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Research Article

Surface Modification of Titanium with Heparin-Chitosan Multilayers via Layer-by-Layer Self-Assembly Technique

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Extracellular matrix (ECM), like biomimetic surface modification of titanium implants, is a promising method for improving its biocompatibility. In this paper chitosan (Chi) and heparin (Hep) multilayer was coated on pure titanium using a layer-by-layer (LbL) self-assembly technique. The Hep-Chi multilayer growth was carried out by first depositing a single layer of positively charged poly-L-lysine (PLL) on the NaOH-treated titanium substrate (negatively charged surface), followed by alternate deposition of negatively charged Hep and positively charged Chi, and terminated by an outermost layer of Chi. The multilayer was characterized by DR-FTIR, SEM, and AFM, and osteoblasts were cocultured with the modified titanium and untreated titanium surfaces, respectively, to evaluate their cytocompatibility *in vitro*. The results confirmed that Hep-Chi multilayer was fabricated gradually on the titanium surface. The Hep-Chi multilayer-coated titanium improved the adhesion, proliferation and differentiation of osteoblasts. Thus, the approach described here may provide a basis for the preparation of modified titanium surfaces for use in dental or orthopedic implants.

1. Introduction

Titanium (Ti) metal and its alloys have been widely used as orthopedic and dental implants. However, Ti is a bioinert material, so that it passively integrated with bone when implanted, and the process of osseointegration between bone and Ti surface needs a long time [1]. Considerable research has been carried out to improve the biocompatibility of titanium implants. Recently, works on the immobilization of biologically active molecules on titanium surfaces and extracellular matrix- (ECM-) like biomimetic surface modification have generated great interest in the dental and orthopedic fields [2–4]. The approach includes passive adsorption, silicate coupling, self-assembled monolayers (SAMs), Langmuir-Blodgett (LB) membranes, and layer-by-layer (LbL) self-assembly methods [3, 5–9]. The LbL deposition process was discovered and developed in the 1990s by Decher and coworkers [10]. This technique is based on the consecutive adsorption of polyanions and polycations via electrostatic interactions. LbL allows for the deposition of homogeneous films with layer thickness controlled on the nanometer scale and with controlled surface structure and charge. Compared with the classic chemical immobilization method, the LbL technique has the least demand for reactive chemical bonds and efficiently keeps molecular activity [8]. Thus, The LbL self-assembly has already been applied extensively in biomaterial fields [11, 12]. In 1995, Cai et al. [8] built a multilayer with chitosan and gelatin (gelatin as the outmost layer) on titanium surface via LbL technique, which was proved to improve titanium biocompatibility. Later reports [13, 14] showed that, if chitosan was the outmost layer, the coating would have similar effect, and would be stable for more than 3 weeks immersed into PBS solution in room temperature. Hu et al. [15] demonstrated that Chi/pGB (plasmid DNA) LbL-modified titanium films were beneficial for sustained *in situ* inducing osteoprogenitor cells to differentiate into mature osteoblasts over a long time.

Glycosaminoglycan (GAG) is an important component of the extracellular matrix (ECM) and has the specific affinity with growth factors and adherence proteins [16, 17]. Heparin (Hep) is an anionic polysaccharide, and can interact with many growth factors to benefitosseointegration indirectly. Heparin-binding growth factors (HBGFs), such as transforming growth factor-β (TGF-β) and bone morphogenetic proteins (BMPs), modulate bone formation and bone resorption by acting as autocrine or paracrine effectors on bone cell proliferation and differentiation [18–20]. Heparan-like molecules, like heparin or heparan sulfates, are able to induce bone regeneration of skull defects and possibly mediated by potentiation of endogenous growth factor activities and/or by neutralization of proteolytic activities [21]. Chitosan (Chi) is a cationic natural biopolymer produced by alkaline N-deacetylation of chitin, with an analogous structure to GAG, and has been extensively used in medicine due to its good biocompatibility, biodegradability, low toxicity, and low cost [22, 23]. Thus, in this study, Chi and Hep were used for an LbL self-assembly system to "build" the bioactive coating on titanium substrate. To our knowledge, little has been studied up to now that Chi and Hep are immobilized on titanium surface via the LbL technique to enhance titanium biocompatibility.

