Construction of Zn-incorporated multilayer films to promote osteoblasts growth and reduce bacterial adhesion

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A B S T R A C T
To improve the biological performance of titanium substrates, a bioactive multilayered structure of chitosan/gelatin pair, containing zinc ions, was constructed via a layer-by-layer self-assembly technique. The successful preparation of zinc ions incorporated multilayer films was demonstrated by scanning electron microscopy, X-ray photoelectron spectroscopy, and contact angle measurements, respectively. The biological behaviors of osteoblasts adhered to modified Ti substrates were investigated in vitro via cytoskeleton observation, cell viability measurement, and alkaline phosphatase activity assay. The cytocompatibility evaluation verified that the present system was capable of promoting the growth of osteoblasts. In addition, Gram-positive (Staphylococcus aureus) and Gram-negative (Escherichia coli) bacteria were used to evaluate antibacterial property of modified Ti substrates. Bacterial adhesion and viability assay confirmed that Zn-loaded multilayer films were able to inhibit the adhesion and growth of bacteria. The approach presented here affords an alternative to reduce bacterial infection and promote osteoblast growth for titanium-based implants.

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1. Introduction

Titanium (Ti) and its alloys have been widely used in orthopedic fields, due to their excellent mechanical property and moderate biocompatibility [1,2]. Despite these excellent properties, the biological performance (e.g. osseointegration) of a Ti-based implant need to be improved [3,4]. Some strategies have been used for surface engineering Ti implants to enhance osseointegration, such as plasma treatment [5,6], micro/nano structures modification [7,8], bioactive molecule immobilization [9,10], as well as alkaline treatment [11].

In addition, bacterial infection of Ti implant remains one of the key challenges in clinic applications, which may result in the failure implantation in clinical applications [12,13]. The increasing use of invasive medical procedures for the implantation of devices leads to an increased risk for the development of device-associated infections. Implant-associated infections are the result of bacterial adhesion to a biomaterial surface. In recent decades, researchers have paid considerable attention to developing antibacterial surfaces to directly kill bacteria upon contact, and/or to reduce the extent of initial bacterial attachment [14–18].

In order to solve the currently existed poor osseointegration and postoperative infection of titanium and its alloys, it is great significance to improve osteoblasts response and endow them with antibacterial function via surface modification.

In present work, multilayer films of gelatin (GEL), chitosan (CHI) and Zn ions were fabricated on Ti substrates via layer-by-layer (LBL) self-assembly technique to enhance osteoblasts response and reduce bacterial adhesion. LBL self-assembly technique is based on the consecutive adsorption of polyanions and polycations via electrostatic interactions. LBL self-assembly technique has emerged as an efficient technique to fabricate biologically active surfaces [19–23]. Previously, chitosan and gelatin were assembled onto the surface of titanium substrates to mimic the extracellular microenvironment [24]. The results showed that the CHI/GEL multilayer films demonstrated good biocompatibility and osteoconductivity. Recently, a bioactive multilayered structure containing bone morphogenetic protein 2 and fibronectin was constructed onto Ti6Al4V (a kind of titanium alloy, 6%Al-4%V with balance Ti) surface via a LBL assembly technique [25]. The results indicated that such multilayers film structure improved osteogenesis and integration of implant/bone. In addition, hybrid multilayers drug-delivery system with gene-activation capabilities were fabricated onto Ti substrates via LBL assembly technique [26]. Such gene-functionalized Ti substrate could
induce the osteogenic differentiation of bone marrow mesenchymal stem cell in situ and enhance bone integration.

Zinc (Zn) ions possess nutritional features important to human health and health care. Zn is an important trace element that has positive impact on bone homeostasis. Zinc is a natural ingredient of human bone and is present at 0.012% to 0.025% by weight in bone. This amount is comparatively high compared to the zinc content in other adult tissue (0.003% by weight in fat free tissue) and plasma (0.78–1 mg/L) [27]. Zinc was reported as an essential trace element involved in diverse metabolic cellular signaling pathways and played an important role in the modulation of gene expression for proteins involved in bone formation [28]. Earlier studies have suggested that slow release of zinc ions has stimulatory effects on cell viability in vitro and stimulate bone formation in vivo [29]. Furthermore, Zn can increase alkaline phosphatase activity, type I collagen formation, osteogenic genes expressions and stimulate mineralization [30,31]. In addition, Zn ions and ZnO nanoparticles have strong antibacterial effect towards a broad range of bacteria in various forms [32,33].

