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Preparation and characterization of chitosan-silver/hydroxyapatite composite coatings onTiO_2 nanotube for biomedical applications

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ABSTRACT

A biocomposite coating containing chitosan, silver, and hydroxyapatite was developed on anodized titanium substrate by electrochemical deposition. Coatings were characterized by field-emission scanning electron microscopy, X-ray diffraction, Fourier transform infrared spectroscopy, X-ray photoelectron spectroscopy and polarisation studies. Results showed that the prepared coatings had compact and dense morphology with a thickness of 6.2 ± 0.7 μm and that silver was evenly distributed. Testing the prepared coatings with Gram-positive and Gram-negative bacterial strains exhibited antibacterial activity because of the synergistic effect of silver and chitosan. The prepared coatings were also found to be nontoxic to MC3T3-E1 cells. These results suggested that chitosan/silver-hydroxyapatite biocomposite coatings can prevent the bacterial infection of implants.

1. Introduction

Hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ (HAp) is an important material for biomedical implants, because its chemical composition similar to that of bone tissue [1]. It has been studied as a coating of dental and orthopedic implants and as a component of biopolymer composite material. HAp can induce formation of bone-like apatite on its surface and strongly bond to bone [2]. Materials with antibacterial properties are ideal for bone regeneration because infection is a predicament during implantation, reduces the success rate of surgery and sometimes increases patient mortality. Antibacterial materials were added into the HAp coating to effectively inhibit postoperative infection. However, cytotoxin is possibly introduced with the addition of antimicrobial materials.

Metallic nanoparticles such as zinc, copper and silver (Ag) exhibit antibacterial properties. Ag is an antibacterial agent that is used in colloidal, metallic and ionic forms. It exhibits excellent and broad-spectrum antibacterial properties at low concentrations without cytotoxin through interacting with the enzymes and proteins of bacteria. Thus, it is widely used in biomedical applications to inhibit the infection [3]. Silver causes structural damage to the bacterial membrane and cell wall. Specifically, it binds to bacteria DNA and RNA, and prevents bacterial reproduction. Ag inhibits electron transport chain of the bacteria cell, resulting in bacterial destruction [4,5]. It has been incorporated into a variety organic materials [6,7]. Ciobanu et al. found the Ag-HAp composite nanoparticles are effective in protecting macrophages from LPS induced cytotoxin in RAW 264.7 macrophage [8]. Our group recently reported that Ag-doped HAp composite coatings on anodized titanium exhibit increased antibacterial activities [9]. Among all antibacterial materials, chitosan (CS) is a widely used biopolymer, generated from deacetylation of chitin. It exhibits nontoxicity, biodegradability, antibacterial and hemostasis properties [10]. CS also exhibits a broad-spectrum of antimicrobial activities against Gram-negative and Gram-positive bacteria, because it binds to the negatively charged bacterial cell wall, attaches to the DNA and inhibits its replication [11]. As the cationic polysaccharide, CS is composed of N-acetylglucosamine and glucosamine residues. It easily forms chelate complexes with metals, such as zinc, Ag [12,13]. It has been reported that Ag ions and CS form complexes and show long-term antibacterial effectiveness against *Escherichia coli* and *Staphylococcus aureus* [13,14]. CS can

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be used in combination with HAp to further enhance bioactivity and tissue osteoconductivity [15,16]. Tang et al. pointed out that CS/carbonated HAp composite coatings show improved cell morphology, adhesion, spreading and proliferation of human bone mesenchymal stem cells than carbonated HAp *in vitro*.

Considering the importance of CS, Ag and HAp, the present study aimed to prepare the chitosan-silver/hydroxyapatite (CSAgHAp) composite antibacterial coatings on titanium (Ti) in aqueous solutions by electrochemical deposition. This method exhibits the following advantages: deposition from solution, low deposition temperature that introduces biomolecules into the coating, controllable phase composition and coating morphology and addition of antimicrobial compounds or drugs into the coating. Morphology, phase composition and antibacterial properties of deposited CSAgHAp composite coatings, as well as the bioactivity and biocompatibility of coatings are discussed.

2. Materials and methods

2.1. Preparation of Ti sheets

Medical pure Ti plate was cut into 10 mm × 10 mm × 0.9 mm sheets, polished using SiC paper from 1200 to 400 grit, and ultrasonically cleaned with alcohol, acetone and distilled water, respectively. The Ti sheets were etched in mixed acid solution (3 mL of nitric acid, 1 mL of hydrofluoric acid and 10 mL of distilled water) for 20 s, washed in distilled water, and dried at room temperature. Before the deposition, Ti sheets were anodized to form evenly arranged TiO_2 nanotubes (TNs) in 5 wt% HF electrolyte for 60 min, with a 15 mm × 15 mm × 0.1 mm platinum foil as cathode and a Ti sheet as anode, the foil and the sheet are 4 cm apart. A constant voltage of 20 V was applied by a direct current (DC) power source. After anodization, the Ti sheets were washed in distilled water and dried at room temperature.