Titanium carries net negative charge at a physiological pH [24] and can be used as substrates for LbL assembly. Furthermore, surface properties and structure of titanium play an essential role in protein adsorption. The increase of hydroxyl groups, surface energy, and submicrometer morphology of titanium surface would enhance the reactivity of titanium with the protein and reduce the release of the biomacromolecules [25, 26]. Titanium treated with alkali solution has the surface property with large hydroxyl groups, higher surface energy, hydrophilicity, and submicrometer porous morphology [27]. Thus, we chose NaOH-treated titanium as the substrate surface to build a multilayer coating composided of chitosan and heparin. We expect that this Hep-Chi multilayer coating can improve Ti biocompatibility.

2. Materials and Methods

2.1. Materials. Commercial pure titanium was purchased from Baoji Non-ferrous Metal Co. Ltd. (Shanxi Province, China). Chitosan (degree of deacetylation 96.5%; viscosity 55 mPa.s) was from Yuhuan Ocean Biochemical Co. Ltd. (Zhejiang province, China). Heparin was from Suolaibao Biochemical Co. Ltd. (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM), trypsin and fetal bovine serum (FBS) were from GIBCO Life Technologies (Grand Island, NY, USA). Poly-L-lysine (PLL, 15–30 kDa), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), dimethyl sulfoxide (DMSO) were from Sigma-Aldrich Inc. (USA). the ALP activity kit was from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Other chemical reagents (A. R.) were from Changzheng Chemical Agent Company (Chengdu, China).

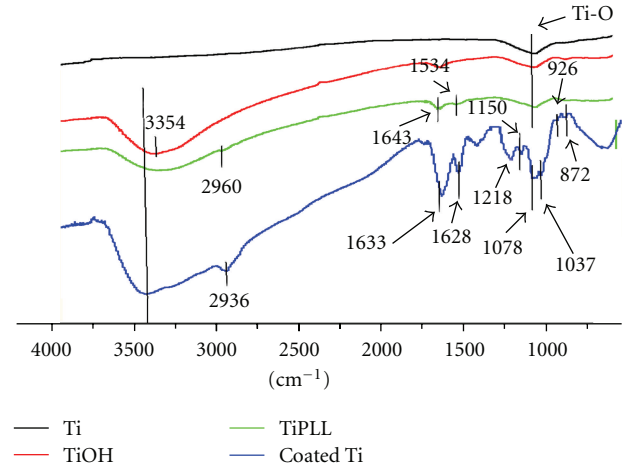


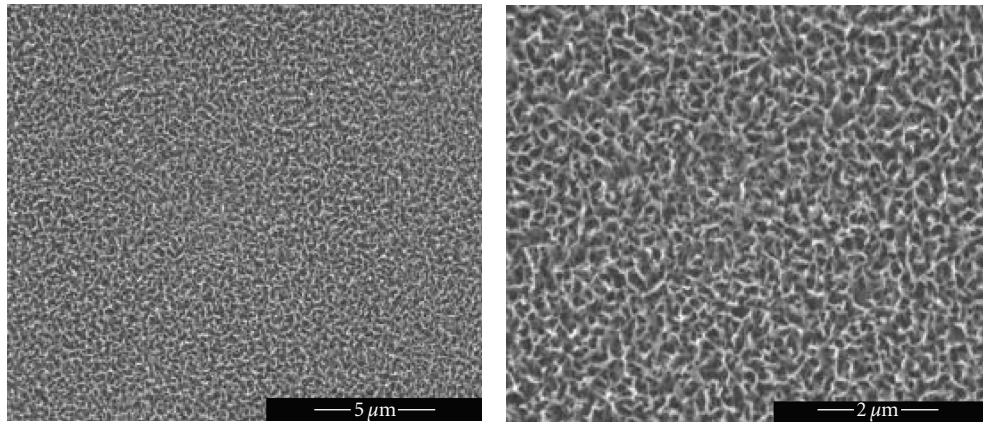
Figure 1: DR-FTIR spectra of untreated titanium (Ti), NaOH-treated titanium (TiOH), PLL-coated titanium (TiPLL), and Ti/PLL/(Hep/Chi)$_{18}$(coated Ti).

2.2. Preparation of Titanium Surface with NaOH. Titanium (Non-ferrous Metal Co. Ltd., Baoji, China) disks (10 mm in diameter and 2 mm thick) were polished to a reflective mirror-like finish. Firstly the specimens were ultrasonically cleaned in a detergent solution, then in acetone, ethanol, and de-ionized water, and finally dried at 60°C. These original titanium samples would be used as the control ones in the later cell experiments. The cleaned specimens were soaked in 5 M NaOH solution at 60°C for 24 hours and followed by de-ionized water at 80°C for 24 hours, then were cleaned with de-ionized water, and at last dried at 60°C.