In the present study, Zn-incorporated multilayer films were fabricated onto Ti substrate to enhance osseointegration and inhibit bacterial growth. By employing CHI and GEL as polycation and polyanion, the multilayered structures were constructed by LBL self-assembly technique. The rationale to select CHI is that it is the analogue of glycosaminoglycan, which presents in extracellular matrix [24]. Since GAG could specifically interact with growth factors, receptors and proteins, CHI with analogous structure may share some similar biological properties to that of GAG [34]. For GEL, it is a derivative of collagen, which is the main component of extracellular matrix [24]. Furthermore, there are rich amino groups and carboxyl groups in CHI and GEL molecules. Thus, Zn ions were embedded into GEL/CHI multilayer films via metal-ligand coordination bonds. Meanwhile, Zn ions can be loaded through electrostatic interactions with oppositely charged moieties (GEL, polyanion). Such multilayer films containing Zn ions would be helpful for improving osseointegration and reducing bacterial infection of Ti substrates. Therefore, the influence of such surface modification of Ti substrates on the growth of osteoblasts and antibacterial potentials in vitro was investigated as well.

2. Experimental section

2.1. Materials

Bare titanium disks (diameter: 15 mm; thickness: 3 mm) were kindly supplied by Northwest Institute for Non-ferrous Metal Research (China). Cell counting kit-8 (CCK-8) kit was obtained from Beyotime Biotechnology Co. (China). Poly (ethylene imine) (PEI), CHI, and GEL were purchased from Sigma Chemical Co. (USA). Other chemicals were purchased from Oriental Chemical Co. (China).

2.2. Fabrication of polyelectrolyte multilayer films

Firstly, a droplet of 5 mg/mL PEI solution was dropped onto Ti substrate and rotated (2000 rpm for 3 s and 3000 rpm for 20 s). The substrates were then rinsed with distilled water. Then, the multilayer construction was then accomplished by successively spin-coating 5 mg/mL GEL solution, 5 mg/mL CHI solutions and zinc acetate (ZnAc₂) solution with different concentrations (5, 10, 20 and 40 mg/mL) on the substrates (2000 rpm for 3 s and 3000 rpm for 20 s) in the same manner, respectively. Finally, the
process was repeated until the desired layers of (GEL/CHI/Zn)6CHI film were achieved onto the substrates in the present study. Zn ions were captured in multilayer films through the formation of metal-ligand coordination bonds with GEL and CHI as well as electrostatic interactions with oppositely charged moieties (GEL). The sample with different Zn ions was denoted as Ti-LBL-Zn5, Ti-LBL-Zn10, Ti-LBL-Zn20, and Ti-LBL-Zn40 substrates, respectively. In addition, (GEL/CHI)6 multilayer films without Zn ions were also fabricated on Ti substrates, which was denoted as Ti-LBL substrates.

2.3. Surface characterization

The field-emission scanning electron microscopy (FE-SEM) (Quanta 200, Philips-FEI Corporation, Netherlands) with 7.5 mm working distance and high vacuum type was employed to observe surface morphologies of the samples. The chemical composition of the coating was analyzed through X-ray photoelectron spectroscopy (XPS) (Axis Ultra, Kratos Analytical Ltd., England) using monochromatic Al Kα radiation (1486.6 eV). The analyzer was operated in the constant energy mode for all measurements. XPS survey spectra over a binding energy range of 0–1100 eV were acquired. Each spectrum was recorded at 20 eV pass energy with a 45° take-off angle. Argon ion sputtering was performed during XPS to estimate the thickness and structure of the surface layers. The hydrocarbon peak maximum in the C1s spectra was set as 284.8 eV to reference the binding energy scales for the samples. Intensity ratios were converted into atomic concentration ratios by using the sensitivity factors proposed by the manufacturer.

Static contact angle with respect to double-distilled water was measured by a Model 200 video based optical system (Future Scientific Ltd. Co. Taiwan, China) in the sessile drop method. Every water drop was deposited to the sample surface. After 10 s, an image of the drop was captured and then analyzed. At least five points were obtained with the deviation range within ±1°, and the averaged value was used to evaluate their wettability. To quantify the film thickness, multilayer films were deposited on silicon wafers and measured using a spectroscopic ellipsometer (M-2000, Woollam, USA).

