The effects of titania nanotubes with embedded silver oxide nanoparticles on bacteria and osteoblasts

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A versatile strategy to endow biomaterials with long-term antibacterial ability without compromising the cytocompatibility is highly desirable to combat biomaterial related infection. TiO2 nanotube (NT) arrays can significantly enhance the functions of many cell types including osteoblasts thus having promising applications in orthopedics, orthodontics, as well as other biomedical fields. In this study, TiO2 NT arrays with Ag2O nanoparticle embedded in the nanotube wall (NT-Ag2O arrays) are prepared on titanium (Ti) by TiAg magnetron sputtering and anodization. Well-defined NT arrays containing Ag concentrations in a wide range from 0 to 15 at % are formed. Ag incorporation has little influence on the NT diameter, but significantly decreases the tube length. Crystallized Ag2O nanoparticles with diameters ranging from 5 nm to 20 nm are embedded in the amorphous TiO2 nanotube wall and this unique structure leads to controlled release of Ag+ that generates adequate antibacterial activity without showing cytotoxicity. The NT-Ag2O arrays can effectively kill Escherichia coli and Staphylococcus aureus even after immersion for 28 days, demonstrating the long lasting antibacterial ability. Furthermore, the NT-Ag2O arrays have no appreciable influence on the osteoblast viability, proliferation, and differentiation compared to the Ag free TiO2 NT arrays. Ag incorporation even shows some favorable effects on promoting cell spreading. The technique reported here is a versatile approach to develop biomedical coatings with different functions.

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1. Introduction

Biomaterial-related infection is one of the most serious post-operative complications of medical implants resulting in patient suffering, financial burden, and even fatalities [1]. Infection may result from incomplete pre-operation disinfection, non-standard protocols during surgical procedure, or transfer of bacteria from infected, adjacent tissues and hematogenous sources to the implant surface after surgery. Despite strict sterilization and aseptic procedures, bacteria contamination cannot be completely avoided. After the bacteria reach the implant surface, they will aggregate in the extracellular viscous polysaccharide secreted by themselves to form a biofilm. The biofilm makes the bacteria highly resistant to the host defense and antibacterial agents thereby leading to persistent and chronic infections [2]. An effective measure to combat the infection is to endow the implants with antibacterial ability to inhibit initial bacterial adherence and subsequent formation of the biofilm [3]. In particular, many implants are susceptible to bacterial invasion throughout the lifetime and so long-term antibacterial ability is desirable. At the same time, the process to endow biomaterials with the desirable antibacterial ability should not compromise the cytocompatibility.

Topographical modification on the nanoscale can effectively improve the biological performance of biomaterials [4]. Recently, highly ordered and vertically oriented TiO2 nanotube (NT) arrays fabricated by electrochemical anodization of titanium (Ti) and its alloys have captured much interest as biomedical coatings, and their diameter and length can be precisely controlled by varying the anodic parameters [5,6]. Owing to their self-organizing nature, even a surface with a complex shape can be coated relatively easily [6]. Many in vitro and in vivo studies have demonstrated that these
TiO2 NT arrays have excellent biological performance. For instance, the TiO2 NT arrays have beneficial effects on the functions of many kinds of cells such as endothelial cells [7–9], vascular smooth muscle cells [8], human mesenchymal stem cells [9–12] and osteoblasts [13–15]. Particularly, their effectiveness in promoting osseointegration in vivo is quite encouraging in orthopedic and orthodontic applications [16].

TiO2 NT arrays have inadequate antibacterial ability and efforts have been made to improve their antibacterial properties. Considering that TiO2 NT arrays are potential drug carriers, antibacterial agents can be loaded. Popat et al. [17] have loaded antibiotics into TiO2 NTs and mitigated adhesion of Staphylococcus epidermis is observed. However, release of antibiotics from the NTs is too fast to maintain the long-term antibacterial ability and the use of antibiotics increases the resistance to antibiotics. Antimicrobial peptides are suggested to be a safer choice because of the smaller possibility of developing resistant strains, but the release rate after introduction to NTS is still too fast to sustain the long-term antibacterial activity and in vivo decomposition of the antimicrobial peptides is another drawback [18]. In comparison, inorganic silver (Ag) may be a better choice because of its broad-spectrum antibacterial property, low cytotoxicity, good stability, and small possibility to develop resistant strains [3,19]. Ag nanoparticles (NPs) or salts are currently used in a variety of medical materials and devices to prevent infection, for instance, wound dressing [20], burn ointments [21], catheters [22,23], vascular grafts [24], and bone fixation devices [25,26]. We have previously incorporated Ag NPs into TiO2 NT arrays (denoted as NT-Ag) by a simple photo-reduction method and the Ag NPs are attached to the NT sidewalls [27]. Long-term antibacterial ability has been observed from the NT-Ag arrays, but unfortunately, cytotoxicity is observed due to fast release of silver ions (Ag⁺). A more cytocompatible Ag incorporated TiO2 NT array structure with controlled Ag⁺ release is therefore highly required. Instead of loading Ag into the as-formed TiO2 NT arrays, a strategy to deposit a TiAg coating on Ti followed by anodization is utilized to fabricate the Ag loaded TiO2 NT arrays in this work. This process produces Ag₂O NP embedded TiO2 NT arrays (NT-Ag₂O arrays) with a unique structure of crystallized Ag₂O NPs embedded in amorphous titania nanotube wall and owing to the barrier effect rendered by the titania, the NT-Ag₂O arrays show slower Ag⁺ release.