2.3. Fabrication of Multilayers of Heparin-Chitosan. Chitosan (Chi, a polycation) was dissolved in 1% (V/V) acetic acid with a concentration of 5 mg/mL, and its pH value was adjusted to about 4 with 1% (V/V) acetic solution and 0.1 M NaOH. Heparin (Hep, a polyanion) was dissolved in de-ionized water with concentration of 5 mg/mL, and its pH value was also adjusted to about 4. Poly-L-lysine solution of 2.5 mg/mL was dissolved in a phosphate buffer solution (PBS, pH = 7.4).

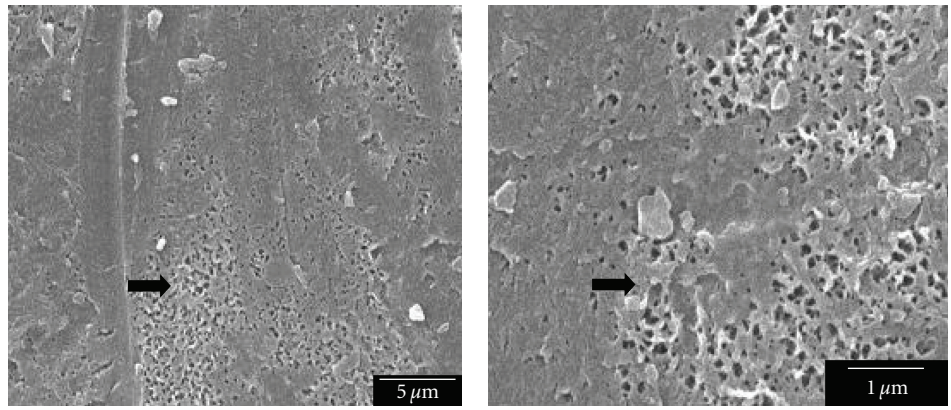
The NaOH-treated samples were immersed in PLL solution for 30 minutes, thus, obtaining a precursor layer with the stable positive charge to initiate the LbL assembly process. The multilayers were fabricated by alternately and successively immersing the titanium samples in the heparin and chitosan solution, followed by 10 minutes of adsorption and subsequently rinsing two times with de-ionized water for 1 minute every cycle. The cycle was repeated n times to obtain the desired film, terminated with a layer of Chi—denoted as Ti/PLL/(Hep/Chi)$_n$—and dried at ambient environment. The surface-modified titanium samples with eighteen-layer films, Ti/PLL/(Hep/Chi)$_{18}$, were achieved by such alternative deposition for the further cell experiments.

2.4. Analysis of the Morphology and Chemical Character of the Surfaces. The morphology of all the specimen surfaces



(a) (b)

Figure 2: SEM images of sample surfaces: NaOH treated titanium.



(a) (b)

Figure 3: SEM images of sample surfaces: Ti/PLL/$(Hep/Chi)_{10}$.

was characterized by scanning electron microscopy (SEM; QUANTA 200, FTI, Holland) at the accelerating voltage of 20 KV and atomic force microscopy (AFM; SPI3800N Seiko, Japan) with tapping mode and standard Si tips. The chemical character of the surfaces was studied by diffuse reflectance Fourier transform infrared spectroscopy (DR-FTIR; agna-IR 550, Nicolet, Madison, Am) at the resolution of 4 cm^{-1}.

