Materials Science and Engineering C 71 (2017) 93–99

Contents lists available at ScienceDirect

Materials Science and Engineering C

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

Antibacterial ability and angiogenic activity of Cu-Ti-O nanotube arrays

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ARTICLE INFO

Article history:
Received 25 May 2016
Received in revised form 9 September 2016
Accepted 29 September 2016
Available online 30 September 2016

Keywords:
Anodization
TiO_2 nanotubes
Bacteria
Endothelial cells

ABSTRACT

Bacterial infection and loosening of orthopedic implants remain two disastrously postoperative complications. Angiogenesis is critical important to facilitate implant osseointegration *in vivo*. TiO_2 nanotubes arrays (NTAs) with proper dimensions possess good osseointegration ability. Accordingly, the present work incorporated copper (Cu) into TiO_2 NTAs (Cu-Ti-O NTAs) to enhance their antibacterial ability and angiogenesis activity, which was realized through anodizing magnetron-sputtered TiCu coatings with different Cu contents on pure titanium (Ti). Our results show ordered Cu-Ti-O NTAs can be produced under proper Cu content (<15.14%) in TiCu coatings. The NTAs possess excellent long-term antibacterial ability against *Staphylococcus aureus* (*S. aureus*), which may be ascribed to sustained release of Cu^{2+}. The cytotoxicity of Cu-Ti-O NTAs to endothelial cells (ECs) could be negligible and can even promote cell proliferation as revealed by live/dead staining and MTT. Meanwhile, Cu-Ti-O NTAs can up-regulate nitric oxide (NO) synthesis and vascular endothelial growth factors (VEGF) secretion of ECs on the sample surfaces compared with that of pure TiO_2 NTAs (control). Furthermore, the angiogenic activity is also enhanced in ionic extracts of Cu-Ti-O NTAs compared with the control. The excellent long-term antibacterial ability and favorable angiogenic activity render Cu-Ti-O NTAs to be promising implant coatings.

1. Introduction

Titanium (Ti) and its alloys have been widely used as orthopedic implant materials on account of their excellent biocompatibility, corrosion resistance, mechanical properties and magnetic resonance imaging (MRI) compatibility [1]. Biological processes peri-implants mainly involve osteogenesis and angiogenesis, among which osteogenesis is critical for implant long-term stability [2]. Unfortunately, Ti and its alloys show poor osteogenic activity thus possibly leading to implant loosening. Angiogenesis is a parallel process to osteogenesis during formation of new bone and bone fracture healing [3–5], as well as bone regeneration and osseointegration of implanted materials [6]. It closely interacts with osteogenic process *in vivo*. Recent studies have indicated that ingrowth of blood vessels can markedly stimulate osteogenesis, since a functional vascular network could offer adequate oxygen and nutrients to promote growth and differentiation of relevant cells [7–10]. During the bone regeneration, insufficient angiogenic capacity may hinder osteogenic process directly and finally compromise implant osseointegration ability and long-term stability [11,12]. Accordingly, Ti-based implants with both enhanced osteogenic and angiogenic activities are highly desired. Moreover, the implants based on Ti and its alloys are susceptible to suffer from bacterial infections during their lifetime [13]. Once initiated, thoroughly eradication of the infections is extremely difficult by traditional antibiotic therapy because of compromised host immune system near infected sites originated from insufficient vascular network peri-implants. Hence, surface modification of implants to enhance their neovascularization contributes to combat implant infections.

TiO_2 nanotubes prepared by anodization of Ti and its alloys are independent, closely arranged, and vertically aligned to the surface of the substrate thus forming the TiO_2 nanotube arrays(NTAs), which have drawn increasing attention as biomedical coatings [14]. Their beneficial effects on osteoblast proliferation, differentiation *in vitro* and osseointegration *in vivo* have been well documented [15–19]. TiO_2 NTAs with proper dimensions have been reported to enhance endothelial cells (ECs) adhesion, proliferation, and motility [20–22]. However, poor antibacterial activity of TiO_2 NTAs invalidates them to combat bacterial infections. Incorporation of metallic elements such as silver (Ag) [23], zinc (Zn) [24], and copper (Cu) [25] into TiO_2 NTAs could solve this problem since one-end opening geometry of TiO_2 NTAs could make themselves serve as local drug delivery systems [26]. It has been reported that Cu possesses satisfactory antibacterial properties against scores of bacteria [27–29] with low cytotoxicity compared with Zn and Ag [30]. Moreover, Cu^{2+} can not only enhance mobility and proliferation of ECs, but also facilitate angiogenesis and vascular maturation through up-regulating expression of vascular endothelial growth factor (VEGF) [31,32]. Therefore, TiO_2 NTAs with sustained release of Cu^{2+} have great potential as a promising material to promote osteogenesis, angiogenesis, and antibacterial activities of Ti-based implants to better meet clinical needs.

