Applied Surface Science 389 (2016) 7–16

Contents lists available at ScienceDirect

Applied Surface Science

journal homepage: www.elsevier.com/locate/apsusc

Antimicrobial design of titanium surface that kill sessile bacteria but support stem cells adhesion

Chen Zhu, Ni-Rong Bao, Shuo Chen, Jian-Ning Zhao*

Department of Orthopaedic Surgery, Jinling Hospital, Nanjing University School of Medicine, Nanjing, Jiangsu 210002, China

ARTICLE INFO

Article history:
Received 27 March 2016
Received in revised form 30 June 2016
Accepted 12 July 2016
Available online 15 July 2016

Keywords:
Antibacterial
Biocompatibility
Bioactivity
Silver
Nanostructure

ABSTRACT

Implant-related bacterial infection is one of the most severe postoperative complications in orthopedic or dental surgery. In this context, from the perspective of surface modification, increasing efforts have been made to enhance the antibacterial capability of titanium surface. In this work, a hierarchical hybrid surface architecture was firstly constructed on titanium surface by two-step strategy of acid etching and H_2O_2 aging. Then silver nanoparticles were firmly immobilized on the hierarchical surface by ion implantation, showing no detectable release of silver ions from surface. The designed titanium surface showed good bioactivity. More importantly, this elaborately designed titanium surface can effectively inactivate the adherent *S. aureus* on surface by virtue of a contact-killing mode. Meanwhile, the designed titanium surface can significantly facilitate the initial adhesion and spreading behaviors of bone marrow mesenchymal stem cells (MSCs) on titanium. The results suggested that, the elaborately designed titanium surface might own a cell-favoring ability that can help mammalian cells win the initial adhesion race against bacteria. We hope the present study can provide a new insight for the better understanding and designing of antimicrobial titanium surface, and pave the way to satisfying clinical requirements.

1. Introduction

Titanium and its alloys possess excellent mechanical property and corrosion resistance, and intrinsic biocompatibility, which make them extensively utilized in orthopaedic and dental implants [1–4]. However, implant-related infections have always been a huge challenge that both clinicians and materials scientists face since titanium-based biomaterials do not possess antibacterial ability. In fact, bacterial infection has become one of the most severe postoperative complications and may cause implant failures [5–8]. Once adhering to titanium surface, bacteria will colonize and form biofilms to protect them from external stimuli, thus causing many types of persistent and chronic infections [9]. In this context, it is of great necessity and importance to improve the surface anti-infection capability of titanium-based biomaterials by virtue of organic and inorganic antimicrobials [10–12].

Nowadays, surface modification and functionalization can provide an effective strategy to endow titanium-based biomaterials with desired antimicrobial ability. Although the *in situ* loading of antibiotics onto titanium surface can be achieved by "nano-reservoir" effect [13] or chemical bonding [14] to reduce bacterial infections, increasing attentions have been paid to the problem of antibiotic resistance [15–17]. In this context, "nanoantibiotics", *i.e.* nanomaterial-based antimicrobials, hold great promise for treatment of implant-related infections [16]. As a good example, silver nanoparticles possess excellent broad-spectrum antimicrobial activity [18–20]; while meantime, the potential cytotoxicity of free nanoparticles or ions should not be ignored. On the other hand, the design of desired implant surface should be emphasized on balancing the antibacterial and cellular functions [21,22]. In terms of this, as implant surface exert the bacteria-killing function, it should not impair the cell normal functions. Excitingly, nanotechnology as a powerful tool holds great promise for this purpose [23,24].

From the balance perspective, researchers have made positive attempts to incorporate silver nanoparticles onto titanium surface by means of polydopamine reduction [25], UV-irradiation reduction [26], magnetron sputtering [7,27], ion implantation [28,29], *etc.* Among these strategies, silver ion implantation can effectively enhance the bacteria-killing property of titanium surface and meanwhile restrict the release of silver ions to minimize potential cytotoxicity [30]. On the other hand, from the biomimetic perspective, a hierarchical titanium surface with micro- and nanostructures

* Corresponding author at: Department of Orthopaedic Surgery, Jinling Hospital, Nanjing University School of Medicine, No. 305 East Zhongshan Road, Nanjing, Jiangsu 210002, China.
E-mail address: zhaojianning.0207@163.com (J.-N. Zhao).

http://dx.doi.org/10.1016/j.apsusc.2016.07.080
0169-4332/

can better simulate the hybrid structures of natural bone tissue and extracellular matrix, thus better promoting cell functions [31–33].

The surface chemical treatment of titanium-based materials with hydrogen peroxide (H_2O_2) has been widely used to enhance their bioactivity and biocompatibility [8,34]. Meanwhile, it was found that the nanostructures produced by H_2O_2–H_2SO_4 treatment could impart good bacteriostatic ability to titanium surface [35,36]. In this context, one can consider that an elaborate combination of silver nanoparticles and hierarchical micro-nanostructure may endow titanium surface with desired antibacterial and cellular functions.

Based on the above considerations, in this study, a hierarchical hybrid micro-nanostructure was fabricated on titanium surface by two-step method of acid etching and H_2O_2 aging. After that, ion implantation was conducted to stabilize silver nanoparticles on titanium surface. Subsequently, the antimicrobial ability of this designed titanium surface was evaluated. At the same time, the initial adhesion behavior and proliferation activity of mesenchymal stem cells on the designed surface were investigated. And the bioactivity (apatite-forming ability) of the designed surface was also estimated.