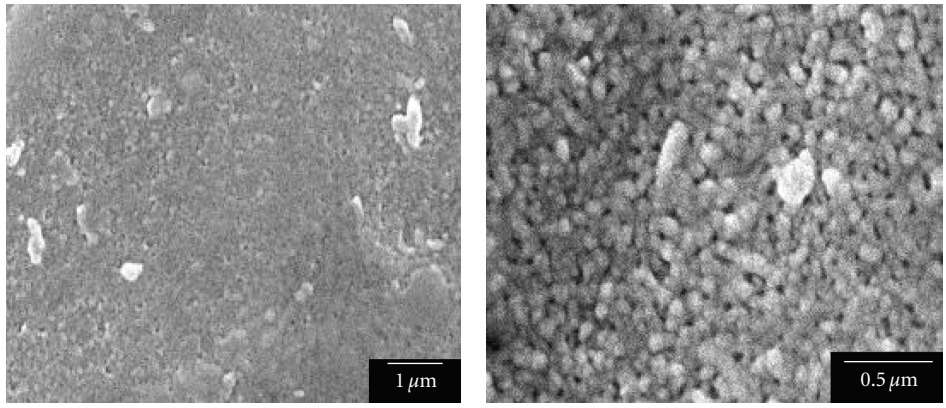
2.5. Cell Culture. Osteoblasts were isolated via sequential collagenase digestions of neonatal rat calvaria according to the established protocol [28]. They were cultured in 25 mL culture bottles containing 5 mL DMEM 10% FBS, and incubated at a humidified 5% CO_2 atmosphere at 37°C. The medium was changed every third day and for sub-culture. Osteoblasts at passage no. 2-3 were used in the following experiments.

2.6. Cell Adhesion. At passage no. 2 cells were seeded onto the original titanium samples and LbL-modified titanium samples at a density of 5×10^4 cells/cm^2 in a 24-well plate. The samples were sterilized with ethylene oxide in West China Hospital affiliated to Sichuan University.

After being cultured on the samples for 4 hours and 24 hours, respectively, the cells adhered to the samples were gently washed with PBS 3 times, fixed with 2.5% glutaraldehyde solution for 2 hours at 4°C, dehydrated through a series of graded ethanol solution, critical point dried, and then sputter gold coated in vacuum. The two-group samples were observed by SEM (Philips, FEI inspect F, Holland) for the morphologic change of cell attachment and spreading.

2.7. Cell Proliferation. Proliferation of the cells was studied by the MTT assay. Yellow MTT reagent can be converted to a dark blueformazan by mitochondrial succinate dehydrogenase of viable cells [29]. Therefore, the production offormazan may reflect the number of viable cells or cell viability.

Osteoblasts were seeded at a density of 8×10^4 cells/cm^2 in a 24-well plate containing the original titanium samples and LbL-modified titanium samples. The culture medium in



(a) (b)

Figure 4: SEM images of sample surfaces: Ti/PLL/$(Hep/Chi)_{16}$.

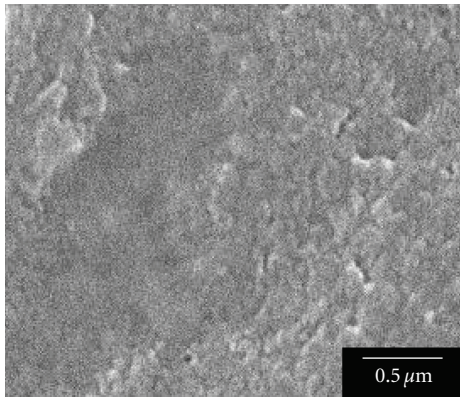


Figure 5: SEM images of sample surfaces: Ti/PLL/$(Hep/Chi)_{18}$.

wells was refreshed every 3 days. After 1, 4, and 7 days of the culture, respectively, 40 μL MTT (5 mg/mL) was added to each well containing 1 mL culture medium and incubated for 4 hours at 37°C. Then, the blue formazan reaction product was dissolved by adding 420 μL DMSO and transferred to a 96-well plate (200 μL every well). The absorbance was measured at 570 nm using a Bio Assay Reader (HTS7000 plus, PERKIN ELMER, USA). Four replicates were read for each sample, and the mean value was used as the final result.

2.8. Cell Differentiation. Alkaline phosphatase (ALP) activity is one of the most widely used early osteodifferentiation markers for osteoblasts [30]. ALP can convert the substrate p-nitrophenyl phosphate into yellow p-nitrophenol and phosphate.

Osteoblasts (2×10^5 cells/cm^2) were seeded onto the LbL-modified titanium samples and control ones in two 6-well plates and cultured for 7 days. Cells were removed off the samples by trypsinization and centrifuged (1000 r/min, 8 minutes). We moved away the supernatant, added 5 mL PBS solution to be a suspension, and counted the cell number and adjusted to the same concentration between the control and experimental groups. Then, aliquot of cell suspension was centrifuged (1000 r/min, 8 minutes) again, resuspended in 120 μL PBS solution, and maintained at −20°C. These suspensions were experienced three freeze-thaw cycles, and used for determining the ALP activity with p-nitrophenyl phosphates substrate as the kit instruction. The absorbance at 405 nm was measured by a Bio Assay Reader (HTS7000 plus, PERKIN ELMER, USA).