2.4. Zn ion release test

Modified Ti substrates were immersed in 5 mL simulated body fluid (SBF) solution and kept in an incubator at 37 °C for 6, 12, 24, 48, 72, 96, 120, 144, 168 and 192 h. The concentrations of released zinc ions at different intervals of time were measured by an inductive coupled plasma-atomic emission spectroscopy (ICP-AES, Vista AX, Varian, USA).

Fig. 2. XPS survey spectra of (a) bare Ti, (b) Ti-LBL-Zn5, (c) Ti-LBL-Zn10, (d) Ti-LBL-Zn20, and (e) Ti-LBL-Zn40 substrates.
2.5. Cytocompatibility evaluation in vitro

2.5.1. Cell culture

Osteoblasts were isolated via sequential collagenase digestions of neonatal rat calvaria according to established protocol [35]. They were cultured at 37 °C in a humidified atmosphere of 5% CO$_2$ in air, in 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.

2.5.2. Cell viability assay

Osteoblasts were seeded onto different Ti substrates, and tissue culture polystyrene (TCPS) at a density of 1.5 × 10$^4$ cells/mL. After culture for 4 and 7 days, 200 μL of new medium and 20 μL of CCK-8 solution were added to each well of a 24-well plate and then incubated for another 1 h. Finally, the incubated solution was measured was measured with a spectrophotometric microplate reader (Bio-Rad 680, USA) at a wavelength of 450 nm.

2.5.3. Alkaline phosphatase (AKP) assay

Osteoblasts (1.5 × 10$^4$ cells per disk), were seeded onto different Ti substrates, and TCPS at a density of 1.5 × 10$^4$ cells/mL and cultured for 4 and 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. AKP kit (Jiancheng, China) was employed to determine the AKP activities of osteoblast cultured on different substrates. The absorbance at 520 nm wavelength was measured with a spectrophotometric microplate reader (Bio-Rad 680). Total protein content in the cell lysates was determined using a commercially available BCA kit (Sigma, USA). The AKP activity was normalized by total intracellular protein production.

2.5.4. Cell morphology observation

Osteoblasts were seeded onto substrates at a density of 5000 cells per disk. After culturing for 2 days, the cell layers were rinsed with PBS (phosphate-buffered saline) three times. The cells were fixed in a 5% glutaric dialdehyde diluent at 4 °C for 30 min followed by three rinses with PBS. The cells were permeabilized with 0.2% Triton X-100 at 4 °C for 5 min followed by three rinses with PBS. The cells were stained with rhodamine phalloidin (Invitrogen, USA) at room temperature overnight followed by three rinses with PBS and then stained with Hoechst fluorescent dyes (Sigma, USA) for 5 min. The cytoskeletal actin and cell nuclei were observed with confocal laser scanning microscopy (CLSM) (Leica DMI 6000, Germany).

2.6. Antibacterial test

2.6.1. Bacteria viability assay

Both Staphylococcus aureus (S. aureus) and Escherichia coli (E. coli) were purchased from ATCC and cultured according to previous report [18]. Bacteria were cultured onto bare and treated Ti substrates at an initial density of 1 × 10$^6$ cells/mL at 37 °C for 24 h. After rinsing with phosphate buffered saline (PBS) three times, 200 μL of CCK-8 solution were added and incubated at 37 °C for another 1 h. The optical density of the solution was determined with a spectrophotometric microplate reader (Bio-Rad 680, USA) at a wavelength of 450 nm.
2.6.2. Bacteria adhesion morphology observation

The bacteria adhering to different substrates were observed by FE-SEM. First, bacteria were cultured onto bare and treated Ti substrates at a density of $1 \times 10^6$ cells/mL at 37 °C for 4 h. Then, paraformaldehyde (4 wt%) was added and incubated at 4 °C for 30 min. The bacteria were dehydrated with gradient dehydration of ethanol (20%, 50%, 80%, and 100%), each for 20 min. The samples were dried with a vacuum freezing dryer (FD-1A-50, BiLon, China) and observed with FE-SEM. Finally, the number of adherent bacteria was performed by counting bacteria in 3 individual images per sample type.

2.7. Statistical analysis

Six replicate samples were performed in vitro assays. All data were expressed as means ± standard deviation (SD). The statistical analysis was performed with OriginPro (version 6.1) at confidence levels of 95% and 99%. Multigroup comparisons of the means were carried out by one-way analysis of variance (ANOVA) test with post hoc contrasts by Student-Newman-Keuls test.

