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Surface modification of titanium thin film with chitosan via electrostatic self-assembly technique and its influence on osteoblast growth behavior

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Abstract Chitosan (Chi) and poly (styrene sulfonate) (PSS) were employed to surface modify titanium thin film via electrostatic self-assembly (ESA) technique in order to improve its biocompatibility. The surface chemistry, wettability and surface topography of the coated films with different number of deposited layers were investigated by using X-ray photoelectron spectroscopy (XPS), water contact angle measurement and atomic force microscopy (AFM), respectively. The results indicated that a full surface coverage for the outmost layer was achieved at least after deposition of five layers, i.e., PEI/$(PSS/Chi)_2$ on the titanium films. The formed multi-layered structure of $PEI(PSS/Chi)_x$ ($x \geq 2$) on the titanium film was stable in air at room temperature and in phosphate buffered solution (PBS) for at least 3 weeks. Cell proliferation, cell viability, DNA synthesis as well as differentiation function (alkaline phosphatase) of osteoblasts on chitosan-modified titanium film (PEI/$(PSS/Chi)_6$) and control sample were investigated, respectively. Osteoblasts cultured on chitosan-modified titanium film displayed a higher proliferation tendency than that of control ($p < 0.01$). Cell viability, alkaline phosphatase as well as DNA synthesis measurements indicated that osteoblasts on chitosan-modified titanium films were greater ($p < 0.01$) than those for the control, respectively. These results suggest that surface modification of titanium film was successfully achieved via deposition of PEI/$(PSS/Chi)_x$ layers, which is useful to enhance the biocompatibility of the titanium film.

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Introduction

Although biomedical implants for fixation and stabilization of fractured bones during healing already represent millions of orthopedic devices annually [1], bone-implant integration (i.e., direct bone-implant bonding [2]) and long-term stabilization is still a common clinical problem [3]. The functionality lifetime of the orthopedic materials (including titanium, Ti6Al4V, etc.) used as bone fixation devices was estimated as short as less than 15 years [4]. Therefore, how to maintain good bone/device interface for a long time is crucial to the success of orthopedic application.

Titanium and its alloys are one of the most successful materials that were widely used in dental [5] and orthopedic [6] fields for manufacturing biomedical devices. However, titanium based implants only passively integrate with bone due to their bio-inert feature in nature. To improve the biological performance of an implant, one needs to enhance the cellular activities (cell adhesion, proliferation, and differentiation) of the bone-forming cells, osteoblasts, between the healthy bone and the implant by surface modification. This represents one of the focuses in biomedical materials research in the future [7].

To this end, a variety of strategies have been implemented to modify the surface of titanium-based implants and enhance bone growth and their initial stability. Immobilization of biologically active peptides (normally well known RGD containing peptides) on titanium surfaces is a common approach [8]. Coating hydroxyapatite on titanium surfaces is another surface modification method to improve

the biocompatibility of titanium-based materials [9]. An electrochemical method, also known as anodization or anodic oxidation is a well-established surface modification technique for metals like titanium to produce protective layers, which are effective to enhance bone growth [10].

We present here another approach, electrostatic self-assembly (ESA) technique, to surface modify titanium film. This technique is based on the consecutive adsorption of polyanions and polycations via electrostatic interactions [11]. In recent years, ESA technique has emerged as a versatile, inexpensive yet efficient technique to "build" biologically active surfaces [12]. It is a flexible strategy that affords nanometre-scale control in spatial distribution of ionized species within films.

Electrostatic self-assembled multi-composites make it possible to surface engineer materials by combing two or more desirable properties of biomaterials (e.g., bioactivity or biodegradability). In this study, chitosan and poly (styrene sulfonate) were employed as polycation and polyanion to fabricate bioactive coatings on titanium films, respectively. Chitosan was extensively investigated for its applications in tissue engineering [13, 14] and drug delivery systems [15] in recent years. For surface modification purpose, chitosan has been recently used to surface engineer titanium via covalent immobilization [16]. Compared with the classic chemical immobilization method, the ESA technique has the least demand for reactive chemical bonds. Moreover, the efficiency of chemical immobilization method still needs to be considered. Chitosan was also utilized as composite coating with calcium phosphate/hydroxyapatite [17] to modify titanium substrate due to its good biocompatibility. Although the ESA technique has been applied on titanium oxide in a previous study [18], it mainly focused on the production of composite with titanium oxide. To the best of our knowledge, this is the first study that employs ESA technique to construct the outmost layer with chitosan on titanium films in order to improve their biocompatibility.