2. Materials and methods

2.1. Preparation of TiAg coatings by magnetron sputtering

Pure Ti rods were cut into thin sheets (Φ14 mm × 2 mm) and used as substrates. The specimens were polished to a mirror finish followed by sequentially ultrasonic cleaning in acetone, ethanol, and deionized water for 5 min, respectively, and drying in air before introducing into the deposition chamber. The TiAg coatings were deposited on Ti by pulsed DC magnetron sputtering with TiAg targets at ambient temperature. The Ag concentrations in the coatings were varied by using five TiAg targets with different Ag contents. A high purity Ti target was used as the control. Prior to deposition, the substrates were sputter-cleaned for 30 min at a bias of −800 V, duty factor of 40%, pulsing frequency of 60 kHz, and working pressure of 5.0 Pa. Afterwards, deposition of TiAg coatings was conducted at a target power of 200 W, bias of −800 V, duty factor of 50%, pulsing frequency of 60 kHz, working pressure of 0.8 Pa, and deposition time of 3 h.

2.2. Anodization of TiAg coatings

The as-deposited TiAg coatings were subjected to electrochemical anodization to produce NT-Ag₂O arrays with different Ag contents. An ethylene glycol solution containing 0.3 wt % NH₄F and 2.0 vol % H₂O was used as the electrolyte. Anodization was carried out at room temperature at a constant DC potential of 30 V on a two-electrode setup with a platinum (Pt) foil counter electrode for 4 h. After anodization, the specimens were immediately washed with deionized water and ultrasonically cleaned to remove the remaining electrolyte as well as the undesired irregular oxide layers on the surface.

2.3. Sample characterization

Field-emission scanning electron microscopy (FE-SEM, JSM-7001F, JEOL) on an accelerating voltage of 10 kV was performed to examine the surface and cross-sectional morphology of the as-deposited TiAg coatings and as-anodized NT-Ag₂O arrays. The cross section of TiAg coatings were obtained by breaking apart the TiAg coated glass slides. The cross-sectional images of NT-Ag₂O were taken from fragments of nanotube arrays after scratching the as-anodized samples using a sharp metallic tip. The Ag concentration in the as-deposited TiAg coatings was determined by inductively coupled plasma mass spectroscopy (ICP-MS, Agilent 7500, Agilent). The atomic structure of NT-Ag₂O arrays was characterized by high-resolution transmission electron microscopy (HR-TEM, JEM-2100F, JEOL) at an accelerating voltage of 200 kV and selected area electron diffraction (SAED). Contact angle measurement was conducted by the sessile-drop method at room temperature on a contact angle meter (SL200B, Solon). Two different liquids, distilled water and diiodomethane, were employed in the contact angle measurements and the surface free energy was obtained by applying the Owens-Wendt/Young equation [28].

2.4. Ag⁺ release

To examine the release behavior of Ag⁺ from the NT-Ag₂O arrays, each anodized specimen was immersed in 10 mL of phosphate buffered saline (PBS) at 37 ± 0.5 °C. The solution was refreshed every day to mimic the physiological conditions inside the human body for 28 days. The PBS containing the released Ag⁺ was sampled at time points of 1, 7, 14, 21, and 28 days and analyzed by inductively-coupled plasma mass spectroscopy (ICP-MS, Agilent 7500, Agilent).

2.5. Antibacterial assay

The in vitro antibacterial activity of the NT-Ag₂O arrays against gram-negative Escherichia coli (E. coli, ATCC 25922) and gram-positive Staphylococcus aureus (S. aureus, ATCC6538) was assessed by the plate-counting method. The bacteria were cultured in the beef extract-peptone (BEP) medium under agitation for 18 h at 37 ± 0.5 °C. After dilution with BEP to a concentration of 1.0 × 10⁶ CFU/mL, 50 μL of the bacteria suspension was introduced onto each sample surface. The samples with the bacteria suspension were incubated at 37 ± 0.5 °C for 12 h at a relative humidity >90% in darkness. At the end of the incubation period, each sample was rinsed in PBS and ultrasonically agitated to detach the bacteria from the sample. The viable bacteria in the PBS were quantified by standard serial dilution and plate-counting. The samples were sterilized, ultrasonically cleaned, and incubated in 10 mL of PBS at 37 ± 0.5 °C for 6.5 days with the PBS refreshed every day. The samples were reinvestigated using the bacterial incubation assay. This process was repeated for a total of 28 days. The antibacterial activity at days 1, 7, 14, 21, and 28 was calculated using the following formula: R = (B – A)/B × 100% where R is the antibacterial rate and B and A are the mean numbers of viable bacteria (CFU) on the TiO2 NT arrays (control) and the NT-Ag₂O arrays (sample), respectively.

Moreover, the antibacterial activity of the NT-Ag₂O arrays at day 28 was qualitatively assayed by fluorescent staining. After final incubation with the bacteria suspensions for 12 h, each sample was rinsed with PBS to remove non-adherent bacteria. The adherent bacteria on each sample were stained by an acridine orange (AO)/propidium iodide (PI) mixture for 10 min and observed by fluorescence microscopy (BM-20AWC, BM). The morphology of S. aureus on the samples was observed by the FE-SEM. At days 1 and 28, after incubation with the bacteria suspensions for 12 h, the sample surfaces were rinsed with PBS, fixed with 2.5% glutaraldehyde, dehydrated in graded ethanol series, freeze dried, sputter-coated with thin platinum layers, and observed by the FE-SEM.