3. Results and discussion

3.1. Preparation and characterization of Zn-incorporated multilayer films

Firstly, surface morphologies of bare and modified Ti substrates were shown in Fig. 1. Bare Ti substrates displayed visible scratches, which was attributed to polishing abrasion (Fig. 1a). After deposition of GEL/CHI multilayer films, the Ti-LBL substrate substrates still displayed rough surface morphology with less scratches (Fig. 1b). For Zn ions-incorporated multilayer films modified substrates (Ti-LBL-Zn5, Ti-LBL-Zn10, Ti-LBL-Zn20, and Ti-LBL-Zn40), with the increase in Zn concentration, the treated Ti substrates gradually displayed smooth surface morphology and clear film structure. This change in surface morphology suggests that Zn ions-infiltrating multilayer films were successfully deposited onto the surface of Ti substrates.

Furthermore, to confirm the successful construction of Zn-incorporated multilayer films onto Ti substrate, the chemical compositions of bare and modified Ti substrate were determined by XPS. For bare Ti substrates, there were only peaks of C, Ti and O elements (Fig. 2a). Carbon was attributed to the environmental contamination. After deposition of multilayer films containing Zn ions, the treated Ti substrates (Ti-LBL-Zn5, Ti-LBL-Zn10, Ti-LBL-Zn20, and Ti-LBL-Zn40) displayed four elements of C, N, O and Zn (Fig. 2b, c, d and e). The C, N and O elements were from CHI and GEL. The signal of Zn element was attributed to Zn ions loaded in the multilayer films. In addition, the peaks of Ti were not observed, which indicated the thickness of multilayer films may be beyond the detection depth of XPS. The surface chemical composition of multilayer films modified Ti substrates was listed in Table 1. Furthermore, with the increase in Zn concentration during the LBL process, the Zn content in the multilayer film coatings increased (Table 1). The results provided evidence for the successful formation of Zn-incorporated multilayer film onto the surfaces of Ti substrates.

Contact angle (CA) measurement was performed in this study to investigate the effects of Zn-incorporated multilayer film on the wettability of Ti substrates (Fig. 3). Bare Ti substrates displayed contact angle of about 85.50°. The contact angle value for Ti-LBL substrates decreased to 66.30°. The contact angles for Ti-LBL-Zn5, Ti-LBL-Zn10, Ti-LBL-Zn20, and Ti-LBL-Zn40 substrates were 60.09°, 54.60°, 61.60°, and 62.10°, respectively, indicating that Zn-incorporated multilayer film apparently enhanced surface wettability of Ti substrate. It has been shown that chemical heterogeneities has a critical influence on the wetting behavior. Shen et al. reported that a series of Zn-incorporated coatings fabricated on the microrough Ti substrates via sol-gel method decreased
contact angles from 104° to 81° [30]. In addition, Hu et al. reported that Zn-incorporated TiO₂ coatings fabricated by plasma electrolytic oxidation enhanced surface wettability of Ti substrate [31]. Therefore, Zn-incorporated multilayer film could enhance surface wettability of Ti substrate. In addition, surface roughness has an impact on wettability of substrate. From SEM images, the Ti-LBL-Zn10 sample presented rougher surface compared to other samples, which could lead to a lower contact angle value.

A spectroscopic ellipsometer was used to quantify the film thickness with different amounts of Zn ions. In detail, the thickness of Ti-LBL substrates was 10.32 ± 0.47 nm. For Ti-LBL-Zn5 substrates, the thickness was 14.91 ± 0.97 nm. The film thickness of Ti-LBL-Zn10 samples was 16.33 ± 0.34 nm. For Ti-LBL-Zn20 samples, the film thickness was 16.79 ± 0.35 nm. The film thickness of Ti-LBL-Zn40 substrates was 17.72 ± 0.63 nm.

The release behaviors of Zn ions from modified Ti substrates in SBF solution were measured by ICP-AES (Fig. 4). Previous study reported that for Zn-incorporated coatings fabricated on the microrough titanium via sol–gel method by spin-coating technique, approximately 1.08, 1.65, and 2.06 ppm of zinc ions released from different substrates within four weeks [30]. For Zn incorporated into titanium substrate by plasma electrolytic oxidation, the accumulated Zn concentrations in the solutions after immersion for 14 days ranged from 1.18 to 3.62 ppm [31]. In this experiment, Zn ions released rapidly during the first 12 h for all the substrates. Then, the rate slowed down. After 7 days (168 h), the amount of released Zn ions did not increase. For Ti-LBL-Zn5, Ti-LBL-Zn10, Ti-LBL-Zn20, and Ti-LBL-Zn40 substrates, Zn ions release concentrations were 0.8, 1.6, 3.4 and 4.4 ppm, respectively. The multilayer films would become unstable in a neutral or basic environment due to the lower electrostatic attraction between the deprotonated chitosan chains and the negatively charged GEL, which resulted in the dissociation of the film. Then, Zn ions released from the surface because of the dissociation of the film.