The objective of this study was to fabricate and characterize surface modified titanium films with PSS/Chi via ESA technique. We hypothesized that this PSS/Chi pair coating would be beneficial for improving osteoblast growth on titanium substrates. Thus, the influence of such surface modification of titanium films on osteoblast growth behavior was investigated in vitro as well.

Materials and methods

Materials

Poly (ethylene imine) (PEI) (M_w = 75,000) was purchased from Aldrich. Chitosan (Chi, medium molecular weight, deactylation degree >85%) were obtained from Sigma-Aldrich. Poly (styrene sulfonate, sodium salt) (PSS, M_w = 70,000) was obtained from Aldrich. All chemicals were used without further purification. Flat titanium thin films were deposited on borofloat glass substrates via electron-beam deposition. More details on the titanium film deposition and characterization can be found elsewhere [19].

Fabrication of multi-layer

Chitosan (Chi) was dissolved in 2 vol% acetic acid with a concentration of 5 mg/mL. Poly (styrene sulfonate, sodium salt) (PSS) solution of 5 mg/mL was prepared by dissolving it in distilled water as polyanion. PEI solution with a concentration of 5 mg/mL was prepared with distilled water. The deposited titanium films were initially immersed in PEI solution for 20 min. The multi-layer construction was then accomplished by successively dipping the titanium films in the PSS and Chi solutions (alternating between PSS and Chi), followed by 10 min adsorption. Distilled water was used to rinse two times each for 1 min after PSS adsorption; while 0.2% acetic acid solution was first used and then distilled water to rinse the multi-layered films after chitosan adsorption. After rinsing with distilled water and PBS buffer, the substrates were dried at ambient environment. Thirteen layers, terminated with a layer of chitosan, i.e., Ti/PEI/$(PSS/Chi)_6$, were achieved by such alternative deposition in the present study for further cell culture investigations.

Contact angle measurement

Contact angle measurement was performed by using an OCA40 video-based optical system (Dataphysics Instruments GmbH, Filderstadt, Germany) in this study. The images of the water spreading on the sample surface were recorded by a camera, and then analyzed using the software supplied by the manufacturer. The initial media (distilled water or dimethicone) volume of 5 μL was used in each measurement after 15 s exposure in ambient temperature. Four samples were measured in each group. Three different points were measured for each sample.

XPS

For chemical composition analysis, samples were characterized by using XPS (Perkin Elmer 5600, USA) with a focused monochromatic Al Kα source (1486.7 eV) for excitation. The electron take-off angle was 45° for all measurements. The hydrocarbon peak maximum in C1s spectrum was set to 285.0 eV for reference of the binding energy scales. For stability characterization, PEI/$(PSS/Chi)_6$ coated titanium films were characterized after

immersion into phosphate buffered saline (PBS) solution for 3 weeks. The PBS media was changed every 3 days. Samples were dried at ambient temperature before characterization.

AFM

All samples were imaged with an atomic force microscopy (Nanoscope IIIa system, Digital Instruments, Santa Barbara, CA) in tapping mode. The 512×512 pixels were chosen for data acquisition at a scan speed of 1 Hz at ambient conditions. Samples were measured in air after drying at room temperature.

Cell culture

Osteoblasts were isolated via sequential collagenase digestions of neonatal rat calvaria according to established protocol [20]. They were cultured at 37 °C in a humidified atmosphere of a 5% CO_2 in air, in 75 cm^2 flasks containing 5 mL Dulbecco's Modified Eagle Medium (DMEM; Gibco), 10% fetal bovine serum (FBS; Gibco). The medium was changed every third day and for sub-culture; the cell monolayer was washed twice with PBS and incubated with trypsin-EDTA solution (0.25% trypsin, 1 mM EDTA; Gibco) for 10 min at 37 °C to detach the cells. The effect of trypsin was then inhibited by adding the complete medium at room temperature. These cells were washed twice by centrifugation and re-suspended in complete medium for re-seeding and growing in new culture flasks. Osteoblasts at population numbers 2–3 were used in the experiments.

