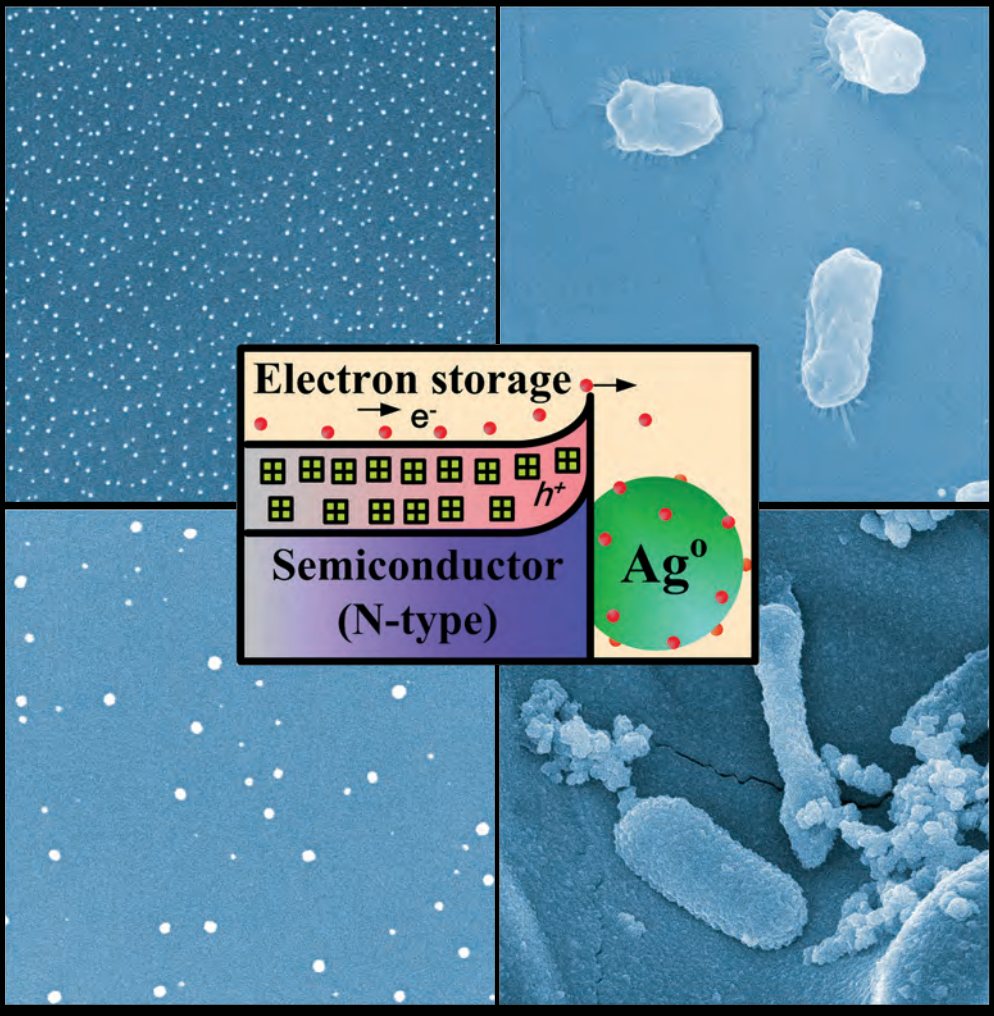
Silver Nanoparticles for Antibacterial Devices

Biocompatibility and Toxicity



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4

Titania Nanotubes as Silver Nanoparticle Carriers to Prevent Implant-Associated Infection

