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Antibacterial activity and increased osteoblast cell functions of zinc calcium phosphate chemical conversion on titanium

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ABSTRACT

To enhance the osteointegration and antibacterial property of titanium (Ti) implant, zinc calcium phosphate chemical conversion (Zn-Ca-PCC) was fabricated on pure Ti by phosphate chemical conversion technique. The surface of the coating was verified by combined techniques of scanning electron microscopy, energy-dispersive X-ray spectrometry, atomic force microscopy and water contact angle measurements. Plate-like Zn-Ca-PCC coatings are built up on the surface of Ti. The results obtained from the antibacterial studies suggest that the Zn-Ca-PCC coatings can greatly inhibit the growth of both Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Pseudomonas aeruginosa*) bacteria. Moreover, the in vitro evaluations demonstrate that the adhesion, proliferation and differentiation of osteoblasts on Zn-Ca-PCC coatings were significantly enhanced compared with pure Ti. In conclusion, innovative Zn-Ca-PCC coatings on Ti with excellent antibacterial activity and biocompatibility are promising candidates for orthopedic and dental implants.

1. Introduction

Titanium (Ti) and its alloys have been widely used to fabricate implantable devices because of their good mechanical properties, corrosion resistance, and biocompatibility [1]. However, some shortcomings (e.g., bioinertness, wears, etc.) of native Ti have been found to block its osseointegration and even influence the long-term survival of Ti-based implants [2–4]. Another bigger complication of Ti-based implant is implant-associated infections. The study suggested that the incidence of prosthesis infections per year is was about 4.3% in the USA [5]. A biofilm would be formed onto the surface of implant when the infection occurs, which is hardly destroyed by antibacterial agents coming from outside [6]. Such infections would inhibit the osteointegration between implants and bone tissue and then give rise to implant failure [7]. Therefore, how to enhance osseointegration and simultaneously prevent bacterial adhesion of Ti-based implants have drawn much attention in the orthopedic field.

It is well established that surface chemistry is also a pivotal factor in the regulation of the interactions between the biomaterial and cells/tissue [8], such as calcium (Ca) and zinc (Zn). Previous studies have shown that Ca-implanted Ti increased cell adhesion, spreading and proliferation of osteoblasts [9]. Furthermore, Ca ion has also been demonstrated to promote the expression of bone-related gene [10]. Carbajal et al. added ZnO to biphasic β/α-tricalcium phosphate (TCP) to developed Zn—TCP and it had good interaction with osteoblasts [11]. Sudheesh et al. reported that the chitosan hydro-gel/nano ZnO composite showed good antibacterial property and interaction with fibroblasts [12]. Hu et al. suggested that Zn-incorporated TiO_2 coatings could not only inhibit bacteria adhesion but also promote the proliferation and differentiation of mesenchymal stem cells [13].

Therefore, we assume that zinc-calcium-modified implant surface would have many advantages, such as promoting cell growth and inhibiting bacterial adhesion. Our next consideration would then be the selection of an appropriate method of surface modification. In the last decade, phosphate chemical conversion (PCC) technology has been introduced as a new surface modification method to enhance the surface performance of metallic implants, which has many advantages such as low-cost, easy operation, rapid coating formation, and usage for treatment of irregular surface [14]. PCC has been reported to be applicable to magnesium and iron, and the test results indicated that cells showed good adherence, high growth rate and proliferation characteristics on PCC coating surface [15,16]. However, whether zinc-calcium-PCC

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coatings on Ti could enhance the osteoblast proliferation has not been explored. In this work, zinc-calcium-PCC coatings were developed on Ti, and the antibacterial activity and the osteoblast response on the coatings were examined.

2. Materials and methods

2.1. Sample preparation

Pure Ti was machined to disks with a diameter of 10 mm and a thickness of 5 mm as substrates. The surfaces of the disks were ground using a series of water-resistant emery papers with various degrees of coarseness up to 1200 grits. Then the polished surface was rinsed using de-ionized water before PCC process. The pretreated disks were incubated into the phosphate solution with compositions of 0.05 mmol/L ZnO, H_3PO_4, 0.1 mmol/L $Zn(NO_3)_2 \cdot 6H_2O$ and 0.075 mmol/L $Ca(NO_3)_2 \cdot 4H_2O$. The pH of phosphate solution was adjusted to 3.0–5.0 using NaOH or H_3PO_4 and the treatment time was 30 min. The pure Ti was used as control. All disks were finally rinsed with distilled and deionized (Milli-Q) water and sterilized in an autoclave prior to cell culture.

2.2. Sample characterization

The morphology of the surface was observed by field emission scanning electron microscopy (FESEM, FEI NOVA NanoSEM). The element composition of the sample was characterized by energy-dispersive X-ray spectrometry (EDS). The phase composition of the coating was examined by X-ray diffractometer (XRD). The surface roughness (Ra) was measured using atomic force microscopy (AFM, Nanoscope 3; Bruker, Germany). The contact angle was determined using a video contact-angle measurement system (Model 200, Future Scientific, Taiwan, China).