2.6. Cell culture

Newborn mouse calvaria-derived MC3T3-E1 subclone 14 pre-osteoblastic cells were used in the biological assays of the NT-Ag₂O NT arrays. The cells were cultured in α-MEM ( Gibco) supplemented with 10% calf bovine serum (CBS), 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO₂ at 37 °C. When sub-confluence was reached, the cells were harvested by mild trypsinization, centrifuged, resuspended in the complete culture medium and reseeded.

To assess the cell proliferation, cytotoxicity, and morphology, MC3T3-E1 cells were seeded on the samples at a density of 2 × 10⁴ cells/cm². In the osteoblast differentiation assay, the cells were seeded on the samples at 2 × 10⁴ cells/cm² and cultured for 3 days in the complete culture medium, additional 10 μM β-glycerophosphate, 50 μg/mL ascorbic acid and 10 nM dexamethasone were added to the medium for osteogenic induction. The media were refreshed every other day.
2.7. Cytotoxicity assay

The cytotoxicity of the samples was assayed by the LIVE/DEAD viability/cytotoxicity kit for mammalian cells (Invitrogen). The kit contained two probes: calcein AM and ethidium homodimer (EthD-1). Calcein AM could be converted to calcein by active intracellular esterase of living cells to generate green fluorescence. EthD-1 could only enter dead cells with broken cell membrane and produced red fluorescence after binding to the nucleic acids. After the cells were seeded and incubated for 1, 3, and 5 days, the samples were taken from the 24-well plates and rinsed with PBS thrice. Then 50 μl of the work solution was immediately added to each sample and incubated in darkness at 37 °C for 1 h. Subsequently, all samples were rinsed with PBS and observed by fluorescence microscopy.

2.8. Cell proliferation

The cell proliferation and viability were evaluated quantitatively by the alamarBlue (Invitrogen) method. After the cells were cultured for 1, 3, and 5 days, they were transferred to a new aseptic 24-well plate with 500 μl of the fresh medium containing 10 vol% alamarBlue in each well and incubated for 2 h. After incubation, 100 μl of the medium in each well was transferred to a 96-well plate and the absorbance was measured at 570 nm and 600 nm on a microplate reader (Infinite F50, TECAN). The reduction rate (%) of the alamarBlue was calculated following the formula provided by the manufacturer.

2.9. Cell morphology

After culturing for 1 and 3 days, all the samples were rinsed with PBS, fixed with 2.5% glutaraldehyde, dehydrated in graded ethanol series, freeze dried, sputter-coated with thin platinum layers, and observed by FE-SEM.

2.10. Alkaline phosphatase activity assay

The intracellular alkaline phosphatase (ALP) activity was evaluated qualitatively and quantitatively using commercially available kits. In the qualitative assay, after osteogenic induction for 3 and 7 days, the cells on the specimens were washed three times with PBS, fixed with 4% paraformaldehyde, and stained with BCIP/NBT ALP Color Development Kit (Beyotime). In the quantitative assay, the cells were lysed in 0.5 ml of distilled water through four standard freeze–thaw cycles. The ALP activity in lysate was evaluated colorimetrically with the Alkaline Phosphatase Assay Kit (Beyotime), which was based on the conversion of colorless p-nitrophenyl phosphate (pNPP) to colored p-nitrophenol after co-incubation for 30 min at 37 °C. The results were normalized to the total intracellular protein content determined by the bicinchoninic acid (BCA) Protein Assay Kit (Beyotime) and expressed in nanomoles that at %.

2.11. Sirius red staining assay

Sirius red staining-based colorimetric assay was employed to quantify collagen secretion from the MC3T3-E1 cells on specimens. After osteogenic induction for 7 and 14 days, the specimens were rinsed thrice with PBS, fixed with 4% paraformaldehyde, and stained in 0.1% solution of Direct Red 80 (Sigma) in aqueous saturated picric acid for 18 h. After rinsing with 0.1 M acetic acid to remove the unbound stain totally, the stain on specimens was eluted in 1 ml of destaining solution (0.2 M NaOH/methanol 1:1) and the absorbance was measured at 570 nm on the microplate reader.

2.12. Extracellular matrix mineralization assay

The degree of extracellular matrix mineralization was determined by Alizarin Red S (Sigma) staining. Briefly, after osteogenic induction for 7 and 14 days, the specimens were fixed in 75% ethanol for 1 h followed by staining with 40 mM Alizarin Red S (pH 4.2) for 30 min at room temperature. Afterwards, the unbound stain was totally removed by rinsing with distilled water. Quantitative analysis of Alizarin Red S staining was performed by eluting the bound stain with 500 μl of 10% cetylpyridinium chloride in 10 mM sodium phosphate (pH 7.0) for 2 h and the absorbance of the resulting solution was measured using the microplate reader at a wavelength of 570 nm.

