$ cells/$cm^2$ at 10 $\mu$g/mL rhBMP-2, significantly higher than the other groups ($p < 0.001$). The trend remained after 8 days of culture. Under non-osteogenic culture, incorporation of rhBMP-2 at 1 $\mu$g/mL did not affect cell attachment and proliferation compared with Ti-DA/RGD/HAp. On the other hand, the deposition of 10 $\mu$g/mL rhBMP-2 initially increased cell attachment after 2 days of non-osteogenic culture, but on Day 8, the cell number ($9.17 \times 10^4$ cells/$cm^2$) was smaller than the values for Ti-DA/RGD/HAp and Ti-DA/RGD/HAp/BMP-2 (1 $\mu$g/mL) ($p < 0.001$). The results indicate that cell proliferation is retarded at high surface deposition of rhBMP-2 under the culture condition without the supplement of osteogenic reagents.

Next, cellular alkaline phosphatase (ALP) activity, an early marker of osteodifferentiation, was investigated under osteogenic and non-osteogenic culture conditions. After 8 days of osteogenic culture, the ALP activities were elevated on all the substrates without significant difference, except that the value on Ti-DA/RGD/HAp/rhBMP-2(10) was significantly higher (154 fmol/cell/h, $p < 0.01$ vs. the other groups, Figure 7B). On the other hand, after 8 days of non-osteogenic culture, ALP activities were not elevated on all the surfaces except Ti-DA/RGD/HAp/rhBMP-2(10) (43 fmol/cell/h, $p < 0.001$ vs. the other groups, Figure 7B), although this value was still much lower than those under osteogenic culture ($p < 0.001$). The results suggest the osteoinductivity of surface-bound rhBMP-2 via poly(dopamine) deposition.

We next further analyzed the expression of several osteogenic genes under osteogenic and non-osteogenic culture using RT-PCR. The electrophoretic images of electrophoresis were shown in Figure S5 (osteogenic culture) and Figure S6 (non-osteogenic culture), Supporting Information. After 3 days of osteogenic culture, the expression of RunX2 and ALP genes was elevated on all the surfaces without significant difference between different substrates, while the expression of OPN, BSP, and OCN was not detected yet (Figure 8A). After 5 days of osteogenic culture, all the studied genes were expressed on all the substrates. The expression of RunX2, ALP, and OPN was at a similar level on all the substrates, while the expression of BSP and OCN was higher on Ti-DA/RGD/HAp/rhBMP-2(10)
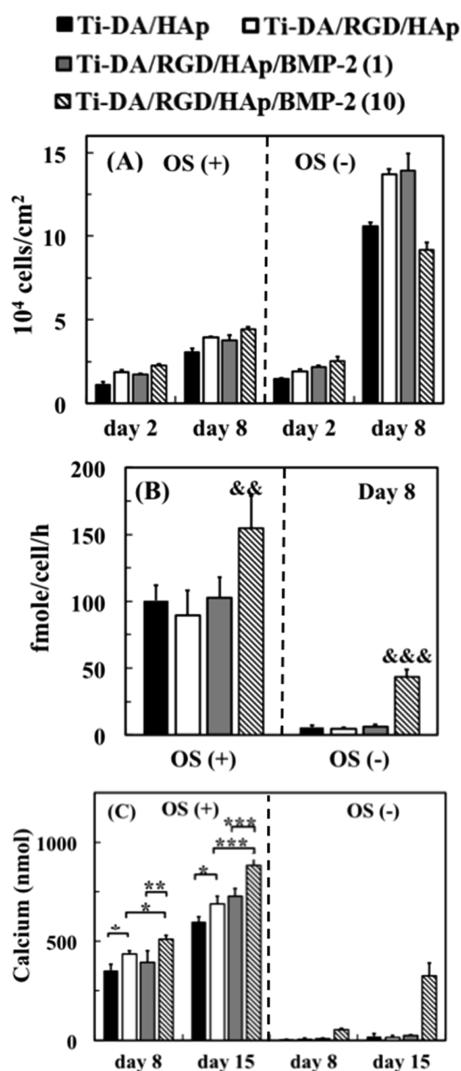
■ Ti-DA/HAp  □ Ti-DA/RGD/HAp
▨ Ti-DA/RGD/HAp/BMP-2 (1)
◪ Ti-DA/RGD/HAp/BMP-2 (10)



**Figure 7.** 3A6 cells were cultured on the titanium substrates with different coatings: dopamine/HAp nanoparticle (Ti-DA/HAp), dopamine/PEI-g-RGD/HAp nanoparticle (Ti-DA/RGD/HAp), and dopamine/PEI-g-RGD/HAp nanoparticle/rhBMP-2 (1 or 10 ng/mL) (Ti-DA/RGD/HAp/BMP-2 (1 or 10)). OS(+) and OS(−) represent the osteogenic and non-osteogenic culture conditions, respectively. (A) The cell numbers, (B) alkaline phosphatase activities in terms of the amount of $p$-nitrophenol formation, and (C) calcium deposition were determined after cell culture. && and &&& represented $p < 0.01$ and 0.001 vs. the other conditions, respectively. *, **, and *** represented $p < 0.05$, 0.01, and 0.001, respectively. Value = mean ± standard deviation; $n = 4$.