2.3. Zn ion release from Zn-Ca-PCC coatings

The release rates of Zn ions from the Zn-Ca-PCC coatings to phosphate buffered saline (PBS) was determined by ICP-AES (inductively-coupled plasma atomic emission spectrometry, Varian Liberty 150). The samples were placed in sterile microcentrifuge tubes (15 mL), rinsed with 10 mL of new medium, and incubated at 37 °C for 1, 4, 7, 14 and 21 days. At the end of each incubation period, all the leachates were removed and replaced with fresh medium aliquots. Six replicates were conducted in the experiment.

2.4. Bacteria culture

Both *Staphylococcus aureus* (*S. aureus*) and *Pseudomonas aeruginosa* (*P. aeruginosa*), purchased from ATCC, were cultured in Mueller–Hinton Broth (MHB) medium with shaking (150 rpm) at 37 °C. A suspension containing the bacteria at a concentration of 10^7 cfu/mL was introduced onto the sample surface to a density of 60 μL/cm^2. After incubating 24 h at 37 °C, the suspended and non-adhered bacteria were removed by rinsing with NaCl solution and a LIVE/DEAD BacLight Viability Kit (Invitrogen) was used to stain the bacteria. Pictures of the bacteria were assessed at random on a fluorescence microscope with a green filter (excitation/emission, 420–480 nm/520–580 nm) and a red filter (excitation/emission, 480–550 nm/590–800 nm).

2.5. Bacteria viability

Bacteria were seeded on the samples with a density of 10^7 cfu/mL for culturing 12, 24 and 48 h. At the prescribed time point, the samples were rinsed with PBS for three times, and 200 μL of fresh MHB medium and 20 μL of CCK-8 solution were added and incubated at 37 °C for another 1 h. The optical density of the solution was obtained using an ELISA reader at a wavelength of 490 nm.

2.6. Cell culture

Osteoblasts were isolated from neonatal rat calvaria. All animals were obtained from the Qilu Hospital Animal Center (Jinan, China), and the animal process protocols were approved by the Animal Research Committee of Shandong University Qilu Hospital. The osteoblasts were cultured using Dulbecco's Modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, USA) at 37 °C in an atmosphere of 5% CO_2. Cell culture media was initially replaced after 24 h and every 3 d thereafter. The following experiments were performed with the osteoblasts at the third passage.

2.7. Cell attachment

Osteoblasts were seeded on the samples at a density of 1×10^4 cells/cm^2. The non-adherent cells were removed with phosphate-buffered saline (PBS) after 4 h. The adherent osteoblasts were fixed with 95% alcohol and stained with 4′,6′-diamidino-2-phenylindole (DAPI). The cells in 5 random fields were counted at low magnification under a fluorescence microscope (Leica DM400).

2.8. Cell morphology

Osteoblasts were seeded on the samples at a density of 1×10^4 cells/cm^2 for 12 h. And then the non-adherent cells were rinsed with PBS and fixed in 2.5% glutaraldehyde in 0.1 M PBS buffer (pH = 7.4) for 20 min. Cells were subsequently rinsed with PBS three times, followed by sequential dehydration in an ethanol series of 30%, 50%, 70%, 90%, 95% and 100% for 5 min in each concentration. Specimens were sputter-coated with gold and the morphology of the osteoblasts were obtained by scanning electron microscope (SEM).

2.9. Cell proliferation

Cell proliferation was assessed using a colorimetric assay based on the conversion of the tetrazolium salt into a soluble formazan using a Cell Titer 96 Aqueous assay (MTS, Promega). Osteoblasts were cultured on samples in a 24-well culture plate at a density of 1×10^4 cells/cm^2 and allowed to grow for 1, 4, 7 and 14 days. At the specified time-points, the substrates were rinsed with PBS and transferred to a new 24-well plate. One hundred microliters of prewarmed MTS solution mixed with 1 mL culture medium was added to each well and incubated for 4 h at 37 °C and 5% CO_2. Then 100 μL of the reacted reagent from each well was transferred to 96-well plates and the absorbance was measured using an ELISA reader at a wavelength of 490 nm.

2.10. Cell differentiation

Osteoblasts were cultured on samples at a density of 1×10^4 cells/cm^2 for 4, 7 and 14 days in differentiation medium comprising DMEM medium supplemented with 50 mg/mL ascorbic acid (Sigma-Aldrich) and 10 mM b-glycerophosphate (Sigma-Aldrich). ALP activity was determined using stable *p*-nitrophenol phosphate substrate. At each time point, cells were washed with PBS and lysed with 0.1% Triton X-100 (Sigma) using a standard freeze-thaw protocol. ALP activity in the supernatants was measured following addition of *p*-nitrophenyl phosphate substrate and the reaction was stopped using 0.1 N NaOH. The optical density was quantified by measuring the absorbance at 405 nm in an ELISA reader. ALP activity was normalized to the total protein content, which was assessed by a BCA protein assay kit and calculated from a standard curve of bovine serum albumin standards.

2.11. Cell mineralization

Mineralization was determined using an Alizarin red staining method after culturing the cells onto the samples for 7 or 14 days. The cells were