2.13. Statistical analysis

Three samples were used in each group and the results were reported as means ± standard deviations. One-way analysis of variance (ANOVA) followed by Student-Newman-Keuls post hoc test was performed to determine the statistical significance of the data. Difference at p < 0.05 was considered to be significant and that at p < 0.01 was considered to be highly significant.

3. Results

3.1. Sample characterization

The surface and cross-sectional morphology of the as-deposited pure Ti and TiAg coatings with different Ag concentrations are shown in Fig. 1. The pure Ti coating (Fig. 1a) exhibits a densely populated and vertically aligned columnar structure with an average column diameter of about 200 nm. Regarding the TiAg coatings, as the Ag content is increased (Figs. 1b–f), the columnar structure gradually transforms to a layered structure and bright spots arising from Ag NPs as verified by EDS become more abundant on the surface. EDS reveals that the Ag concentrations in the TiAg coatings vary from 1.27–27.23 at %. The TiAg coatings on Ti with different Ag concentrations are denoted as TiAg1.27, TiAg4.67, TiAg7.53, TiAg14.63 and TiAg27.23 where the Arabic numerals imply the Ag atomic percentages.

Fig. 2 depicts the top-view and cross-sectional SEM images of the self-organized NT arrays prepared by anodizing the pure Ti and TiAg coatings, which are denoted as TiO2–NT, NT-Ag1.27, NT-
Ag$_2$O4.67, NT-Ag$_2$O7.53, NT-Ag$_2$O14.63 and NT-Ag$_2$O27.23, respectively. As shown in Figs. 2a–e, TiO$_2$-NT, NT-Ag$_2$O1.27, NT-Ag$_2$O4.67, NT-Ag$_2$O7.53 and NT-Ag$_2$O14.63 have homogeneous and uniform NTs with a diameter of approximately 80 nm and wall thickness of around 10 nm. The insets in Figs. 2a–e indicate that the length of the NTs decreases with the increase of Ag contents in the deposited coatings, from 6 μm for TiO$_2$-NT to 2 μm for NT-Ag$_2$O14.63. With regard to TiAg$_2$O27.23, anodization does not give rise to a regular

![Fig. 2. Surface morphology of the as-anodized TiO$_2$ NT array (a) and NT-Ag$_2$O arrays (b–f) observed by FE-SEM with the insets showing the respective cross-section. The number behind NT-Ag$_2$O is the atomic concentration of Ag in the corresponding TiAg coating.](image)

![Fig. 3. XPS spectra of the NT-Ag$_2$O arrays: (a) XPS survey spectra; (b) high-resolution XPS spectra of Ag 3d; (c) high-resolution XPS spectra of Ti 2p.](image)
nanotubular structure but instead a porous structure with a thickness of 1 mm (Fig. 2f). Hence, NT-Ag$_{27.23}$ is not used in the subsequent experiments.

The elemental composition and chemical states of the NT-Ag$_2$O arrays are determined by XPS. The XPS survey spectra and high-resolution Ag 3d and Ti 2p spectra are shown in Fig. 3 and the atomic concentrations are listed in Table 1. Ti, O, C, N, F, and Ag are detected from the NT-Ag$_2$O arrays. The presence of C and N is attributed to surface contamination from the organic electrolyte. A small amount of F originates from the use of fluoride-containing electrolytes. The Ag 3d$_{5/2}$ peak at 367.7 eV in Fig. 3b corresponds to the binding energy of Ag$^+$, indicating that Ag exists exclusively in the oxidized state in NTs. The high-resolution Ti 2p spectra in Fig. 3c indicates that Ti is in the form of TiO$_2$. Therefore, it can be inferred that the NT-Ag$_2$O arrays are composed of stoichiometric TiO$_2$ and Ag$_2$O. The distributions of Ti, O, and Ag in the NTs along the longitudinal direction obtained by EDS elemental mapping are shown in Fig. 4. The images indicate that Ti, O, and particularly Ag are uniformly distributed on the entire NTs.

Fig. 5 shows the TEM results of the TiO$_2$-NT and NT-Ag$_2$O arrays. The low-magnification images in Figs. 5a–e show that in the NT-Ag$_2$O arrays, dark NPs are randomly dispersed over the relatively light NTs, whereas no NP is observed from the TiO$_2$ NT array. The HR-TEM images (local enlargement of the squared region in each figure) show that the NPs are well encapsulated or embedded in the amorphous TiO$_2$ substrate. The lattice spacing of the NPs of 0.246, 0.234 and 0.181 nm match the (002), (011) and (012) crystallographic planes of hexagonal Ag$_2$O, respectively. The SAED pattern (inset in Fig. 5c) taken at the corresponding region exhibits four relatively sharp diffraction rings corresponding to (002), (011), (110) and (112) crystallographic planes of hexagonal Ag$_2$O, further confirming the HR-TEM results. Ag$_2$O NPs with two different sizes can be observed, namely the one with a diameter larger than 10 nm (indicated by the black arrow in insets) and the other less than 5 nm (indicated by the white arrow in insets). The quantities of the two kinds of Ag$_2$O NPs increase gradually with Ag concentrations in the TiAg coatings (Figs. 5b–e). According to the contact angle measurement and surface free energy calculation (Table 2), all the samples are highly hydrophilic but there is no appreciable difference among each other.