(Figure 8B, $p < 0.05$ vs. the other groups). After 8 days of osteogenic culture, the expression of ALP and OCN on Ti-DA/RGD/HAp/BMP-2(10) was significantly higher compared to the other groups (Figure 8C, $p < 0.05$). The RT-PCR analysis indicates that the incorporation of rhBMP-2 enhances the osteodifferentiation of 3A6 cells even in the osteogenic medium.

When the cells were cultured under the non-osteogenic condition, the osteogenic genes studied here were all silent on all the substrates throughout the culturing period except the surface deposited with 10 μg/mL rhBMP-2 (Figure 9A–D). RunX2 was activated on Ti-DA/RGD/HAp/BMP-2(10) after 3 days of culture (Figure 9A), while ALP was activated after 5 days of culture (Figure 9B). The other genes were activated
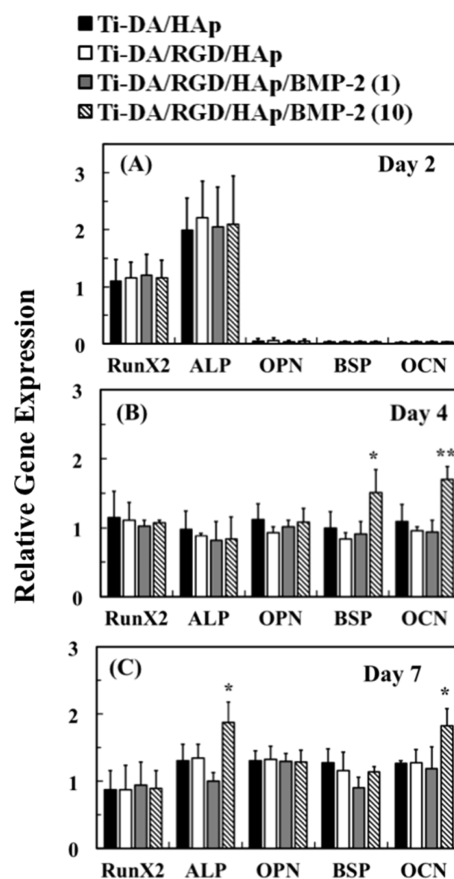
■ Ti-DA/HAp
□ Ti-DA/RGD/HAp
▨ Ti-DA/RGD/HAp/BMP-2 (1)
▨ Ti-DA/RGD/HAp/BMP-2 (10)



**Figure 8.** 3A6 cells were cultured in the osteogenic medium on the titanium substrates with different coatings: dopamine/HAp nanoparticle (Ti-DA/HAp), dopamine/PEI-g-RGD/HAp nanoparticle (Ti-DA/RGD/HAp), and dopamine/PEI-g-RGD/HAp nanoparticle/rhBMP-2 (1 or 10 ng/mL) (Ti-DA/RGD/HAp/BMP-2 (1 or 10)). The expression of several osteogenic genes, such as RunX2 (Runt-related transcription factor 2), ALP (alkaline phosphatase), OPN (osteopondin), BSP (bone sialoprotein), and OCN (osteocalcin), was analyzed by RT-PCR. The intensity of each gene was normalized to the value of GADPH. * and ** represent $p < 0.05$ and 0.01 vs. Ti-DA/RGD/HAp. Value = mean ± standard deviation; $n = 3$.

after 8 days of culture medium (Figure 9C). The expression of OCN reached a maximal level after 15 days of culture, while the other genes seemed to be waning (Figure 9D). The RT-PCR results indicate that the titanium surface becomes osteoinductive when rhBMP-2 is immobilized on the titanium surface via dopamine polymerization.