Cell proliferation

Osteoblasts were seeded onto native titanium films and modified films as well as tissue culture polystyrene (TCPS) at a density of 5,000 cells/cm^2 in a 24-well plate. The cells were maintained in complete medium that was refreshed every 3 days. Proliferation assay was performed at 1, 4, and 7 days, respectively. At each time interval, plates were rinsed twice with PBS solution and then the cells were detached from the substrate by 0.25% trypsin in PBS. The number of adhered cells was determined by hemocytometric counting [21]. The mean value of four replicates was used as the final result for each sample.

Cell viability

Cell activity was determined by using MTT assay, which is based on the mitochondrial conversion of the tetrazolium salt, 3-(4,5-dimethylthiazol-2yl)-2, 5-diphenyl tetrazolium bromide (MTT). Briefly, after the osteoblasts were incubated on different films or TCPS for 7 days, 100 μL of MTT (5 mg/mL) was added to each well and incubated for 4 h at 37 °C. At the end of the assay, the blue formazan reaction product was dissolved by adding 0.5 mL DMSO and transferred to a 96-well plate. The absorbance was measured by using a microplate reader (BIO-RAD, model 550) at wavelength 490 nm, blanked with dimethyl sulfoxide solution. Four replicates were read for each sample; the mean value was used as the final result.

DNA assay

Osteoblasts adhered to modified films and native titanium films as well as TCPS after 7 days of culture were gently rinsed with 1% (v/v) PBS, free of calcium and magnesium, overlaid with 1 mL of 0.25% trypsin/0.1% EDTA per well and incubated at 37 °C for 10 min. The loosely detached cell layer was then scraped off the substrate, centrifuged (500*g*, 7 min) and re-suspended in 1 mL distilled water with 1% Triton X-100. These cell suspensions were experienced three freeze-thaw cycles. A 100-μL aliquot of the cell suspension was used to determine DNA content. The DNA in the homogenate was measured using the fluorometric method described as previous study [22].

Total intracellular protein content

This procedure is performed according to established protocol [20]. Osteoblasts (5,000 cells/cm^2) were seeded onto the modified titanium films, control one as well as TCPS, and cultured in DMEM with 10% fetal bovine serum for 7 days. At the end of the prescribed time interval, osteoblasts were lysed using distilled water with 1% Triton X-100 by three freeze-thaw cycles. Total protein content in the cell lysates was determined spectrophotometrically using a commercially available kit (BCA, Sigma). Light absorbance of these samples was measured at 570 nm on a Bio-Rad 550 spectrophotometric microplate reader. Total intracellular protein (expressed as mg) synthesized by osteoblasts cultured on the films was determined from a standard curve of absorbance versus known concentration of albumin run in parallel with experimental samples.

Alkaline phosphatase assay

Cell lysates was prepared as mentioned above. The supernatant was used for determining the ALP activity with *p*-nitrophenyl phosphate (Sigma) as substrate. The absorbance at 405 nm was measured via a spectrophotometer in a 96-well microplate reader (Bio-Rad 550). The ALP activity (expressed as nmols of converted *p*-nitrophenol/min) was normalized by total intracellular protein synthesis

(determined as described in the total intracellular protein content section) and thus expressed as μmol *p*-nitrophenol/min/mg protein. ALP activity of osteoblast cultured on untreated titanium films served as control.

Statistical analysis

All data were expressed as means ± standard deviation (SD) for $n = 4$. Single factor analysis of variance (ANOVA) technique was used to assess the statistical significance of results between groups. The statistical analysis was performed with the software OriginPro (version 6.1) at a confidence level of 95% and 99%.

Results and discussion

Contact angle measurement

ESA technique offers the possibility to vary the wettabilities of coated substrates since various polyanion or polycation components can be intentionally chosen in practical use. The sequential deposition of multi-layer is expected to greatly change the wettability of ESA-modified titanium films. This was confirmed by measuring the contact angles with distilled water and dimethicone (Fig. 1), respectively. The contact angle gradually decreased with increasing the layer number for the initial four layers. This may be related to the fact that the substrate surface was not fully covered by the outermost layer component such as PEI, Chi or PSS during the first 4-layer coating. Thus, the contact angles for these films may result from a combination of different interfaces of PEI/Chi/PSS and native titanium substrate. For water contact angle measurements, the chitosan ending films showed a constant contact angle around 63°, in contrast to a constant value around 53 for the PSS-ending films after deposition of five layers. This result suggests that at least five layers are necessary to build-up a constant interface. The alternative change of contact angles from layer 5 to layer 13 indicates the existence of discrete and fully covered outmost layer, which dominates the wettability of the modified titanium surface. For the contact angle measurements using dimethicone as the medium, similar alternative change of contact angle related to the outmost layer component was also observed. These results in the present study were consistent with the previous study [23].