3.2. Ag$^+$ release

Ag$^+$ release from the NT-Ag$_2$O arrays as a function of immersion time is shown in Fig. 6a. Generally, relatively large amounts of Ag$^+$ are released initially. Afterwards, the amounts of released Ag$^+$ diminish gradually finally reaching a steady value after 21 days. NT-Ag$_{27.23}$ that has a larger Ag content releases less Ag than the other three NT-Ag$_2$O structures in the beginning. As time elapses, the amount of Ag released by NT-Ag$_{27.23}$ gradually exceeds those from the other samples with smaller Ag concentrations. According to our data, NT-Ag$_{27.23}$ shows the best Ag$^+$ release profile in a controlled mode with the lowest burst release.

3.3. Antibacterial activity of the NT-Ag$_2$O arrays

The antibacterial activity of the NT-Ag$_2$O arrays against S. aureus and E. coli is evaluated up to 28 days and the results are shown in Figs. 6b and c, respectively. On day 1, all the NT-Ag$_2$O arrays generate high antibacterial rates of 100% for both bacterial species.

Table 1

<table>
<thead>
<tr>
<th>Sample</th>
<th>Atomic concentrations (at.%)</th>
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<tr>
<td></td>
<td>Ti</td>
</tr>
<tr>
<td>TiO$_2$-NT</td>
<td>15.85</td>
</tr>
<tr>
<td>NT-Ag$_{01.27}$</td>
<td>11.87</td>
</tr>
<tr>
<td>NT-Ag$_{04.67}$</td>
<td>14.31</td>
</tr>
<tr>
<td>NT-Ag$_{07.53}$</td>
<td>15.29</td>
</tr>
<tr>
<td>NT-Ag$_{14.63}$</td>
<td>15.29</td>
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</tbody>
</table>

Fig. 4. EDS elemental mapping of the area enclosed by a square in SEM image showing the distribution of Ti, O and Ag elements over the cross-section of the TiO$_2$-NT and NT-Ag$_2$O arrays.
and there is no obvious decrease in the antibacterial rates up to days 7 and 14. Afterwards, only a slight decrease in the antibacterial rates is observed and the NT-Ag2O arrays with smaller silver concentrations diminish relatively faster. Even up to day 28, a high antibacterial rate of at least 97% is still maintained, suggesting effective and long lasting antibacterial activity against both S. aureus and E. coli.

The excellent long-term antibacterial activity of the NT-Ag2O arrays is further confirmed by fluorescent staining at day 28, as shown in Fig. 6d. After exposure to the fluorescent stain, the viable bacteria are green while the dead ones are bright red. The amounts of adhered bacteria are obviously less on the NT-Ag2O arrays than that on TiO2-NT arrays. The vast majority of the bacteria adhered to the TiO2-NT arrays are viable, whereas quite a few or no live bacteria can be found on the NT-Ag2O arrays. The results indicate that the NT-Ag2O arrays can not only retard bacteria adhesion, but also effectively kill the adhered bacteria.

Fig. 7 depicts the morphology change of S. aureus cultured on the sample surfaces at days 1 and 28. A large number of bacteria with smooth and intact cell membrane are observed on the TiO2-NT arrays and most of them undergo the binary or multiple fission process. On the contrary, only few bacteria can be found on NT-Ag2O arrays with damaged membranes. These bacteria with irregular spherical morphology are surrounded by cell fragments, indicating the deformation, cytosolic leakage, and lysis of bacteria.

### 3.4. Cytotoxicity and cell proliferation

The cytotoxicity of the NT-Ag2O arrays is qualitatively assayed using the LYE/DEAD viability/cytotoxicity kit after culturing for 1, 3, and 5 days and the results are presented in Fig. 8a. Almost no dead cell can be observed from the samples. As the culturing time is increased, the number of cells increases linearly, indicating that the NT-Ag2O arrays have nearly no cytotoxicity and support cell proliferation.

The results of cell proliferation on NT-Ag2O arrays quantitatively assayed using alamarBlue reagent as shown in Fig. 8b are consistent with the live/dead staining results. The reduction rates of alamarBlue increase with culture time on account of continuous cell proliferation. No significant difference in cell proliferation can be observed among the TiO2-NT and NT-Ag2O arrays throughout the culturing period.

### 3.5. Cell morphology

The SEM images in Fig. 9 show the morphology of cells cultured on the TiO2-NT and NT-Ag2O arrays for 1 and 3 days. At day 1, as shown by the lower-magnification images, the cells distribute evenly and well spread on all the TiO2-NT and NT-Ag2O arrays with a typical polygonal osteoblastic shape. The higher-magnification images disclose that on all the surfaces, abundant pronounced finger-like protrusions and filopodia stretch out from the cell body to interact with the nanotopography. No difference in cell morphology can be observed at this time point. At day 3, generally the cells spread better on all the surfaces than those at day 1. Specifically, the cells on the NT-Ag2O arrays appear to spread larger than those on TiO2-NT, especially NT-Ag2O1.27, NT-Ag2O4.67 and NT-Ag2O7.53.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Contact angle (deg.)</th>
<th>Surface free energy (mJ/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Distilled water</td>
<td>Diiodomethane</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>Polar</td>
</tr>
<tr>
<td>TiO2-NT</td>
<td>21.71 ± 1.76</td>
<td>8.82 ± 0.53</td>
</tr>
<tr>
<td>NT-Ag0.127</td>
<td>20.16 ± 1.59</td>
<td>8.12 ± 0.68</td>
</tr>
<tr>
<td>NT-Ag0.467</td>
<td>17.25 ± 2.16</td>
<td>7.34 ± 1.25</td>
</tr>
<tr>
<td>NT-Ag0.753</td>
<td>19.57 ± 1.63</td>
<td>8.78 ± 0.93</td>
</tr>
<tr>
<td>NT-Ag0.1463</td>
<td>17.94 ± 2.13</td>
<td>7.51 ± 1.03</td>
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</tbody>
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Fig. 5. TEM images of the NTs obtained from the as-anodized samples and their corresponding high-magnification or HR-TEM images taken from the area enclosed by a square: (a) TiO2-NT; (b) NT-Ag0.127; (c) NT-Ag0.467; (d) NT-Ag0.753; (e) NT-Ag0.1463. The inset in (c) is SAED pattern taken at the corresponding region.
3.6. Osteoblastic differentiation