Previous studies demonstrated that the materials’ surfaces at appropriate dose of Zn ions show excellent biocompatibility and antibacterial property [27–31]. However, zinc ions is toxic at high concentrations, although the threshold concentration of zinc ions was dependent on cell types [36–38]. Brauer et al. demonstrated that zinc concentrations between 50 and 300 μM could significantly increase the metabolic activity of MC3T3-E1 mouse osteoblast, and concentrations near 350 μM would lead to cytotoxicity [36]. Watjen et al. reported that viability in eight cell lines exposed to Zn ions at concentrations between 50 and 600 μM, and found that the mouse fibroblast cell line (NIH3T3) showed zinc toxicity at only 70 μM, while human lung adenocarcinoma cell line (A549) maintained viability at zinc concentrations of up to 600 μM [37]. Aina et al. confirmed that 2 ppm Zn ions was in the safe range without obvious cytotoxicity to human MG-63 osteoblasts, but 4 ppm showed the
3.2. Assessment of cytocompatibility of modified Ti substrates

Due to their excellent mechanical properties, chemical stability, biocompatibility, and bioactivity, Ti and its alloys are widely used as surgical implants in the orthopedic and dental fields. However, insufficient bonding of implants to bone tissues leads to implant loosening over time and, ultimately, to implant failure. Here, Zn-incorporated multilayer films were used to modify Ti substrates. Furthermore, we investigated the proliferation, differentiation, and morphology of osteoblasts grown onto different substrates in vitro.

To evaluate cell viability of osteoblasts cultured on both bare and modified Ti substrates, the CCK8 assay was employed in the present study. Fig. 5 shows cell viability of osteoblasts adhered to different substrates after culture for 4 and 7 days, respectively. It can be seen that the Ti-LBL-Zn5 ($p < 0.05$), Ti-LBL-Zn10 ($p < 0.01$), and Ti-LBL-Zn20 ($p < 0.05$) substrates lead to significantly increased cell viability in comparison with bare Ti substrates after 4 days culture. However, the Ti-LBL-Zn40 substrates showed decreased cell viability, compare to bare and modified Ti substrates ($p < 0.01$). After 7 days culture, the Ti-LBL-Zn10 substrates exhibited higher cell viability ($p < 0.01$) compared to bare Ti substrates. The Ti-LBL-Zn20 substrates showed significantly reduced values of cell viability, compared to Ti, Ti-LBL, Ti-LBL-Zn5, Ti-LBL-Zn10 substrates ($p < 0.01$). The Ti-LBL-Zn40 substrates display clearly lower cell viability, compare to bare Ti substrates and other modified substrates ($p < 0.01$). Released reactive oxygen species (ROS) from the Zn-related materials partly did damage to surrounding cell or tissue. Zn ions could induce a significant decrease in intracellular glutathione (GSH), which could be partly attributable to GSH oxidation to intracellular glutathione disulfide, and partly to GSH efflux out of the cells. In addition, high amount of Zn ions was needed to induce a significant leakage of lactate dehydrogenase in the culture medium [36-38].

Since alkaline phosphatase (AKP) is a Zn-dependent enzyme, previous studies have suggested that zinc ions could enhance osteogenic differentiation of mesenchymal stem cells and AKP activity [39]. AKP activity measurement was also performed in this study. AKP activity is widely used as a transient early osteo-differentiation marker for osteoblasts. Fig. 6 shows AKP activity of osteoblasts adhered to different Ti substrates. After culture for 4 days, osteoblasts grown on Ti-LBL-Zn10, Ti-LBL-Zn20, and Ti-LBL-Zn40 substrates exhibited significantly higher AKP activity than that of corresponding bare Ti substrates ($p < 0.05$). In addition, Ti-LBL-Zn10 substrates showed higher AKP activity in comparison with Ti-LBL-Zn5 substrates ($p < 0.05$). After 7 days culture, Ti-LBL-Zn10 substrates exhibited higher AKP activity compared to the negative controls and the Ti-LBL-Zn20 substrates ($p < 0.05$). The Ti-LBL-Zn40 substrates showed significantly lower AKP activity, compared to bare Ti substrates ($p < 0.05$), Ti-LBL-Zn10 ($p < 0.01$) and Ti-LBL-Zn20 ($p < 0.05$) substrates after 7 days culture. In short, the Ti-LBL-Zn10 substrates showed the highest AKP activity among all bare and modified Ti substrates.