Ruiqiang Hang, Ang Gao, Long Bai and Paul K. Chu

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4.1 Introduction

Titanium (Ti) and its alloys are widely used in the orthopedic and dental fields because of their good mechanical properties, corrosion resistance and biocompatibility (Chu et al. 2002; Geetha et al. 2009; Liu et al. 2004). However, implant-associated infection remains one of the most prevalent and catastrophic postoperative complications (Zhao et al. 2009). Although the infection rate has been reduced to less than 5% on account of more thorough disinfection, strict aseptic surgical protocols and intraoperative systemic prophylactic treatment (Lee and Murphy 2013), the total number of people infected continues to increase because of growing medical demand for prosthetic replacements by the increasing aging population and prevalence of

joint degenerative and periodontal diseases (Kurtz et al. 2012). Exogenously virulent bacteria such as *Staphylococcus aureus* and *Escherichia coli* and endogenously low-virulent ones such as *coagulase-negative Staphylococci* (*CoNS*) and *Propionibacterium acnes* (*P. acnes*) may serve as pathogens. The infection is mainly ascribed to bacteria adhesion, colonisation and finally formation of biofilms on the implant surface. Accurate diagnosis of the infection is sophisticated and time consuming, and it is difficult to treat such infection because bacteria in the biofilms are highly resistant to antibiotics (Mah and O'Toole 2001). Usually, extraction of the contaminated implant is the only viable option to eliminate the infection.

Owing to the large resistance of biofilms to systemic antibiotic therapy, endowing the implant surface with the ability to resist bacteria adhesion, colonisation and formation of biofilms through active release of antibacterial agents is a promising strategy. To this end, various surface modification techniques such as ion implantation, physical vapor deposition, micro-arc oxidation and anodisation have been utilised (Liu et al. 2004). In addition, construction of size-adjustable titania (TiO_2) nanotubes (NTs) on Ti-based implants by anodisation has drawn tremendous attention since the first report in 1999 (Zwilling et al. 1999). TiO_2 NT can not only improve osteoblast functions *in vitro* and osseointegration ability *in vivo* compared with pure Ti but also serve as drug carriers to prevent implant-associated infection (Popat et al. 2007a; von Wilmowsky et al. 2009; Yu et al. 2010). Silver (Ag) possesses excellent broad-spectrum antibacterial properties, good long-term stability, low effective concentration and satisfactory cytocompatibility for a proper dose and thus is considered an ideal bactericide (Chernousova and Epple 2013). Compared to metallic Ag, Ag nanoparticles (NPs) possess more effective antibacterial activity because of their extremely large specific surface area, which provides more contact with aqueous solutions to release more Ag^+. Therefore, loading Ag NPs into TiO_2 NTs and controlling the Ag^+ release profiles constitute an effective strategy to prevent infection of Ti-based implants.

In this chapter, we briefly review the synthesis method and biological properties of TiO_2 NTs and focus on recent progress on TiO_2 NTs loaded with Ag NPs as anti-infective coatings. Finally, some critical concerns on this topic are highlighted.

4.2 Electrochemical Synthesis of TiO_2 NTs

In 1999, Zwilling and co-authors first reported the synthesis of TiO_2 NTs on Ti and Ti6Al4V alloy by electrochemical anodisation in an aqueous electrolyte containing chromic acid and hydrofluoric acid, and they observed that the presence of fluoride ions (F^-) was essential to the formation of nanotubular

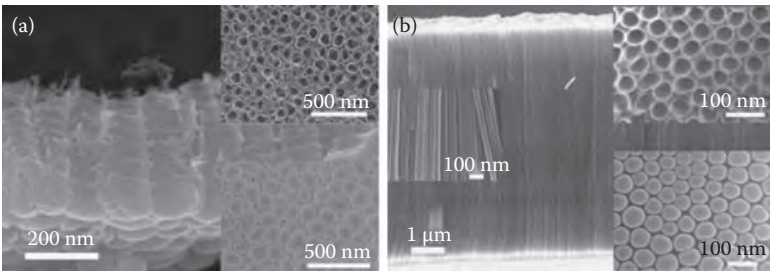

FIGURE 4.1
SEM images of TiO_2 NT layers prepared by different anodisation processes of Ti: (a) morphology obtained in the HF electrolytes; and (b) morphology obtained in the glycerol/F electrolytes. (Reprinted with permission from Macak, JM, H Tsuchiya, A Ghicov, K Yasuda, R Hahn, S Bauer, and P Schmuki. 2007. TiO_2 nanotubes: Self-organized electrochemical formation, properties and applications. *Current Opinion in Solid State and Materials Science* 11 (1):3–18.)