The MC3T3-E1 osteoblastic differentiation on different samples is assessed in terms of ALP production, collagen secretion, and extracellular matrix mineralization. The qualitative and quantitative assay results of the ALP produced by the MC3T3-E1 cells after osteogenic induction for 3 and 7 days are shown in Fig. 10. The ALP production increases with prolonged osteogenic induction whereas no significant difference can be observed from the TiO2-NT and the NT-Ag2O arrays. The results of collagen secretion of the MC3T3-E1 cells and extracellular matrix mineralization are shown in Fig. 11. After osteogenic induction for 7 days, as the Ag content in the NT-Ag2O arrays is increased, the amount of collagen secreted by the cells gradually decreases. However, prolonging the osteogenic...
induction time to 14 days diminishes this discrepancy except for NT-Ag2O14.63. The result of extracellular matrix mineralization is similar to that of collagen secretion. After osteogenic induction for 14 days, no significant difference can be observed among the different groups.

4. Discussion

The proper approach that can endow biomaterials with long-term antibacterial ability while not impairing the biological properties of biomaterials is being actively pursued [29]. We have fabricated NT-Ag arrays on Ti with long-term antibacterial activity but cytotoxicity is also observed due to excessive release of Ag⁺ [27]. In this work, we report the NT-Ag2O array structure with crystallized Ag2O NPs embedded in amorphous TiO2 nanotube wall. The materials show slower Ag⁺ release because the TiO2 barrier is surrounded thereby minimizing the cytotoxicity induced by burst and/or large Ag⁺ release. Our process produces an even distribution of the Ag NPs along the entire length of the NTs and the amount of incorporated Ag can be tuned in a certain range. The NT-Ag2O arrays show good antibacterial characteristics and cytocompatibility and are attractive in preventing implant associated infection. Moreover, the fabrication process of the NT-Ag2O arrays is simple, economical, and versatile.

One technique used in this work is magnetron sputtering. The microstructure of the deposited coatings mainly depends on the relative temperature between the substrate during deposition ($T_s$) and fusion point of the materials to be deposited ($T_f$). Here, $T_s$ is typically below 150 °C and $T_f$ of pure Ti is 1668 °C. When $T_s/T_f < 0.3$, surface/bulk diffusion is generally very small and the films are composed of columnar crystals [30]. When the atomic concentration of Ag in the TiAg coatings is less than 3%, a solid solution of Ag in α-Ti is formed and a columnar structure is maintained. However, when the Ag content is large enough, Ag presents an additive phase which blocks grain growth and stimulates renucleation of the grains [31]. Further increase in the Ag content results in nucleation of Ag NPs in the coatings because of the low solubility of Ag in Ti. The other preparation technique is anodization and to the best of our knowledge, anodization of TiAg alloy has not yet been reported. Our results show that in an aqueous electrolyte, it is difficult to achieve well-defined NT arrays on the TiAg coatings with a relatively large Ag concentration (Fig. S1). However, in organic electrolytes such as

![Fig. 8. (a) Reduction percentage of alamarBlue for MC3T3-E1 cells after culturing for 1, 3 and 5 days on the TiO2-NT and NT-Ag2O arrays; (b) Fluorescence images of Live/dead staining of MC3T3-E1 cells after culturing for 1, 3 and 5 days on the TiO2-NT and NT-Ag2O arrays.](image-url)
ethylene glycol used in this study, a regular nanotubular architecture can be produced on the TiAg coatings with a wide range of Ag contents. Our data show that the presence of Ag in TiAg coatings has little influence on the NT diameter, presumably because the growth rate of Ag oxide is close to that of Ti oxide. The growth of NTs is the result of a competition between electrochemical anodization and chemical dissolution of the oxide layer in the fluoride-containing electrolyte [6]. Incorporation of Ag accelerates dissolution of the oxide layer due to high solubility of Ag–F compounds in the electrolytes thereby resulting in the decreased NT length.

