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Protocols

Surface modification of titanium substrates for enhanced osteogenetic and antibacterial properties

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ABSTRACT

The insufficient osseointegration and bacterial infection of titanium and its alloys remain the key challenges in their clinic applications, which may result in failure implantation. To improve osteogenetic and antibacterial properties, TiO_2 nanotube arrays were fabricated on titanium substrates for loading of antibacterial drug. Then, TiO_2 nanotube arrays were covered with chitosan/sodium alginate multilayer films. The successful construction of this system was verified *via* scanning electron microscopy and contact angle measurement. The cytocompatibility evaluation *in vitro*, including cytoskeleton observation, cell viability measurement, and alkaline phosphatase activity assay, confirmed that the present system was capable of accelerating the growth of osteoblasts. In addition, bacterial adhesion and viability assay verified that treated Ti substrates were capable of reducing the adhesion of bacteria. This study may provide an alternative to develop titanium-based implants for enhanced bone osseointegration and reduced bacterial infection.

1. Introduction

Titanium (Ti) and its alloys are extensively used as orthopedic implants because of their good mechanical property and biocompatibility [1,2]. Despite these excellent properties, the bio-inertness of native Ti and its alloys hinder their early integration with surrounding bone tissues, which would have an adverse impact on the long-term survival of Ti implants [3]. The benefits of enhanced bone growth and improved initial stability of bone-contacting implants are clear.

In addition to the insufficient osseointegration, bacterial infection of Ti implants remains one of the key challenges in clinic applications, which may result in the failure implantation in clinical applications [4]. Bacterial infection would occur after the implantation of a Ti-based implant in some cases, which in turn resulting in the formation of biofilms around the Ti implant. It could lead to deleterious side effects, including bacteria-induced inflammation and resistance to antibacterial agents. Post-operation infection is one of the most common reasons that result in the failure implantation of medical devices in clinical applications. Serious infection even would lead to repeated implantation of devices. The increasing use of invasive medical procedures for the implantation of devices leads to an increased risk for the development of device-associated infections. Therefore, to exploit implants with inherent antibacterial property is of particular importance for the successful implantation of medical devices.

As for Ti-based implants, the initial interactions with cells and bacteria occur at their surfaces. Thus, surface modification of titanium so that to promote osseointegration and simultaneously reduce bacterial adhesion is essentially important for both fundamental research and clinical application [5–8]. Some strategies, such as plasma treatment, micro/nano structures modification, bioactive molecule immobilization, as well as alkaline treatment have been used for surface engineering Ti implants to enhance osseo-integration and antibacterial property [9–13].

With the advancement and convergence of materials science and biology, it is possible to construct osteogenetic and antibacterial surfaces by combining functional drug-delivery systems with biomaterials. Herein, we employed TiO_2 nanotube (TNT) arrays on Ti substrates as drug nano-reservoir for loading gentamicin (antibacterial drug). Then, gentamicin-loaded TNT arrays were covered with sodium alginate/chitosan (SA/CHI) multilayer films *via* layer-by-layer (LBL) self-assembly technique. The nanotubular structures of TNT arrays with open volume represent themselves as one of the ideal candidates serving as nano-reservoir for drug storage [14–16]. LBL self-assembly approach has been widely used to construct biofunctional surfaces [17–21]. Compared with the

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classic chemical immobilization method, the LBL technique has the least demand for chemical bonds. The multilayers built by the LBL method afford a more stable coating than that prepared by physical adsorption because of the electrostatic attractions between layer to layer and layer to substrate.

The rationale to choose SA and CHI to fabricate multilayer films is that SA and CHI are the analogues of glycosaminoglycans which are the important components of extracellular matrix (ECM) [22,23]. Therefore, SA/CHI multilayer films were fabricated to improve osteoblasts growth. In addition, in this study, CHI was also utilized as an antibacterial agent [24–26]. As CHI is highly positively charged, the negatively charged bacteria will be strongly attracted. The polycationic nature of chitosan interferes with bacterial metabolism by electrostatic interactions at the negatively charged cell surface of bacteria, which leads to contact killing [25]. Meanwhile, gentamicin-loaded TNT arrays were used to impose antibacterial property on Ti substrate *via* drug-release killing mechanism. Thus, the present system can afford dual-action antibacterial ability. We hypothesized that the combination of TNT arrays and multilayer films 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. Materials and methods

2.1. Materials

Native titanium disks (0.25 mm thickness) were kindly supplied by Tianjin Alfa Aesar Co. (China). Cell counting kit-8 (CCK-8) kit was obtained from Beyotime Biotechnology Co. (China). AKP kit was purchased from Jiancheng Biotechnology Co. (China). Poly (ethylene imine) (PEI), fluorescein diacetate (FDA), propidium iodide (PI), CHI, and SA were purchased from Sigma Chemical Co. (USA). Other chemicals were obtained from Chongqing Oriental Chemical Co. (China).