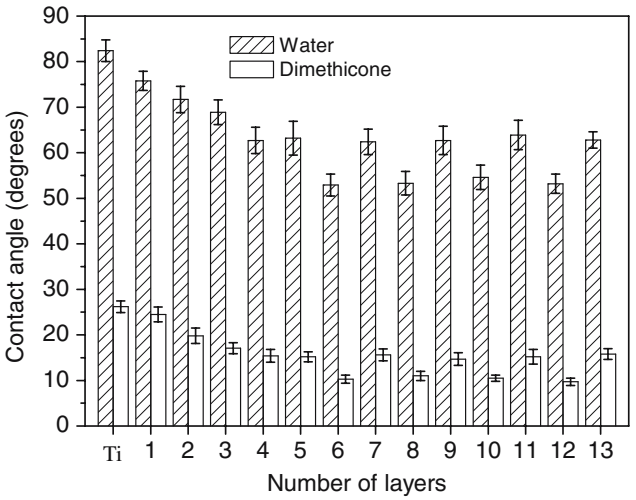

Fig. 1 Contact angles as a function of the layer number coating. The even layer numbers correspond to PSS as the outermost layer; the odd layer numbers correspond to chitosan as the outermost layer except for the first layer of PEI

XPS

Chemical compositions of pure titanium film, pure chitosan film and chitosan-modified (PEI/$(PSS/Chi)_6$) titanium film were characterized by using XPS and the spectra were shown in Fig. 2. The original titanium film demonstrates three elements as shown in Fig. 2a. The binding energies are 285.0 eV for C, 458.0 eV and 463.6 eV for Ti (Ti 2p3/2 and Ti 2p1/2, respectively) and 531.0 eV for O. More details could be found in literature [19]. Figure 2b shows the spectrum of original chitosan film. Characteristic elements of C, O, and N were found with corresponding energies at 285.0, 531.0, and 401 eV, respectively. The C/N ratio was determined as 7.5 for the original chitosan film, which is consistent with our previous study [24]. Figure 2c shows the XPS spectrum of titanium film coated with PEI/$(PSS/Chi)_6$ layers. No titanium signal was detected, which indicates that the substrate was fully covered by the PEI/$(PSS/Chi)_6$ layers. When compared with Fig. 2b, an additional weak peak at the binding energy of 1,072 eV was found in Fig. 2c, corresponding to the sodium element. This peak could be possibly derived from the underlayer PSS molecules.

The stability of the multi-layered structure of PEI/$(PSS/Chi)_6$ on the titanium film was characterized via XPS by immersion of the sample into PBS solution (Fig. 2d) for 3 weeks. Except for the elements of C, O, N, and Na, the Ti element previously not detected (Fig. 2c) was observed with a percentage of 4.9%. This result suggests that the multi-layered structure on the titanium film was partially degraded during the immersion time. The PSS/Chi layers, however, was not fully peeled from the titanium surface, which is supported by the presence of N and Na elements. This result indicates that the multi-layer structure of PEI/$(PSS/Chi)_6$ on the titanium film is relatively stable, which is consistent with the previous study [25].