2.9. Statistical Analysis. All data were expressed as means ± standard deviation (S) for $n = 4$. Single factor analysis of variance (ANOVA) technique was used to assess the statistical significance of the results between the two groups. The statistical assessment was done by the software SPSS 15.0 at a confidence level of 95%.

3. Results and Discussion

3.1. DR-FTIR. In the FTIR spectrum of the NaOH-treated titanium (Figure 1), the broad peak at 3354 cm^{-1} suggested that the surface was rich in hydroxyl groups.

In Figure 1 of PLL-coated sample (Ti/PLL), the distinctive –NH_2(C=O) symmetric vibrational I and II bands were centered at about 1643 cm^{-1}, 1543 cm^{-1}; the peak at 2956 cm^{-1} was attributed to alkyl vibrational band. These indicated that PLL was coated onto the NaOH-treated titanium.

A comparison of the DR-FTIR spectra of untreated titanium (Ti), NaOH-treated titanium (TiOH), and the coated titanium (TiPLL, Ti/PLL/$(Hep/Chi)_{18}$) (Figure 1) confirmed that Hep-Chi were coated on the titanium surface; that is, the distinctive peaks of chitosan at 1150 cm^{-1} and 926 cm^{-1}, sulphonic acid group peaks of heparin at 1218 cm^{-1} and 872 cm^{-1} existed in spectra (Figure 1 blue line).

3.2. SEM. The SEM photos (Figure 2) showed that the surface of NaOH-treated titanium was characterized by uniform submicron pores and a mesh-like morphology.