structure (Zwilling et al. 1999). Early follow-up works showed that by tailoring anodic parameters, TiO_2 NTs with different diameters (15 nm to 120 nm) and lengths (20 nm to 1000 nm) could be successfully fabricated (Bauer et al. 2006; Ghicov et al. 2005). However, the NTs synthesised in aqueous electrolytes possessed limited lengths and inhomogeneous sidewalls because of rapid field-assisted chemical dissolution and current fluctuations during anodisation (Roy et al. 2011). Later works showed that organic electrolytes with high viscosity such as ethylene glycol and glycerol could overcome these limitations by retarding dissolution of NTs and restraining local current fluctuations (Macak et al. 2005). By using various organic electrolytes and tailoring the electrochemical parameters, TiO_2 NTs with diameters ranging from 15 to 800 nm and lengths ranging from less than 1 to 1000 μm have been produced (Lee et al. 2014; Paulose et al. 2007; Roy et al. 2011) and the typical SEM images of TiO_2 NTs fabricated in aqueous and nonaqueous electrolytes are depicted in Figure 4.1. The growth mechanism of TiO_2 NTs in the F-containing electrolyte is mainly ascribed to the attack by F^- of newly formed TiO_2 under an electric field to form water-soluble $[TiF_6]^{2-}$. For more information on this topic, readers are referred to some recent reviews (Lee et al. 2014; Regonini et al. 2013; Roy et al. 2011).

4.3 Biological Properties of TiO_2 NTs

It is well established that surface characteristics, especially morphology, are crucial to the clinical success of biomedical implants and many studies have aimed at constructing various morphologies on biomaterials to improve the biological properties (Geetha et al. 2009; Liu et al. 2004, 2010). In the past

decade, the possibility of using TiO_2 NTs on implant coatings has been extensively explored. A significant finding is the strong dependence of cell functions on the NT diameter. Oh and co-authors reported that human mesenchymal stem cells with multiple differentiation potential maintained their undifferentiated phenotype on TiO_2 NTs with a small diameter (<30 nm), while NTs with a large diameter (>70 nm) could selectively induce them to differentiate into osteoblasts (Oh et al. 2009). Moreover, almost all of the osteoblast functions including cell proliferation, alkaline phosphatase (ALP) activity, secretion of type I collagen and extracellular matrix mineralisation were reported to be promoted on TiO_2 NTs with a large diameter (Brammer et al. 2009; Zhao et al. 2010). These enhanced *in vitro* osteoblast functions were further evidenced by some *in vivo* experiments (Bjursten et al. 2010; von Wilmowsky et al. 2009, 2012). Overall, these results clearly indicate that TiO_2 NTs are promising coatings on Ti-based implant materials.

Although TiO_2 NTs possess good osseointegration ability, they have poor antibacterial activity and so cannot combat implant-associated infection. However, the open hollow structure of NTs suggests that they may serve as reservoirs for anti-infective drugs. Popat and co-authors loaded gentamicin into NTs and good antibacterial effect was observed (Popat et al. 2007a); similar results were observed by loading antimicrobial peptides (Ma et al. 2012). Nevertheless, the poor long-term stability and fast release rate of these organic antibacterial agents cannot meet clinical requirements. In contrast, inorganic antibacterial agents such as silver (Ag), copper (Cu) and zinc (Zn) are relatively stable at low concentrations and loading them into NTs and controlling their release profiles may generate effective and long-term antibacterial activity. The following two sections will focus on TiO_2 NTs decorated with Ag NPs because of their merits mentioned in Section 4.1.