In our previous work, Cu-doped TiO_2 NTAs (Cu-Ti-O NTAs) prepared by anodizing magnetron-sputtered TiCu coatings show satisfactory antibacterial activity and cytocompatibility with osteoblasts [25]. The

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http://dx.doi.org/10.1016/j.msec.2016.09.077
0928-4931/

present work aims at investigating the long-term antibacterial ability and angiogenic activity of the Cu-Ti-O NTAs. The variation of antibacterial rates against *Staphylococcus aureus* (*S. aureus*) as a function of immersion time was evaluated, and EC functions such as adhesion, proliferation, NO synthesis, VEGF secretion, and angiogenic activity were also studied.

2. Materials and methods

2.1. Preparation of TiCu coatings

Commercially pure Ti foils (BaoTi, China) with dimensions of $10 \times 10 \times 0.3$ mm^3 were used as the substrates. After ultrasonic cleaning with acetone, ethanol, and distilled water for 5 min successively, they were dried with cool air before introducing into the deposition chamber. Four TiCu targets with different Cu contents were used to deposit TiCu coatings on Ti foils by pulsed DC magnetron sputtering. Samples and targets were sputter-cleaned for 30 min at a bias of −800 V, duty factor of 40%, pulse frequency of 60 kHz, and working pressure of 5.0 Pa prior to deposition. The deposition parameters were as follows: target power of 320 W, working pressure of 0.8 Pa, substrate bias of −80 V, pulse frequency of 60 kHz, duty factor of 60%, and duration of 3 h.

2.2. Fabrication of Cu-Ti-O nanotube arrays

Cu-Ti-O NTAs with different Cu contents were fabricated through anodizing as-deposited TiCu coatings. The electrolyte was an ethylene glycol solution containing 0.25 wt% NH_4F and 0.8 vol% H_2O. Anodization was carried out at 35 °C for 4 h under a constant voltage of 30 V in a two electrode setup with a carbon rod as the counter electrode and sample as the working electrode. The distance between two electrodes was kept at 20 mm during anodization. After anodization, all samples were immediately ultrasonically cleaned to remove the remaining electrolyte as well as the undesired irregular oxide layers on their surfaces. Anodization of pure Ti was also performed to generate TiO_2 NTAs as a comparison.

2.3. Sample characterization

The surface and cross-sectional morphologies of TiCu coatings and Cu-Ti-O NTAs were observed by a field-emission scanning electron microscopy (FE-SEM, JSM-7001F, JEOL). Energy-dispersive X-ray spectroscopy (EDS, QX200, Bruker) was used to determine Cu contents in TiCu coatings. Cu contents in TiCu coatings were represented by atomic percentage in this work.

2.4. Antibacterial assay

To assess the influence of immersion time on the antibacterial ability of the Cu-Ti-O NTAs, the samples in each group were immersed in 3 ml of PBS for 1, 7, 14, 21, and 28 days with PBS refreshed every day. At each time point, the samples in each group were taken out and antibacterial tests were conducted on them. Spread plate counting method was used to evaluate antibacterial abilities of Cu-Ti-O NTAs. *S. aureus* was shaking cultured in a beef extract peptone (BEP, Sangon, China) medium at 37 ± 0.5 °C for overnight. The concentration of bacteria was adjusted to 5×10^4 CFU/ml in the antibacterial assay. 50 μl of bacteria suspension was introduced onto each autoclave sterilized sample surface. Then all samples were incubated at 37 ± 0.5 °C for 12 h at a relative humidity environment in darkness. At the end of incubation, each sample was rinsed with 1.5 ml of PBS and the viable bacteria in PBS were quantified by standard serial dilution and spread plate method. Three samples in each group were used at each time point. The antibacterial activity at days 1, 7, 14, 21, and 28 was calculated using the following formula: $R = (B - A) / B \times 100\%$. Where R is antibacterial rate, B and A are the mean numbers of viable bacteria (CFU) on TiO_2 NTAs (control) and Cu-Ti-O NTAs, respectively.