2.2. Fabrication of TNT arrays and polyelectrolyte multilayer films

TNT arrays were prepared *via* an anodic oxidation approach. Ti foil (1 cm × 1 cm) was applied as the anode. Platinum foil was used as the cathode. Ti substrates were treated at a 20 V DC potential for 60 min. The electrolyte was a solution mixture of deionized water (500 mL), glycerol (500 mL) and NH_4F (10 g). Then, TNT arrays were employed as nano-reservoirs for loading gentamicin. 5 μL gentamicin solution (10 mg/mL) was dropped onto each TNT arrays modified substrate and dried *via* vacuum filtration for 5 min. The process was repeated 5 times. Next, a droplet of 5 mg/mL PEI solution was dropped onto TNT arrays modified Ti substrate and rotated (1500 rpm for 3 s and 2500 rpm for 30 s). The multilayer film construction was then accomplished by successively spin-coating 5 mg/mL SA solution, and 5 mg/mL CHI solutions on the substrates. Finally, the spin-coating process was constantly repeated until the desired layers of $(SA/CHI)_6$ film were constructed onto TNT arrays. These samples were denoted as LBL substrates. In addition, Ti substrates decorated only with TNT arrays were denoted as TNT substrates.

2.3. Surface characterization

The field-emission scanning electron microscopy (FE-SEM, Quanta 200, Philips Corporation, Holland) was used to characterize surface morphology of the substrates. Static contact angle of pristine and modified Ti substrates were measured by a Model 200 video based optical system (Future Scientific Ltd Co. Taiwan, China) in the sessile drop method. The averaged value was used to evaluate their wettability. X-ray photoelectron spectroscopy (XPS) (Model PHI 5400, Perkin Elmer, USA) were employed to characterize the surface chemistry of different substrates. To quantify the thickness of LBL layer, $(SA/CHI)_6$ films were deposited on silicon wafers and measured using a spectroscopic ellipsometer (M-2000, Woollam, USA).

2.4. *Cytocompatibility evaluation* in vitro

2.4.1. Cell culture

Osteoblasts were isolated *via* sequential collagenase digestions of neonatal rat calvaria according to established protocol [27]. They were cultured at 37 °C in a humidified atmosphere of a 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.

2.4.2. Cell viability assay

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

2.4.3. 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. After culture for 4 and 7 days, osteoblasts were lysed using distilled water with 1% Triton X-100 by three freeze-thaw cycles. The alkaline phosphatase (AKP) activities of osteoblast cultured on different substrates were determined by AKP kit. 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.

2.4.4. Cell morphology observation

Osteoblasts were seeded onto different substrates at a density of 5000 cells per disk. After culturing for 2 days, the cells were stained with rhodamine phalloidin (Invitrogen, USA) at room temperature overnight and then stained with Hoechst 33258 fluorescent dyes (Sigma, USA) for 5 min. The cytoskeletal actin and cell nuclei were observed *via* confocal laser scanning microscopy (CLSM) (TCS-NT, Leica, Germany).

Spreading areas of cells were measured with the ImageJ1.34s software (freely available at www.nih.gov). The approach briefly contains the following procedures: (a) to convert a RGB image to a 8-bit black-white one; (b) to set measurement scale; (c) to threshold the image of cell using manual settings including all of the cell area; (d) to calculate spreading areas of cells. Spreading area of adherent cells was performed by analyzing all the cells in 6 individual fields per sample type. Finally, the number of adherent cells was performed by counting cells in 6 individual fields per sample type.

2.5. *Antibacterial property test* in vitro

2.5.1. Bacteria viability assay

Staphylococcus aureus (*S. aureus*) and *Escherichia coli* (*E. coli*) were purchased from ATCC and cultured according to previous report [28]. Mueller-Hinton broth medium (Beijing Dingguo Biological Technology Co. Ltd.) was used to culture bacteria. Bacteria were