4.4 TiO_2 NTs Loaded with Silver NPs

4.4.1 Preparation Methods

Ag NPs can be incorporated into TiO_2 NTs by physical and chemical methods, and the typical loading techniques are compared in Table 4.1. The physical methods are mainly vacuum-based technology. Magnetron sputtering, an industrial technique to deposit structural and functional coatings on various substrates, has been utilised to introduce Ag NP into TiO_2 NTs (Roguska et al. 2012a,b). During the process, Ag atoms or their clusters that are sputtered from a silver target by bombardment of energetic Ar^+ are deposited onto the NTs to form Ag NPs via spontaneous nucleation and island growth. Because of the line-of-sight motion of sputtered atoms, the Ag NPs are located mainly on the top edges of the NTs (Figure 4.2a). Excessive supply of Ag atoms leads

TABLE 4.1

Loading Methods of Ag NPs into TiO_2 NTs

Loading Methods		Characterisations	References
Physical	Magnetron sputtering	Diameter varies from 2 to 50 nm; located mainly on the top edges of the NTs.	Roguska et al. 2012a
	Electron beam evaporation	Diameter varies from 5 to 20 nm; increased NT diameter leads to decreased aggregation of Ag NPs near the NT surface.	Lan et al. 2013
	Plasma immersion ion implantation (PIII)	~20 nm in diameter; the distribution of Ag NPs can be tailored by varying PIII voltage.	Mei et al. 2014
	Electrophoretic deposition (EPD)	The initial diameter is the same as that in the Ag NP colloids (~6 nm) but increases with EPD time (even larger than 100 nm).	Jiang et al. 2013
Chemical	Photocatalytical reduction	Diameter of 10 to 20 nm; distributed mainly near the top of the NT surface.	Zhao et al. 2011
	Chemical reduction	~20 nm in diameter; distributed along the entire NT length but the content decreases with depth.	Liang et al. 2011
	Electrodeposition	~10 nm in diameter.	Ma et al. 2011
	Thermal decomposition	~40 nm in diameter.	Chen et al. 2013

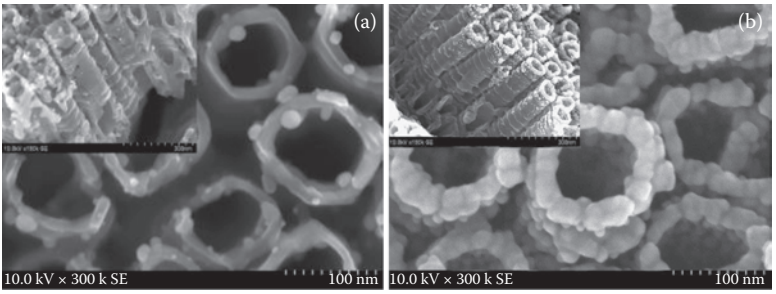

FIGURE 4.2
Typical SEM images of TiO_2 NT layers with different Ag contents: (a) 0.01 mg/cm^2; (b) 0.04 mg/cm^2. Insets show the cross-sectional views. (Reprinted with permission from Roguska, Agata, Anna Belcarz, Tomasz Piersiak, Marcin Pisarek, Grażyna Ginalska, and Małgorzata Lewandowska. 2012. Evaluation of the antibacterial activity of Ag-loaded TiO_2 nanotubes. *European Journal of Inorganic Chemistry* 2012 (32):5199–5206; and Roguska, Agata, Marcin Pisarek, Mariusz Andrzejczuk, Malgorzata Lewandowska, Krzysztof J Kurzydlowski, and Maria Janik-Czachor. 2012. Surface characterization of Ca-P/Ag/TiO_2 nanotube composite layers on Ti intended for biomedical applications. *Journal of Biomedical Materials Research Part A* 100 (8):1954–1962.)

to NP aggregation (Figure 4.2b), thus decreasing the specific surface area and compromising the antibacterial activity. Electron beam evaporation, an ideal technique to deposit materials with high melting point such as oxides, is also used to achieve this purpose (Lan et al. 2013; Uhm et al. 2013). Ag atoms in the target are transformed into the gaseous phase by electron beam bombardment, followed by transferring to substrate, nucleation and precipitation forming Ag NPs. After Ag deposition, the surface morphology of the TiO_2 NTs changes depending on the diameter (Figure 4.3). Another physical method used to introduce Ag NPs into TiO_2 NTs is plasma immersion ion implantation (PIII) in conjunction with filtered cathodic arc (Mei et al. 2014). An outstanding advantage of this technique compared to the previous two is its non–line-of-sight nature, rendering it particularly suitable for biomedical implants with a complex shape. Because energetic ion bombardment may destroy the nanotubular structure, a low implantation voltage (typically less than 1 kV) is desirable. Mei et al. (2014) found that Ag NPs aggregated near the top surface of NTs at a PIII voltage of 0.5 kV, while 1.0 kV led to penetration of the Ag NPs into the NTs. Besides these vacuum-based techniques, liquid-based ones such as electrophoretic deposition (EPD) have also been reported (Jiang et al. 2013). During EPD, negatively charged Ag NPs