2. Materials and methods

2.1. Sample fabrication

Commercially pure titanium (>99.85% purity) plates (size, 10 mm × 10 mm × 1 mm) were ultrasonically cleaned in ethanol and deionized water several times, followed by etching in oxalic acid solution to obtain a rough surface at microscale [37]. According to previous reference [38], H_2O_2 aging method was utilized to fabricate porous titania nanostructure on titanium surface. In brief, titanium plates were firstly soaked in 10% H_2O_2 aqueous solution and then aged for 10 h at 80 °C in an oven. After that, the aged titanium plates were gently rinsed with deionized water and dried in ambient atmosphere. Then heat treatment was carried out on the aged samples at 400 °C for 2 h under ambient atmosphere for film crystallization. After that, metal vapor vacuum arc (MEVVA) ion implantation was conducted on the designed titanium surface (pressure 3.5×10^{-4} Pa, beam energy 40 keV, dose 10^{16} ions/cm^2). The above designed samples were denoted as *micro-Ti*, *nano-Ti* and *Ag-Ti*, respectively.

2.2. Sample characterization

Field-emission scanning electron microscopy (SEM; JEOL JSM-7800F, Japan) was used to investigate the surface morphology of samples. X-ray photoelectron spectroscopy (XPS; Model PHI-5072, Physical Electronics Inc., Eden Prairie, MN) with Mg Kα (1253.6 eV) source was adopted to study the surface chemical compositions and chemical states of samples. X-ray diffractometer (XRD; Model 6100, Shimadzu, Japan) fitted with Cu Kα (λ = 1.541 Å) source was used to investigate the film crystallinity with glancing angle of incident beam of 1° at 100 mA and 40 kV. Raman microscopy (inVia, Renishaw, UK) with Ar-ion laser (excitation 514 nm) was utilized to analyze the Raman spectra. Transmission electron microscope (TEM; JEM-2010, JEOL, Japan) analysis was performed with accelerating voltage of 200 kV. The testing sample was scratched off from the Ag-Ti surface, dispersed in ethanol, and then positioned on holey copper grid covered with porous carbon film.

2.3. Ion release detection

Samples *micro-Ti*, *nano-Ti* and *Ag-Ti* were immersed in 10 ml ultrapure water at 37 °C for 2 weeks at static state. After that, the leaching liquid was collected and the release amount of silver or titanium was detected by inductively-coupled plasma mass spectrometry (ICP-MS; Agilent 7800, Japan). Since the ion release is dependent on the surface areas exposed, all the tested samples should keep the consistent size of 10 mm × 10 mm × 1 mm. Four samples were tested for each group.

2.4. Bioactivity estimation

Simulated body fluid (SBF) was adopted to estimate the bioactivity of samples *micro-Ti*, *nano-Ti* and *Ag-Ti*. According to previous reference [39], the SBF was fabricated by dissolving reagent-grade chemicals NaCl, $NaHCO_3$, KCl, $K_2HPO_4{\cdot}3H_2O$, $MgCl_2{\cdot}6H_2O$, $CaCl_2$ and Na_2SO_4 in deionized water and then buffered at pH = 7.4 by adding Tris and HCl at 36.5 °C. A couple of samples per group were soaked in 20 ml SBF in a plastic vial, and then maintained in a thermostat at 36.5 °C for 7 d at static state. After that, the samples were gently taken out, washed with deionized water and then dried at room temperature for further characterization.

2.5. Adherent bacteria study

Staphylococcus aureus (*S. aureus*, ATCC 25923) grew in trypticase soy broth (TSB; BD Biosciences) medium at 37 °C. The inoculum was prepared by adjusting bacteria suspension concentration to 10^7 colony forming units per ml (CFU/ml) in TSB medium. The samples were sterilized in 75 v/v% ethanol solution for 2 h. After drying, they were placed in 24-well plate, and then 500 μl bacteria-containing medium per well was introduced for the sterile samples. Subsequently, these samples with bacterial solution were statically incubated at 37 °C overnight for the following antimicrobial assay. Firstly, bacteria counting method was adopted to estimate the antimicrobial rate against adherent bacteria, calculated by the following equation,

$$R_a = \frac{(A - B)}{A} \times 100\%$$

in which: R_a, antimicrobial rate against adherent bacteria; A, average number of adherent bacteria dissociated from *micro-Ti* (CFU/sample); B, average number of adherent bacteria dissociated from *nano-Ti* or *Ag-Ti* (CFU/sample). Four samples were tested for each group.

After culturing bacteria on samples for 24 h, the samples were gently rinsed with PBS twice, transferred into a new 24-well plate, and then stained with 1 ml mixed dyes (LIVE/DEAD BacLight Bacterial Viability Kit, Molecular Probes) in darkness for 15 min, and finally observed by confocal laser scanning microscopy (CLSM; Leica, Germany). The viable bacteria with intact cell membrane were stained green while defunct bacteria with impaired cytomembrane were stained red.

2.6. Planktonic bacteria study

After bacteria incubation on samples for 24 h, the floating bacteria in suspensions were collected and assayed by PrestoBlue Cell Viability Reagent (Life Technologies). The operation procedures and viability calculation of bacterial cells strictly followed the instruction of the PrestoBlue assay. Planktonic bacteria viability was estimated by the following equation,

$$R_e = \frac{117,216 \times A^{\lambda_1} - 80,586 \times A^{\lambda_2}}{155,677 \times A'^{\lambda_1} - 14,652 \times A'^{\lambda 2}} \times 100\%$$

in which: R_e, reduction rate of PrestoBlue reagent; A, absorbance of testing wells; A′, absorbance of negative control wells (containing medium with PrestoBlue reagent but without bacteria); λ_1 = 570 nm, λ_2 = 600 nm. Four samples were tested for each group.