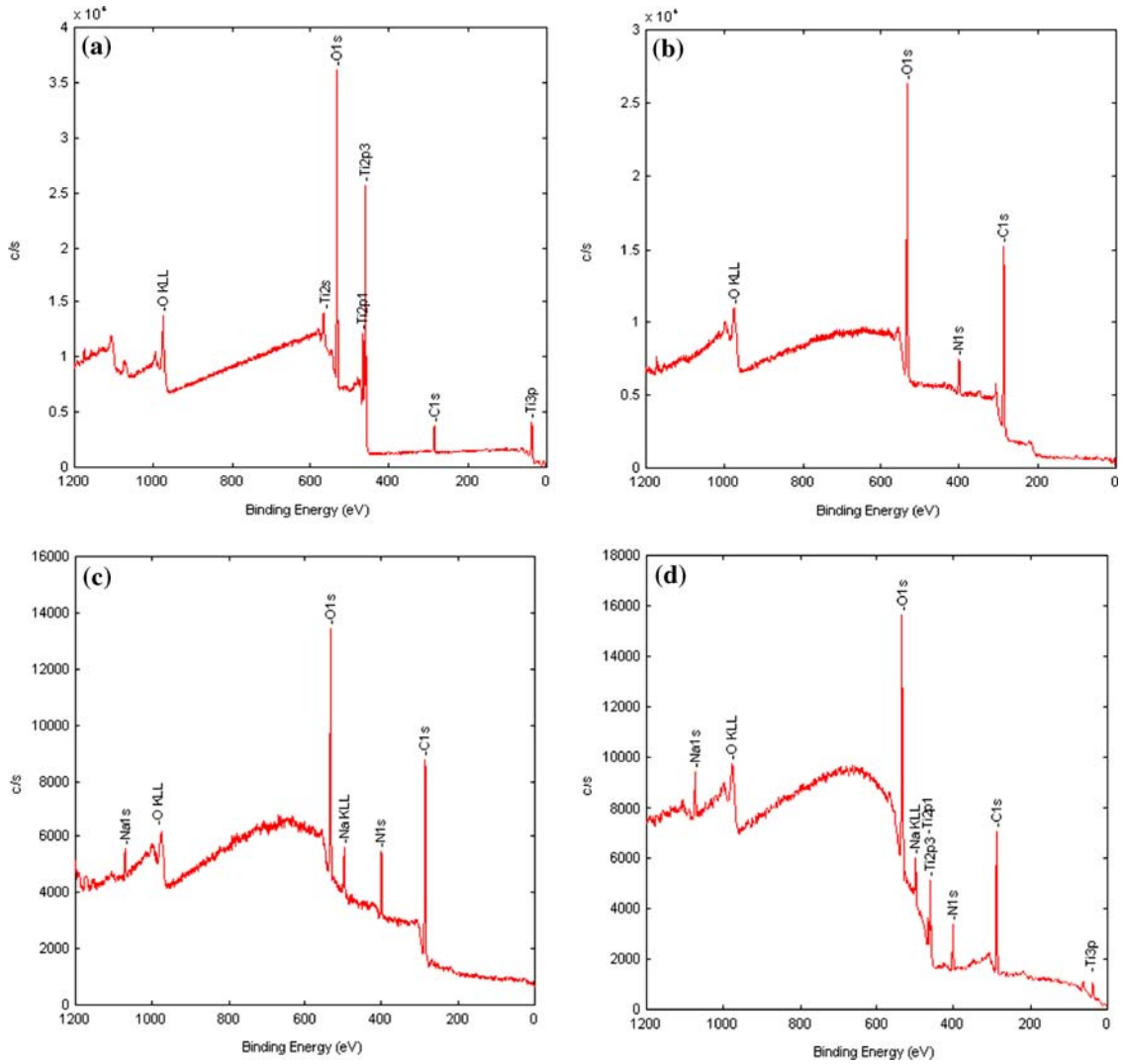

Fig. 2 XPS spectra of original titanium film, chitosan film and chitosan-modified titanium films: (**a**) original titanium film, (**b**) chitosan film, (**c**) PEI/$(PSS/Chi)_6$ coated titanium film, and (**d**) PEI/$(PSS/Chi)_6$ coated titanium film immersed into PBS solution after 3 weeks

AFM

Figure 3 shows the surface morphologies of the pure and chitosan-modified titanium films over an area of 10×10 μm^2 by atomic force microscopy. The original titanium film displays a smooth surface (Fig. 3a), while the chitosan-modified film (PEI/$(PSS/Chi)_6$) shows rough morphological characteristics (Fig. 3b). Granular structure was observed in Fig. 3b and the interface between the granular aggregates is easily discernible. This morphology is related to the physical property of the outmost chitosan component. The high intrinsic chain stiffness (persistence length of the order of 6–11 nm) [26] and high molecular weight for the chitosan molecules affect its diffusion ability during the multi-layer build-up process.