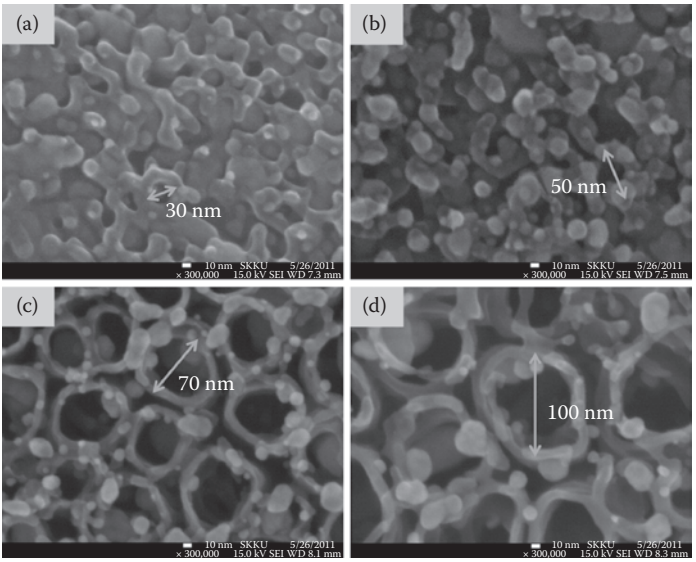

FIGURE 4.3
SEM images of the Ag NPs precipitated on the TiO_2 NTs with diameters of (a) 30 nm, (b) 50 nm, (c) 70 nm, and (d) 100 nm prepared by electron beam evaporation. (Reprinted with permission from Uhm, Soo-Hyuk, Doo-Hoon Song, Jae-Sung Kwon, Sang-Bae Lee, Jeon-Geon Han, Kwang-Mahn Kim, and Kyoung-Nam Kim. 2013. E-beam fabrication of antibacterial silver nanoparticles on diameter-controlled TiO_2 nanotubes for bio-implants. *Surface and Coatings Technology* 228:S360–S366.)

suspended in the colloidal solution are attracted to the anode and deposited onto TiO_2 NTs. Increasing the deposition time is beneficial to the dosage but leads to aggregation and growth of Ag NPs under the electric field.

Chemical methods used to load Ag NPs into TiO_2 NTs are mainly based on the reduction of Ag^+, and photocatalytic reduction is a widely used approach to achieve this purpose. Typically, TiO_2 NTs are first immersed in the $AgNO_3$ solution to adsorb $AgNO_3$ and then illuminated by UV light. During irradiation, Ag^+ is reduced to Ag^0 by the following reaction (Ohtani and Nishimoto 1993):

$$4Ag^+ + 2H_2O \rightarrow 4Ag + O_2 + 4H^+. \tag{4.1}$$

The loaded amount can be adjusted by varying the $AgNO_3$ concentration, soaking time, irradiation intensity and duration (Bian et al. 2013; Zhao et al. 2011). The same as vacuum-based methods, the photocatalytic reaction occurs near the top surface of the NTs because of the line-of-sight property of light. In contrast, liquid-based chemical reduction can produce uniformly distributed Ag NPs on TiO_2 NTs (Figure 4.4). The reaction using $NaBH_4$ as a reducing agent is expressed as follows (Wang et al. 2012):