Finally, calcium deposition on the substrates after long-term cell culture was evaluated. After 8 or 15 days of osteogenic culture, calcium deposition on all the substrates was all elevated (Figure 7C). Incorporation of RGD enhanced calcium deposition compared to Ti-DA/HAp ($p < 0.05$). The deposition of 1 μg/mL rhBMP-2 did not further enhance calcium deposition, while 10 μg/mL rhBMP-2 further increased calcium deposition compared with the other groups ($p < 0.001$). On the other hand, when the cells were cultured in the non-osteogenic medium, calcium deposition was not detectable on all the substrates except the surface deposited with 10 μg/mL rhBMP-2 (55 and 325 nmol Ca²⁺/sample on day 8 and day 15, respectively) (Figure 7C). Therefore, the osteoinductive ability of poly(dopamine)-immobilized rhBMP-2 is demonstrated.
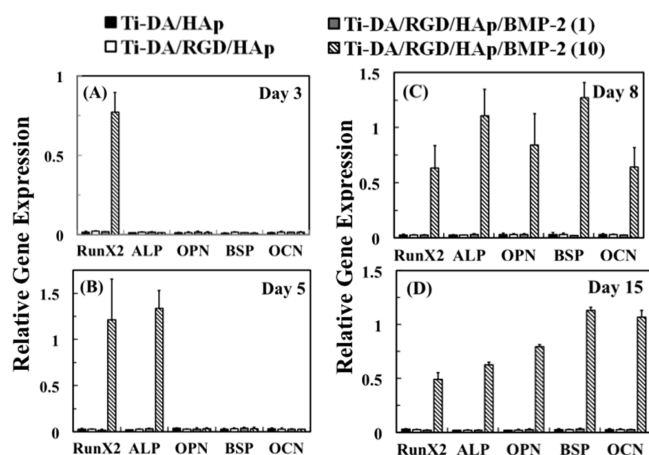
**Figure 9.** 3A6 cells were cultured in the non-osteogenic medium on the titanium substrates with different coatings: dopamine/HAp nanoparticle (Ti-DA/HAp), dopamine/PEI-g-RGD/HAp nanoparticle (Ti-DA/RGD/HAp), and dopamine/PEI-g-RGD/HAp nanoparticle/rhBMP-2 (1 or 10 ng/mL) (Ti-DA/RGD/HAp/BMP-2 (1 or 10)). The expression of several osteogenic genes such as RunX2 (Runt-related transcription factor 2), ALP (alkaline phosphatase), OPN (osteopondin), BSP (bone sialoprotein), and OCN (osteocalcin) was analyzed by RT-PCR. The intensity of each gene was normalized to the value of GADPH. Value = mean ± standard deviation; $n = 3$.

In this study, we used a different protein-immobilization strategy via dopamine polymerization from the methods used in the literature. Post-conjugation is commonly used for protein immobilization on poly(dopamine)-coated surfaces.[27,41−43] Briefly, poly(dopamine) is preformed on a substrate and then chemically heterogeneous poly(dopamine) that presents catechol and quinone groups binding to proteins, probably via the interaction with amino acid residues containing amino or thiol side chains. It is shown that enzymes or growth factors still had their activities after immobilization on poly(dopamine) surfaces.[41,42] Differently, in this study, rhBMP-2 was deposited in a single step with dopamine polymerization. During the deposition process, dopamine may react with peptides and proteins and thus be detrimental to the efficacy of biomolecules, such as the osteoinductive ability of BMP-2. However, our results indicate that the bioactivity of BMP-2 still remains at a level sufficient for the osteodifferentiation of 3A6 cells. Our one-step immobilization strategy only takes less than an hour. On the other hand, the post-conjugation strategy usually takes at least one day for protein immobilization.[41] Therefore, our strategy saves a lot of deposition time.

This study demonstrates that several bioactive macromolecules and nanoparticles could be immobilized on a substrate via poly(dopamine). In this study, three different types of biomolecules, cell adhesive RGD that enhances the adhesion and proliferation of osteoblasts or MSCs, osteoconductive HAp that enhances osteogenesis, and osteoinductive BMP-2 that induces osteogenesis, are coimmobilized on the titanium surfaces. Ti surface is especially suitable for this purpose. Titanium substrates are covered by a layer of titanium oxide once the metal is exposed to air, as indicated in the ESCA data (Table 3). Lee et al. showed that the interaction between a catechol molecule and a $TiO_2$ surface is of surprisingly high strength.[44] The strong interaction facilitates the deposition of poly(dopamine) coating on titanium surface. Three different types of bioactive substances with different forms (polymer, nanoparticle, and protein) are mixed in dopamine solution and

then immobilized on a substrate simultaneously during dopamine polymerization. This "one-pot" strategy successfully incorporates cell adhesive peptides, osteoconductive hydroxyapatite nanoparticles, and osteoinductive BMP-2 colocalized on titanium surfaces. The adhesion, proliferation, and osteodifferentiation of human bone marrow stem cells (3A6 cells) are significantly enhanced by the synergetic effect of RGD, HAp, and BMP-2 on the modified titanium surface. We expect that osteointegration of a titanium implant would be enhanced by our surface modification strategy and protocol. This simple method can even be done in a clinical setting. For example, a dentist can soak a dental implant in the mixture of dopamine/RGD/HAp/BMP-2 for minutes and then insert the modified implant into a implantation site immediately.

## 4. CONCLUSION

In this work, we developed a facile and effective "one-pot" deposition method based on dopamine polymerization for the development of cell-adhesive, osteoconductive, and osteoinductive titanium implants. PEI-g-RGD, HAp, and BMP-2 were immobilization on titanium surfaces together with poly(dopamine). The adhesion, proliferation, and osteodifferentiation of human bone marrow stem cells were greatly enhanced on the modified titanium surfaces. The surface modification technique shows a great potential for the development of osteointegrative orthopaedic and dental implants.

## ■ ASSOCIATED CONTENT

**Ⓢ Supporting Information**
(1) Information regarding recombinant human morphogenetic protein-2 (Figure S1); (2) TEM images of synthesized hydroxyapatite nanoparticles (Figure S2); (3) AFM images of surface topography of various titanium substrates (Figure S3); (4) electrophoresis images for RT-PCR analysis (Figures S4−S6); (5) determination of immobilization of 10 nm gold nanoparticles (Table S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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