Cell proliferation

Figure 4 shows the osteoblast proliferation kinetics on the original titanium film, the pure chitosan film and the chitosan-modified titanium film with (Ti/PEI/$(PSS/Chi)_6$ layers as well as TCPS after 1, 4, and 7 days culture. The numbers of osteoblasts cultured on the modified titanium film were significantly higher ($p < 0.01$) than those of control after 4 and 7 days in culture. However, the number of osteoblasts cultured on the modified titanium film was comparable ($p > 0.05$) to that of control at day 1. The cell growth tendency on the pure chitosan film was higher than that on the chitosan-modified titanium film. However, no statistically significant difference between these two samples was observed. At day 7, the cell numbers were $(4.85 \pm 0.25) \times 10^4$ and $(3.65 \pm 0.2) \times 10^4$ cells/well on

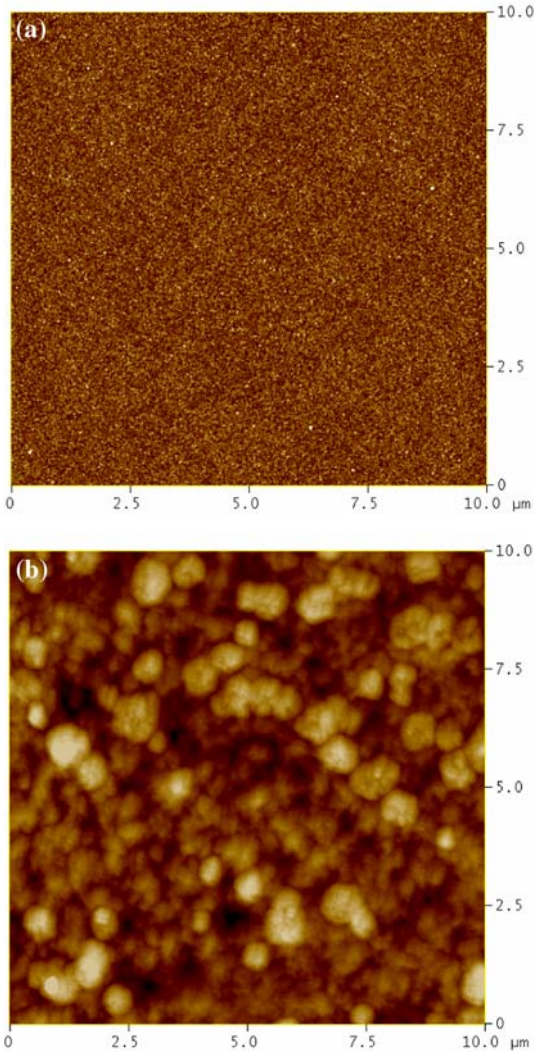

Fig. 3 Topography AFM images of titanium film and chitosan-modified titanium film: (**a**) original titanium film, and (**b**) PEI/(PSS/Chi)$_6$ coated titanium film. Film morphologies were observed in air after drying

the modified titanium film and the TCPS sample, respectively. This result indicates of better proliferation tendency of cells adhered to the chitosan-modified titanium film than that to the control sample. Osteoblasts cultured on the modified titanium film display a similar growth tendency to those on TCPS. No statistical difference was observed between them. The cellular behavior on biomaterials is an important factor for the evaluation of the biocompatibility of a biomaterial. This result suggests that chitosan/PSS coating may be helpful in improving cell growth and in turn osteointegration.

Cell viability

MTT reagent is a pale yellow substance that is converted to a dark blue Formosan product with viable cells. This assay