Based on results of cell viability and AKP activity, it indicated that multilayer films with proper Zn ions (the Ti-LBL-Zn10 substrates) were most beneficial for promoting osteoblasts biological responses. Furthermore, osteoblasts cultured on the bare and Ti-LBL-Zn10 substrates were visualized with a double staining of actin (cytoskeleton) and nucleus to observe cell morphology. Fig. 7 shows that cells distribute evenly and well spread with the features of typical polygonal osteoblastic shapes. However, there were limited cells on the bare Ti substrates after 2 days culture (Fig. 7a). The Ti-LBL-Zn10 substrates showed significantly higher numbers of cells (Fig. 7b). This increase in cell numbers suggests that the Ti-LBL-Zn10 substrates may increase the affinity for osteoblasts on the surface.

In the present study, Zn ions have been incorporated into multilayer films on Ti substrates. Our findings indicated that addition of with proper Zn ions within multilayer films improved the bioactivity and the functionality of osteoblasts. The better integration and osteoconduction of these substrates within host bone could be expected. Thus, surface engineering approach presented here is promising to improve osseointegration of Ti substrates.

3.3. Evaluation of antibacterial property of modified Ti substrates

Another reason for orthopedic and dental implant failure is associated with bacterial infections on the material surface. Unmodified Ti and its alloy are susceptible to bacterial infections from the patient’s own skin and/or mucosa during surgical insertion of implants. Such bacterial infections lead to implant failure, removal of implants, increased patient morbidity, and higher treatment costs.

Based on the results of cytocompatibility, the Ti-LBL-Zn10 substrates might be a promising choice due to promoting osteoblasts biological responses. Hence, its antibacterial property was further investigated. We evaluated the antibacterial property of bare and modified Ti substrates to both Gram-positive (S. aureus) and Gram-negative (E. coli) bacteria via bacterial viability assay and bacteria adhesion experiment.

Firstly, we quantitatively measured bacterial viability regarding S. aureus and E. coli grown onto the bare Ti and Ti-LBL-Zn10 substrates via CCK-8 method (Fig. 8). Compared with bare Ti substrates, Zn-incorporated samples clearly decreased bacterial viability of both S. aureus and E. coli ($p < 0.01$). This decrease in bacterial viability suggests that GEL/CHI multilayers with Zn ions displayed efficient antibacterial activities. Following, the colony formation of bacteria adhered to different Ti substrates was investigated via SEM. SEM images revealed that fewer bacteria were found on the surface of Zn-incorporated substrates (Fig. 9b and d) compared with bare Ti substrates (Fig. 9a and c). Furthermore, the number of bacteria on samples was measured. After statistical analysis, the number of S. aureus and E. coli on bare Ti substrates was 544 ± 26 and 171 ± 13, respectively. While the number of S. aureus and E. coli on Ti-LBL-Zn10 substrates was 256 ± 20 and 90 ± 12, respectively. The results suggest that the Zn-incorporated samples had antibacterial property, which was consistent with CCK-8 assay.

Previous studies confirmed that antibacterial mechanism of Zn-related materials was attributed to zinc ions or production of reactive oxygen species [32,33]. Here, Zn ions were captured in multilayer films via chelated with GEL and CHI, as well as electrostatic interactions with oppositely charged moieties (GEL). As a result, release of zinc ions from substrates has shown antibacterial effects. The method presented here is not limited to the demonstrated Ti substrates. It might be extended to generate antibacterial coatings on other metal and its alloys to meet the requirements of (including but not limited to) biomaterials applications.

4. Conclusion

In summary, Zn ions-incorporated multilayer films were successfully fabricated on Ti substrates via LBL self-assembly method. The optimal modified Ti substrate (Ti-LBL-Zn10) had the greatest potential for promoting osteoblast growth and reducing bacterial adhesion. These findings suggest that Zn is considered to be one promising agent for enhancing the osseointegration and antibacterial property of biomaterial implants. Bioactive Zn ions releasing from Ti substrates into the surrounding bone environment may lead to differentiation and maturation of osteogenesis related cells and new bone formation. This study presented here will provide novel strategies for surface modification of titanium-based implants and accelerate the application of them in tissue repair and regeneration.
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