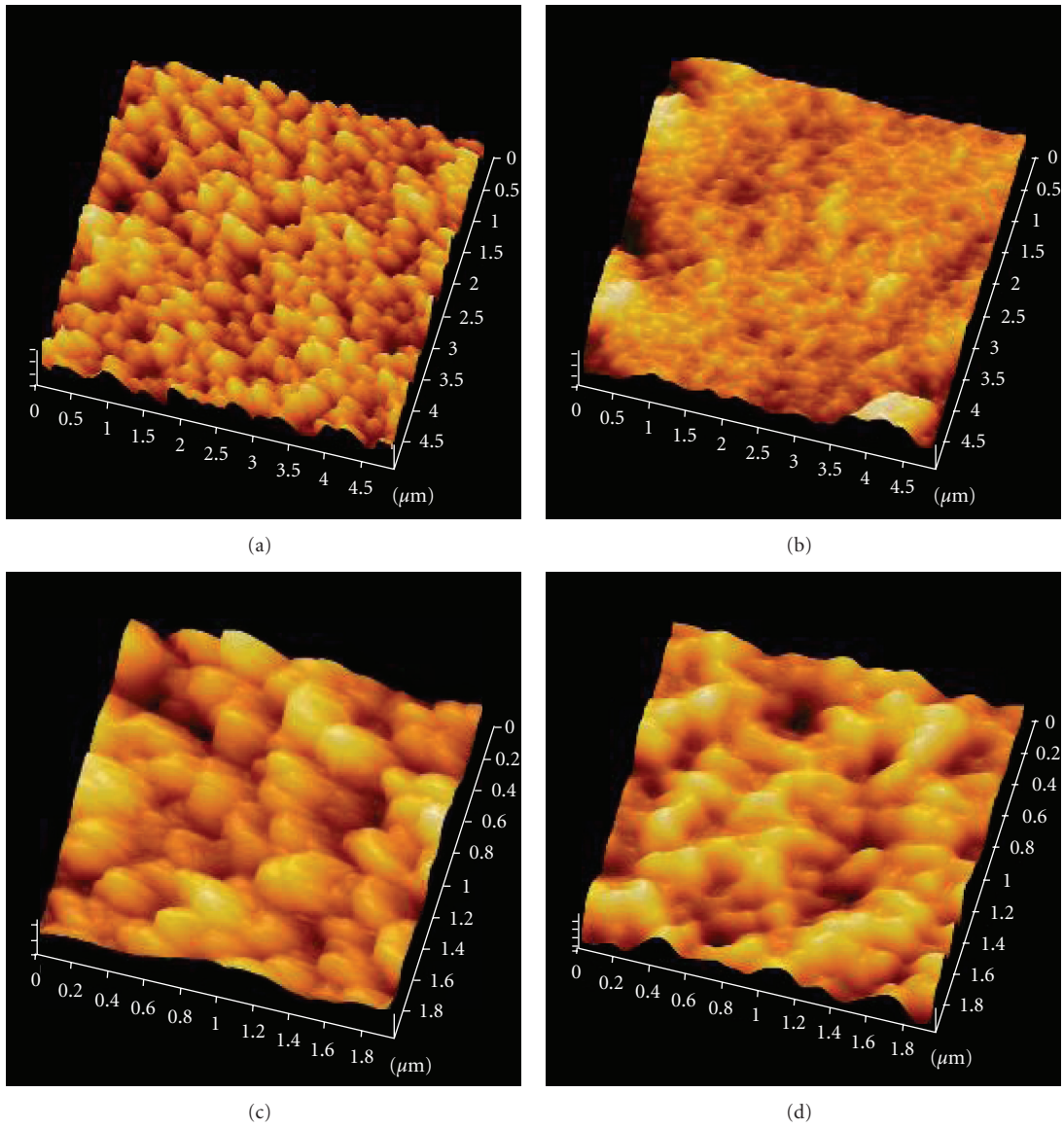
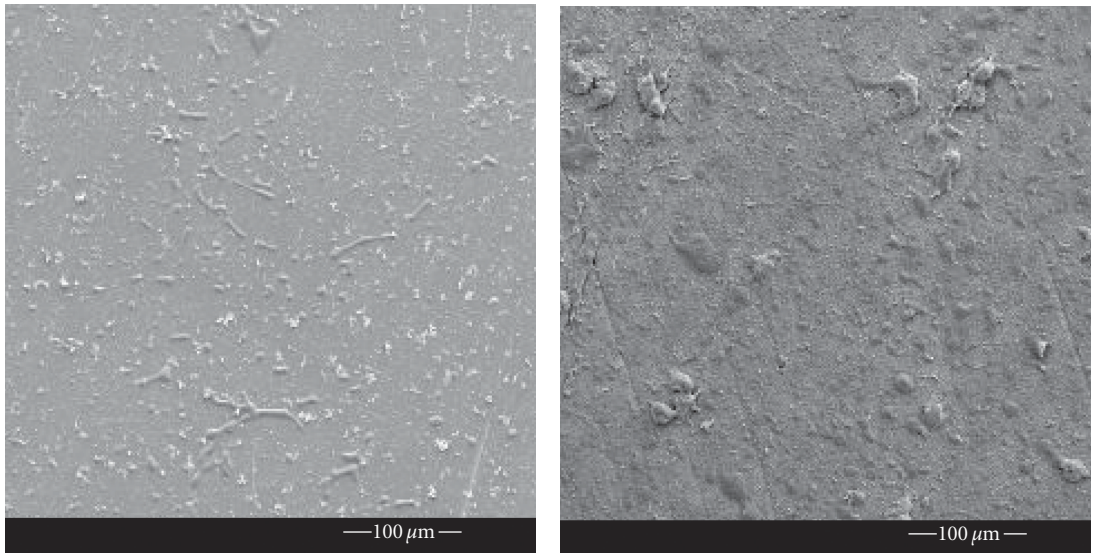


Figure 6: AFM images of sample surfaces: NaOH-treated titanium (Ti-OH) ((a), (c)) and coated titanium (Ti/PLL/$(Hep/Chi)_{18}$) ((b), (d)). The image size is 5 μm × 5 μm ((a), (b)) and 2 μm × 2 μm ((c), (d)).

The SEM images of the multilayers are shown in Figures 3–5. In the increasing of cycles of multilayers, the net structures have disappeared gradually, and submicron pores have changed by Hep and Chi deposition. As the arrow showed in Figure 3, some mesh-like structures were still found in the surface of Ti/PLL/$(Hep/Chi)_{10}$ samples because of the incomplete coverage of coating. But after 16 times cycles of the preparation (Ti/PLL/$(Hep/Chi)_{16}$), displayed in Figure 4, the mesh-like structure could not been seen, replaced by tightly deposited polysaccharides chitosan granules (Figure 4(b)). Up to 18 times cycles (Ti/PLL/$(Hep/Chi)_{18}$), more polysaccharides granules deposited, the coating became denser and smoother, and obvious granules could not been seen via SEM on partial surface (Figure 5).