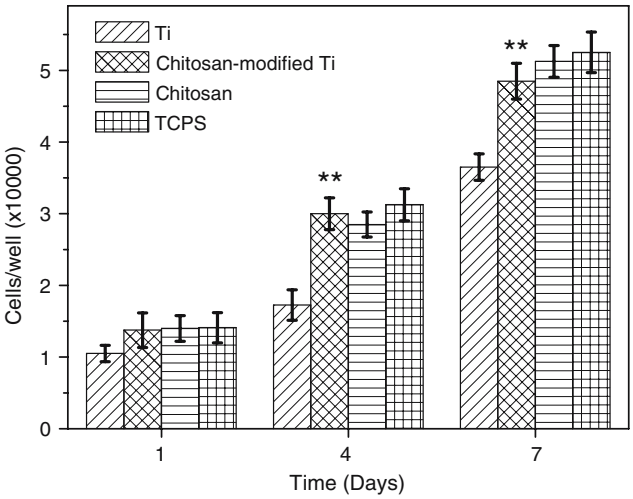

Fig. 4 Proliferation kinetics of osteoblasts cultured on original titanium films, chitosan films and chitosan-modified titanium films (Ti/PEI/(PSS/Chi)$_6$). The initial seeding density was 5,000 cells/cm^2. Error bars represent means ± SD for $n = 4$, $^{**}p < 0.01$ (compared to control)

implies the mitochondrial function of cultured viable cells. Therefore, the production of Formosan indicates the level of viable cell or cell viability. As shown in Fig. 5, osteoblasts adhered to the pure chitosan film and the chitosan-modified titanium film display statistically significant difference ($p < 0.01$) when compared to the control one. Similar cell viability was observed for the pure chitosan film and the chitosan-modified titanium film. Also no statistical difference regarding the cell viability was observed between on the chitosan-modified titanium film and the TCPS surface. The above result suggests that chitosan as the outmost terminating layer in polyelectrolyte multilayered structure is biocompatible and non-toxic for surface modification.

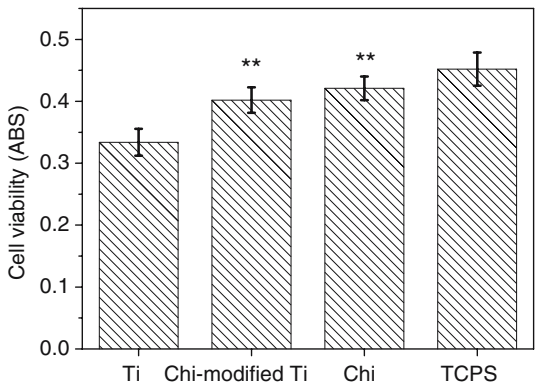

Fig. 5 Total DNA content of osteoblasts cultured on original titanium films, chitosan films and chitosan-modified titanium films (Ti/PEI/(PSS/Chi)$_6$). The initial seeding density was 5,000 cells/cm^2. Error bars represent means ± SD for $n = 4$, $^{**}p < 0.01$ (compared to control)

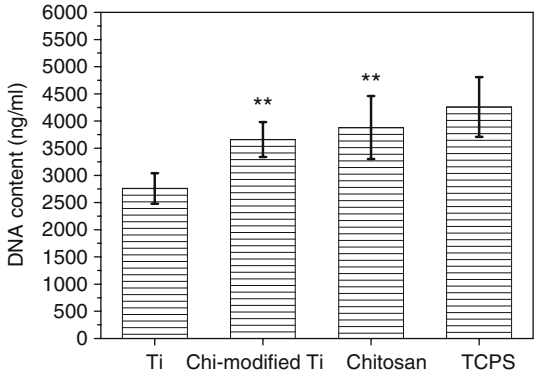

Fig. 6 MTT assay, Formosan absorbance expressed as a measure of cell viability from osteoblasts cultured on chitosan films, chitosan-modified titanium film and control. The initial seeding density was 5,000 cells/cm^2. Error bars represent means ± SD for $n = 4$, $^{**}p < 0.01$ (compared to control)

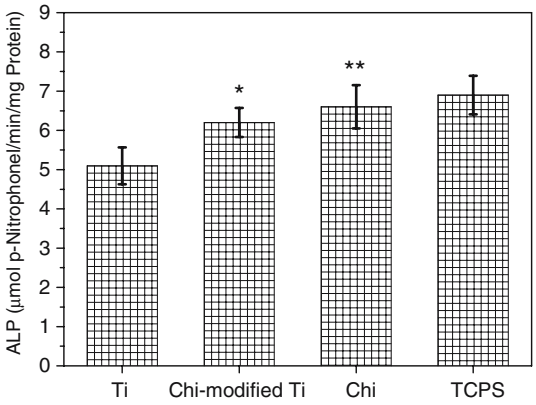

Fig. 7 Alkaline phosphatase activity of osteoblasts cultured on chitosan films, chitosan-modified titanium films (Ti/PEI/(PSS/Chi)$_6$) and control after 7 days culture. The initial seeding density was 5,000 cells/cm^2. Error bars represent means ± SD for $n = 4$, $^{*}p < 0.05$ and $^{**}p < 0.01$ (compared to control)