2.5. Cell culture

ECs (EA. hy926) were used in biological assays of Cu-Ti-O NTAs. The cells were cultured in DMEM (Gibco, America) supplemented with 10% fetal bovine serum (FBS, Sijiqing, China), 100 units/ml penicillin (Weifang Pharmaceutical Co., Ltd., China), and 100 mg/ml streptomycin (Lukang Pharmaceutical corp. Ltd., China) in a humidified atmosphere of 5% CO_2 at 37 ± 0.5 °C. When sub-confluence was reached, the cells

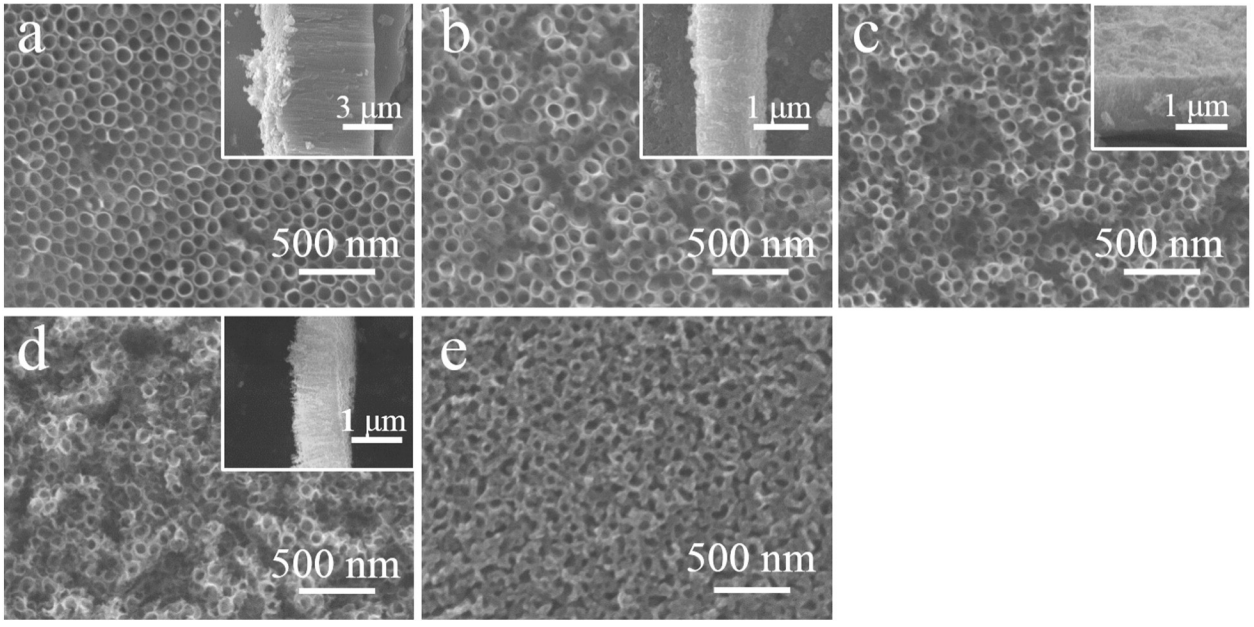

Fig. 1. Surface and cross-sectional (inset photo) FE-SEM images of as-anodized Cu-Ti-O NTAs: (a) anodized on pure Ti, denote as Cu-Ti-O0.00; (b) anodized on TiCu (2.69% Cu) coating, denote as Cu-Ti-O2.69; (c) anodized on TiCu (4.62% Cu) coating, denote as Cu-Ti-O4.62; (d) anodized on TiCu (15.14% Cu) coating, denote as Cu-Ti-O15.14; and (e) anodized on TiCu (20.47% Cu), denote as Cu-Ti-O20.47.