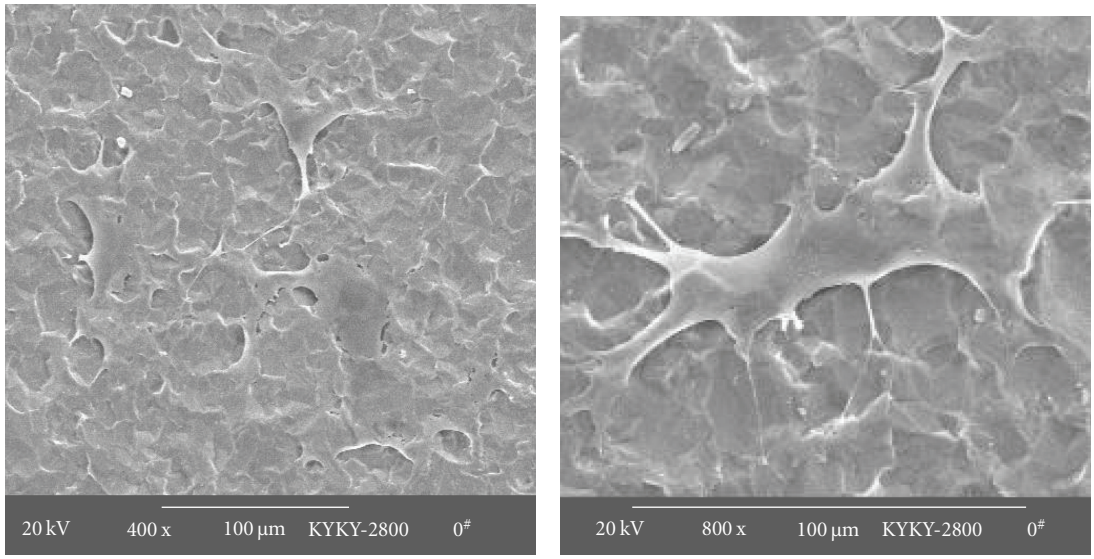
3.3. AFM. The surface morphology of the multilayers was further confirmed by AFM (Figure 6). AFM photos of NaOH-treated Ti (Figures 6(a) and 6(c)) showed that the mesh-like morphology in SEM images was formed of many taper-pointed granules, is which "topography" was like valleys and peaks. Ra (Arithmetic Average Roughness) was about 65.7 nm. For the multilayer-coated samples (Ti/PLL/$(Hep/Chi)_{18}$) (Figures 6(b) and 6(d)), ball or oval granules deposited on the substrate surface, and Ra became about 47.5 nm. The surface was smoother than NaOH-treated Ti, with the similar images observed by SEM.

3.4. Cell Adhesion. The cellular initial behavior on a biomaterial is an important factor for evaluation of its



(a) (b)

Figure 7: SEM images of osteoblasts adhered to original titanium (a) and LbL-modified titanium (Ti/PLL/(Hep/Chi)$_{18}$) (b) after seeding 4 hours.



(a) (b)

Figure 8: SEM images of osteoblasts adhered to original titanium (a) and LbL-modified titanium (Ti/PLL/(Hep/Chi)$_{18}$) (b) after seeding for 24 hours.

biocompatibility. Cell adhesion, spreading, and migration on materials are the first sequential reactions when contacting with a material surface, which is important for cell survival [8].

Figure 7 showed, after seeding for 4 hours, that cells randomly adhered and spread on both LbL-modified titanium surfaces and control ones. The number of adhered cells on modified titanium surfaces were greater than on original ones. Many adhered cells on modified titanium extended their pseudopods and showed a tendency to spread, whereas the cells on original titanium still retained a flat shape.

Compared with that of control ones, osteoblasts adhered to LbL-modified titanium surface fully spread after seeding for 24 hours, as shown in Figure 8, and developed more cellular processes to facilitate cell-substrate and cell-cell interactions [31]. Strong interactions between the cells and modified titanium surfaces would promote cell adhesion and tend to be helpful for cell proliferation. These indicated that