DNA content

Figure 6 shows the DNA contents of osetoblasts cultured on the pure chitosan film, the chitosan-modified titanium film and the original titanium film as well as TCPS, measured with fluorescence assay. DNA synthesis of osteoblasts on the chitosan-modified titanium film is statistically significant higher ($p < 0.01$) than that for the control one. This result indicates that the osteoblasts cultured on the chitosan-modified titanium film have higher DNA synthesis ability than those on the control surface. No statistical difference in DNA synthesis between the pure chitosan film, the chitosan-modified titanium film and the TCPS surface was observed. The DNA content has been shown to directly correlate with the cell number [27]. The general trend observed for DNA contents of osteoblasts cultured on different substrates was consistent with the cell proliferation results by 7th day (Fig. 4).

Alkaline phosphatase (ALP) activity

Alkaline phosphatase (ALP) activity is one of the most widely used as a transient early osteo-differentiation marker for osteoblasts [28]. ALP is a ubiquitous enzyme, which catalyzes the hydrolysis of phosphate esters at an alkaline pH condition. The ALP assay was performed after 7 days proliferation with an initial seeding density of 5,000 cells/cm^2. Figure 7 shows the ALP activity of osteoblasts cultured on the original titanium film, the pure chitosan film and the chitosan-modified titanium film as well as TCPS. It displays statistically significant difference between the chitosan-modified ($p < 0.05$) and the control samples. The osteoblasts adhered to the pure chitosan film display higher ALP expression than that for the chitosan-modified titanium sample. It shows statistically significant difference ($p < 0.01$) on ALP expression compared with that of control. Previous study demonstrated the importance of ALP in bone matrix mineralization [29]. The result of the present study indicates that ESA modification of titanium is beneficial for its bone formation potential. Thus, the surface modification of titanium using chitosan via ESA technique is promising to improve the osteo-integration ability of the titanium-based implants.

These results in the present study suggest that the biological responses such as cell proliferation, cell viability, and DNA synthesis as well as cell differentiation function depend markedly on the surface properties of the substrates. Chitosan was confirmed as a good substrate candidate for the osteoblast growth. The osteoblasts adhered to the pure chitosan substrate display better biological response than that of the chitosan-modified titanium film via ESA technique. The possible reason lies in the fact that poly (styrene sulfonate) is co-existed with chitosan in the multi-layered structure on the titanium film even though chitosan was employed as the outmost layer in this study. Due to the degradation of the multi-layer structure, exposed poly (styrene sulfonate) layer or degraded poly (styrene sulfonate) fragments possibly impose negative effects on the biocompatibility of the whole multi-layered (PSS/Chi pairs) structure. This result implies that how to choose the polycation and polyanion in the surface modification of biomaterials by using ESA technique is one of the most important issues to be considered.

Conclusion

The objective of the present study was to surface modify titanium film in order to improve its biocompatibility. An

ESA technique, based on the electrostatic interaction mediated adsorption of chitosan (Chi) and poly (styrene sulfonate) (PSS), was used leading to the formation of the multi-layered PEI/$(PSS/Chi)_x$ structure on the titanium thin film. The experimental results from the XPS, contact angle and AFM measurements confirmed the successful formation of the multi-layered PEI/$(PSS/Chi)_x$ structure on the titanium surface. The multi-layered structure was stable for more than 3 weeks, which was indicated by the XPS result. The contact angle measurement indicated that a full coverage of coating could be developed only after five layers deposition, i.e., PEI/$(PSS/Chi)_2$. The surface wettability of the modified titanium film was dominated by the outmost coating layer for those fully coated surfaces. The ESA technique using PSS/Chi as the coating pair was proved to be a successful approach to generate bioactive chitosan terminating layer on the hydrophobic titanium film. The results of in vitro investigations demonstrated that such surface modification was helpful to improve the biocompatibility of the titanium substrate. The approach presented here may be employed as an efficient alternative for surface modification of the titanium-based implants.

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