2.2. Electrochemical deposition process

CS solutions were prepared by dissolving appropriate quantities of CS in 2 wt% acetic acid at 50 °C. Electrolyte was prepared with 0.042 mol L^{-1} $Ca(NO_3)_2$, 0.025 mol L^{-1} $NH_4H_2PO_4$, 0.036 g L^{-1} CS and 0.1 mmol L^{-1} $AgNO_3$ in distilled water with pH value adjusted to 4.3 using ammonia solution. Deposition was conducted in 250 mL glass breaker at a constant current density of 0.85 mA cm^{-2}. Deposition with three electrode cell lasted for 35 min at 50 °C by using electrochemical workstation (LK2005A, China), a platinum foil as counter electrode, a Ti sheet as working electrode and a saturated calomel electrode (SCE) as a reference electrode. Distance between the counter and working electrode was kept 2 cm. After that, the coated Ti sheet was carefully removed into distilled water and then air dried at room temperature.

3. Characterization

3.1. Morphology and composition studies

X'Pert Pro DX1000 X-ray diffractometer, which uses Cu Kα radiation at 40 kV and 30 mA at room temperature, was used for X-ray diffraction (XRD) studies. XRD scan rate was fixed at 1°/min. Morphology of samples was examined through JSM 7500F field emission scanning electron microscope (FESEM) with equipped energy dispersive X-ray (EDX). Fourier transform infrared (FTIR) spectroscopic studies (NICONET NEXUS 670, USA) were carried out to investigate compositional characteristics of the coatings. X-ray photoelectron spectroscopy (XPS) spectra were recorded with Al Kα radiation (1486.6 eV) as excitation source.

3.2. Bioactivity test and silver release in SBF solution

The bioactivity of the coatings was investigated by studying the apatite formation ability in a simulated blood fluid (SBF) with ionic concentrations equal to human blood plasma. The SBF was prepared by dissolving reagent grade chemicals such as KCl, NaCl, $NaHCO_3$, $K_2HPO_4{\cdot}3H_2O$, $MgCl_2{\cdot}6H_2O$, $CaCl_2$, and $NaSO_4$. The pH was adjusted to 7.4 using Tris and 1 mol L^{-1} HCl. The chemical composition was as follows (mmol L^{-1}): Na^+, 142.0; K^+, 5.0; Mg^{2+}, 1.0; Ca^{2+}, 2.5; Cl^-, 131.0; HCO_3^-, 5.0; HPO_4^{2-}, 1.0; SO_4^{2-}, 1.0. To induce apatite formation, the coated Ti sheets were immersed in 40 mL SBF solution at 37 °C for 3 days, and the immersion media was updated every day. After the experiment, the sheets were taken out and dried in air at room temperature and then characterized using FESEM.

Concentration of Ag ions was studied after immersion in SBF for different periods at 37 °C. At a preset time, the SBF was analyzed by inductively-coupled plasma atomic emission spectrometry (ICP-AES, IRIS Advantage ER/S). Four samples were tested, and results of average value (M ± SD) were reported.

3.3. Antimicrobial activity

Antimicrobial activity of HAp samples with the addition of CS and Ag was investigated against two bacterial strains, namely *E. coli* (ATCC 25922) and *S. aureus* (ATCC 25923) by plate-counting method. The prepared HAp, CSHAp and CSAgHAp samples were placed into 24-well plates, covered with 50 μL of bacterial suspension in logarithmic growth phase and incubated by rotation for 24 h at 37 °C. After incubation, each sample was taken out, placed into 10 mL of sterilized PBS, and shaken for 5 min. 100 μL of the shaken bacterial suspension was spread evenly on LB medium agar plates. After incubation for 24 h at 37 °C, the number of visible cell was determined by quantifying CFUs. Witness test was applied as a control group; each test was performed in quadruplicate. Antibacterial effect was estimated by the following formula:

$$\text{Antibacterial efficiency}\,(\%) = \frac{\text{Average number in control group} - \text{Average number in testing group}}{\text{Average number in control group}} \times 100\%$$

3.4. Cell culture

Mouse calvarial cells (MC3T3-E1, West China School of Medicine) were cultured in α-minimal essential medium (Hyclone) supplemented with 10% fetal bovine serum (FBS, Hyclone) as well as antibiotic antimycotic solution with 100 U mL^{-1} penicillin and 100 U mL^{-1} streptomycin sulfate (Gibco). Culture conditions consisted of humidified atmosphere of 5% CO_2, 95% air at 37 °C. Prior to the experiment, the samples were sterilized under 121 °C for 25 min.

Cell proliferation was studied using MTT assay. Basically, cells with density of 5×10^4 mL^{-1} were seeded on each sample in 12-well tissue culture plates. Cell proliferation was assessed by 1,3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) after 1, 3, and 7 days of culture. Culture medium was renewed every day. At the predetermined time point, cell-substrates were washed with PBS and placed into another 12-well plate; 900 μL of culture medium and 100 μL of MTT (5 mg mL^{-1}) solution were added and lasted for 4 h. Then 100 μL of the solution was added to a 96-well plate for the absorbance measurement at 570 nm using a microplate reader. Each test was performed four times (M ± SD).