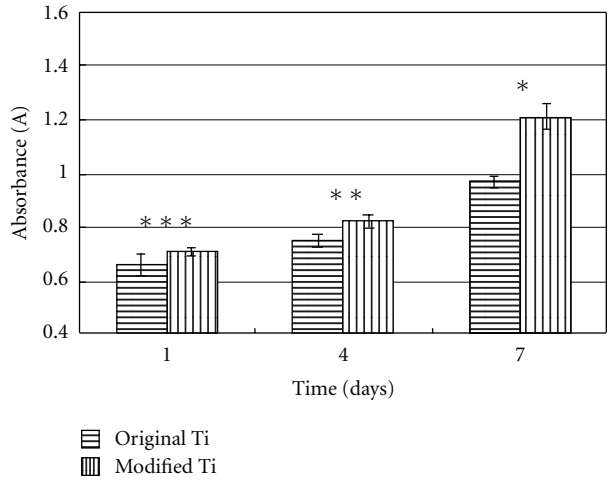


Figure 9: Proliferation of osteoblasts cultured on original titanium and LbL-modified titanium evaluated by MTT test at 1, 4, and 7 days. Error bars represent means ± S for $n = 4$; $^{*}P < .01$; $^{**}P < .05$; $^{***}P > .05$ (compared with the control).

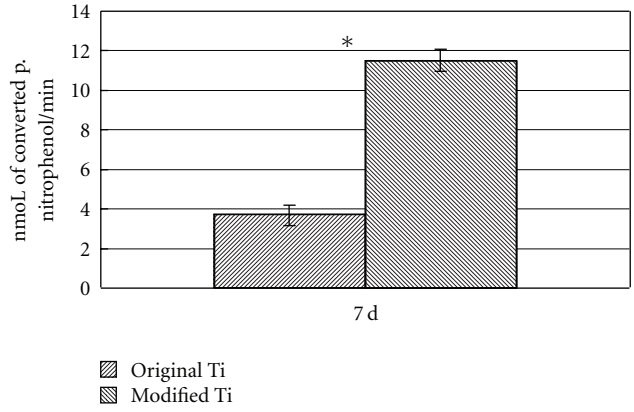


Figure 10: ALP activity of osteoblasts cultured on original titanium and LbL-modified titanium after 7 days of incubation. Error bars represent means ± S for $n = 4$; $^{*}P < .01$ (compared with the control).

the LbL-modified titanium was more favorable for the cell adhesion than the control.

3.5. Cell Proliferation. Figure 9 showed that the absorbance offormazan produced by viable cells adhered to LbL-modified titanium and the control ones at 1 day, 4 days, and 7 days of culture, respectively. MTT test results showed that the number of the cell proliferation had no significant difference between the two groups at 1 day ($P > .05$), the cells cultured on modified titanium surfaces proliferated faster than those on original ones at 4 days and 7 days, and the differences had statistical significance ($P < .05$).

Osteoblasts cultured on LbL-modified titanium showed higher proliferation viability compared with those on original titanium. Tryoen-Tóth et al. [11] revealed that the terminating layer in polyelectrolyte multilayer films influenced the cell adhesion and cell viability. Our study also indicated that Chi as the outmost layer of polyelectrolyte multilayers was biocompatible with osteoblasts.

3.6. Cell Differentiation. When cultured up to 7 days, osteoblasts grow into maturation and osteo-differentiation stage and express a large number of ALP. The ability of cell differentiation could be estimated by ALP activity assay. Figure 10 showed a statistically significant difference of ALP activity between the two groups ($P < .01$). The osteoblasts cultured on the modified group displayed much higher ALP expression than those on the control group, and they owned the stronger ability of cell differentiation. As demonstrated in a previous study, ALP was significant in bone matrix mineralization [32]. Thus, the modification with heparin-chitosan multilayers on titanium surface via LbL technique is promising to improve theosseointegration of the titanium-based implants.

The results in this study regarding osteoblast proliferation and differentiation are consistent with previous studies and indicate that LbL modification of titanium is helpful for the osteoblast growth [8, 13–15]. These results also suggest that the biological responses such as cell adhesion, cell proliferation, as well as cell differentiation depend markedly on the surface properties of the substrates. Chitosan was con?rmed as a good candidate to improve the biocompatibility of the titanium substrate [33].

4. Conclusion

Polyelectrolyte multilayer of chitosan and heparin was successfully coated on titanium substrate using a layer-by-layer self-assembly technique. The multilayer can promote the adhesion, proliferation, and differentiation of osteoblasts *in vitro*. We suggest that heparin-chitosan multilayer on titanium surfaces via LbL method is beneficial to osteoblast biocompatibility.

Acknowledgments

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