Based on SEM, XPS and TEM, the formation mechanism of the NT-Ag2O arrays is proposed. Fig. 12 shows the current density–time curves of TiO2–NT and NT-Ag2O4.67 and schematic diagram of the formation process. The current density–time curve of NT-Ag2O4.67 shows no obvious variations compared to that of TiO2–NT, indicating the similar formation process. In the early stage, Ti and Ag are oxidized simultaneously forming a compact oxide layer (Fig. 12b1), and therefore, the current density is reduced rapidly. Because of the small solubility of Ag2O in TiO2, the nucleated Ag NPs in TiAg coatings are directly oxidized to “big” Ag2O NPs with a
diameter larger than 10 nm, while the solid-dissolved Ag tends to nucleate forming “small” Ag₂O NPs with a diameter of less than 5 nm at the interface of NT/substrate. Under the electric field, F⁻ ions in the electrolyte attack the oxide layer, resulting in the random formation of the irregular nanoporous structure (Fig. 12b2) and increased current density. As anodization continues, the nanoporous structure is gradually converted to the nanotubular structure (Fig. 12b3) and the current density decreases slowly. During prolonged anodization, the initial irregular oxide layer dissolves gradually thereby exposing the highly ordered nanotubular structure (Fig. 12b4) and the current density approaches a constant value. Two different fates of the formed Ag₂O NPs can be characterized during anodization. The Ag₂O NPs formed at the gap of NTs (Fig. S2) dissolve gradually under electric field assisted chemical etching since no Ag₂O NPs can be observed on the outer wall on the bottom of NTs (Fig. S3b), while the Ag₂O NPs formed just under the NTs are embedded into them during the growth of NTs. Meanwhile, because of prolonged chemical etching, the wall at

![Image](A. Gao et al. / Biomaterials 35 (2014) 4223-4235)

Fig. 10. (a) ALP product staining and (b) ALP activity of MC3T3-E1 cells cultured on the TiO₂-NT and NT-Ag₂O arrays after osteogenic induction for 3 and 7 days.

Fig. 11. Quantitative results of collagen secreted by MC3T3-E1 cells and extracellular matrix mineralization after osteogenic induction for 7 and 14 days. *p < 0.05 and **p < 0.01 compared to the TiO₂-NT, *p < 0.05 and ***p < 0.01 compared to the NT-Ag₂O1.27, &&p < 0.01 compared to the NT-Ag₂O4.67.
the top of the NTs becomes thinner compared to that on the bottom and so the Ag$_2$O NPs originally embedded in the TiO$_2$ wall is partly exposed (Fig. S3c).

It is believed that a certain range of Ag concentrations can kill bacteria without impairing the mammalian cell functions [32]. Compared to those of the NT-Ag arrays [27], the amounts of Ag released from the NT-Ag$_2$O arrays are reduced by an order of magnitude thus potential improving the cytocompatibility. Release of Ag$^+$ from the Ag NPs is related to the reaction with the infiltrated water [33]. In the NT-Ag arrays, the Ag NPs attached to the NT sidewalls can reach the water relatively easily thereby releasing a high dose of Ag$^+$. With regard to the NT-Ag$_2$O arrays, because of the barrier effect of the surrounding titania, it is harder for the Ag$_2$O NPs to reach and react with water, finally resulting in a slower Ag$^+$ release. An interesting phenomenon is that NT-Ag$_2$O14.63 that has the highest Ag content releases the least amount of Ag$^+$ during the first several days, showing ideal Ag$^+$ release in a controlled mode. This is related to the tube length. The tube length is inversely
related to the Ag contents in the NT-Ag2O arrays. Longer NTs can provide a larger contact area with the aqueous solution and so the amounts of Ag+ released from NT-Ag2O1.27, NT-Ag2O4.67, and NT-Ag2O7.53 are larger than that from NT-Ag2O-PDMS during initial immersion. It can also be inferred that the remaining TiAg coatings underneath the NT arrays contribute little to Ag+ release. The non-cumulative Ag+ release pattern from the NT-Ag2O arrays shows an initial rapid release followed by the sustained lower release, which is similar to that of the NT-Ag arrays [27]. This release pattern is beneficial to clinical applications [34]. The initial phase after implantation is prone to infection and stronger antibacterial ability is needed. Afterwards, smaller Ag release that can inhibit biofilm formation is adequate to prevent infection conjugated with the host defense. All in all, the NT-Ag2O arrays give rise to controlled low dose Ag+ release that meets the clinical requirement.

Although Ag+ release from the NT-Ag2O arrays is substantially reduced compared to that reported previously, the long-term antibacterial ability of the NT-Ag2O arrays as evidenced here is well maintained. It has been proposed that the antibacterial activity of Ag is dominated by released Ag+, which induces the inactivation of bacterial proteins [35, 36], condensation of DNA [35, 37], and degradation of bacterial cell membranes [38]. In addition, direct contact between Ag NPs and bacteria has been reported to induce structural changes and functional damages of plasma membranes resulting in cytosolic leakage and lysis of bacteria [39–42]. In our experiments, the antibacterial rates and the amounts of released Ag do not match very well. During the 28 days, the amounts of released Ag drop from about 40 ppb to less than 10 ppb, but the antibacterial rate is maintained at over 97%. Therefore, the antibacterial activity of the NT-Ag2O arrays stems from the synergistic effect of released Ag+ and direct contact with Ag NPs. Given the two antibacterial mechanisms, the NT-Ag2O arrays can produce a sustained antibacterial environment on the implant surface. It should be noted that although fewer bacteria are introduced to the sample surface in our antibacterial assay compared to other studies [27, 43], the experimental conditions are still much harsher than the normal situation in vivo. The effective period in which the NT-Ag2O arrays can prevent infection occurrence is expected to be longer than the 28 days experimentally demonstrated here.

It is of interest to determine whether the NT-Ag2O arrays have good cytocompatibility. Although eukaryotic cells are usually larger and show higher structural and functional redundancy compared to prokaryotic cells, they can be impaired by the similar mechanisms if the Ag dose is too high [19, 27]. The diffused Ag NPs can be readily taken up by eukaryotic cells through endocytosis and macropinocytosis [44] and the released Ag can damage intracellular functions [45, 46]. As shown in Fig. 5, the Ag2O NPs are well immobilized into the nanotubular wall thus minimizing the risk of cell uptake. Only some Ag2O NPs immobilized at the top of the NTs can directly interact with the cells. The effect of Ag on cells is dose-dependent. Over-dose of Ag can induce cytotoxicity while a small dose has positive effects on cells [47]. In our experiments, the amounts of Ag+ released from all the NT-Ag2O arrays are only between 2.45 ppb and 44.90 ppb (Fig. 6a). They are much less than all the generally accepted thresholds of toxic concentrations for human cells [19]. As evidenced by the cell experiments, the NT-Ag2O arrays support osteoblast functions. First of all, they do not exhibit appreciable deleterious effects on proliferation and viability of MC3T3-E1 cells and in fact, the NT-Ag2O arrays result in improved cell attachment and spreading after culturing for 3 days. Even though the detailed mechanism still requires further study, cell spreading improvement seems to be a common effect for the incorporation of metals to implant surface. We have observed similar phenomenon from strontium (Sr) or zinc (Zn) incorporated NTs [43, 48]. Larger cell spreading is suggested to be beneficial to osteoblast differentiation [49] and also results in better cell–cell communication reported to be critical to coordinating cell behavior [50, 51]. In addition, good cell attachment and spreading on the biomaterial surface is significant in winning the “race for the surface” against bacteria [52], thereby aiding to combat infection. Finally, we have studied whether the NT-Ag2O arrays influence osteoblast differentiation. The activity of ALP, an early differentiation marker of osteoblasts, is not influenced by the NT-Ag2O arrays. For the collagen synthesis and extracellular matrix mineralization, although the NT-Ag2O arrays with a larger Ag content show some hindering effects in the early stage of cell culturing, nearly no influence can be observed after a prolonged period of culturing. The initial side effects of the NT-Ag2O arrays with larger Ag contents on collagen synthesis and extracellular matrix mineralization can be ascribed to the direct contact between the cells and Ag2O NPs on the sample surface instead of released Ag+, since the samples with larger Ag contents release smaller amounts of Ag initially. The initial side effects of the NT-Ag2O arrays may be easily eliminated by depositing a thin coating such as hydroxyapatite or chitosan. Generally, the NT-Ag2O arrays generate good cytocompatibility, improve osteoblast spreading, and support osteoblast proliferation and differentiation.

The technique reported here can be readily extended to other types of nano-functional coatings. Magnetron sputtering is an industrial low-temperature deposition technique by which uniform and well adherent films can be fabricated on a myriad of materials such as ceramics, polymers, composites, and natural products [53]. Hence, TiAg coating deposition can be readily extended to biomaterials to fabricate the NT-Ag2O arrays. Besides Ti, other valve metals such as hafnium (Hf), zirconium (Zr), tantalum (Ta), and niobium (Nb) can also be deposited and anodized to produce NT arrays. Since the favorable bulk properties of the materials are preserved, nanotubular structures with different compositions and dimensions can be produced to cater to various cell types [7–10, 13–15]. Besides Ag, some other biologically functional elements such as copper, magnesium, zinc, manganese, strontium, and so on, can be loaded into the NTs by this technique to produce the desirable biological effects. The incorporated amounts and release rates can be readily controlled by varying the deposition and anodization parameters.

5. Conclusion

The NT-Ag2O arrays are produced by magnetron sputtering of TiAg coatings and anodization. The materials are composed of crystallized Ag2O NPs embedded in the amorphous TiO2 nanotube wall and controlled Ag+ release at a low dose is observed. The Ag contents in the NT-Ag2O arrays can be changed by varying the deposition parameters of the TiAg coatings. After immersion for up to 28 days, the NT-Ag2O arrays show antibacterial rates higher than 97%, indicating long lasting and effective antibacterial ability. The NT-Ag2O arrays possess good cytocompatibility, improved osteoblast spreading, and good osteoblast proliferation and differentiation. The technique reported here can be extended to other functional coatings and biomaterials.

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.biomaterials.2014.01.058.

References

Supplementary information for

The effects of titania nanotubes with embedded silver oxide nanoparticles on bacteria and osteoblasts

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Fig. S1 SEM images of TiAg coatings anodized in aqueous electrolyte containing 0.5wt% HF at 20 v (vs. Pt) for 30 min at room temperature: (a) TiAg1.27; (b) TiAg4.67.
Fig. S2 SEM images of the metal/oxide interface of the NT-Ag$_2$O arrays. Ag$_2$O NPs distribute at the border of the nanopits after the removal of NTs.
Fig. S3 (a) Cross-sectional image of a bunch of as-anodized NT-Ag$_2$O1.27 arrays; (b) High-magnification image of the bottom of the NTs which are enclosed by a square in (a); (c) High-magnification image of the top of the NTs which are enclosed by a square in (a).