$$4Ag^+ + BH_4^- + 3H_2O \rightarrow 4Ag + B(OH)_3 + 2H_2 + 3H^+. \tag{4.2}$$

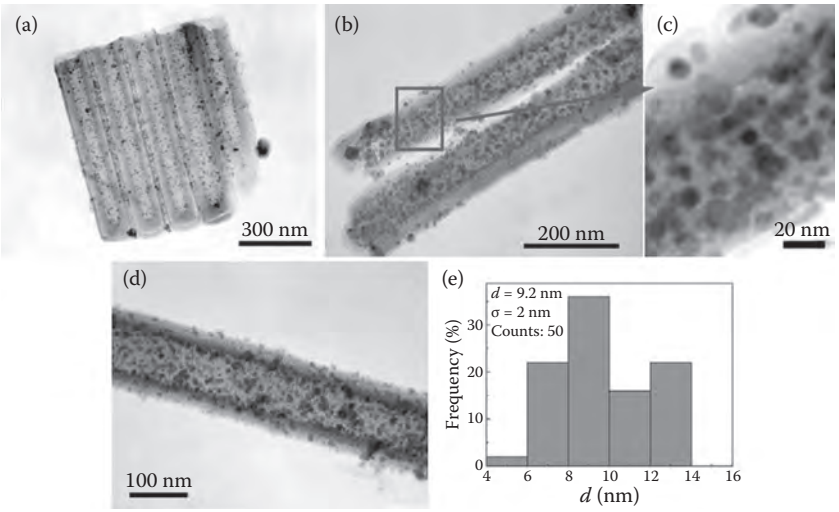

FIGURE 4.4
TEM images of Ag/TiO_2 NTs: (a) overall, (b) front side and (d) middle region; (c) HR-TEM image of Ag/TiO_2 NTs and (e) size distribution of the Ag nanoparticles. (Reprinted with permission from Wang, Qingyao, Xiuchun Yang, Dan Liu, and Jianfu Zhao. 2012. Fabrication, characterization and photocatalytic properties of Ag nanoparticles modified TiO_2 NTs. *Journal of Alloys and Compounds* 527:106–111.)

Another liquid-based technique is electrodeposition in which the TiO_2 NTs act as the cathode and Ag^+ is attracted and reduced to Ag^0 on the surface. The reduced Ag atoms evolve into Ag NPs by field-assistant nucleation and growth. A prominent advantage of this technique is the short deposition time (typically less than 60 s) (Ma et al. 2011; Zhang et al. 2011). Thermal decomposition has also been reported to introduce Ag NPs into TiO_2 NTs (Chen et al. 2013; He et al. 2010). Briefly, the samples are soaked in the $AgNO_3$ solution to adsorb $AgNO_3$ and then annealed under nitrogen or argon. The decomposition reaction is shown in the following (Lin et al. 2009):

$$2AgNO_3 \rightarrow 2Ag + 2NO_2 + O_2. \tag{4.3}$$

Since there is no line-of-sight limitation, Ag NPs can distribute evenly across the entire length of the NTs.

4.4.2 Antibacterial Activity of the Ag NP–Loaded NTs

Ag NPs are widely used as antibacterial agents in the biomedical and consumer products industry. However, the mechanism in which they interact with biological systems remains controversial. It has been shown that compared to their 'nano effects', release of Ag^+ plays a decisive role (Xiu et al. 2012). In aqueous solutions with dissolved oxygen, Ag^0 on the surface of Ag NPs can be oxidised to Ag_2O (Equation 4.4), leading to the release of Ag^+ under acidic conditions (Equation 4.5) (Liu and Hurt 2010):

$$4Ag + O_2 \rightarrow 2Ag_2O \tag{4.4}$$

$$2Ag_2O + 4H^+ \rightarrow 4Ag^+ + 2H_2O. \tag{4.5}$$

The released Ag^+ may induce toxicity by deactivating bacterial synthetase, disturbing cell membranes, interfering with nucleic acid replication and destroying intracellular respiratory and transport systems (Chernousova and Epple 2013; Lee and Murphy 2013). Accordingly, with regard to Ag NP–loaded TiO_2 NTs, the Ag^+ release profiles and antibacterial activity are primarily determined by the total surface area of the Ag NPs in contact with H_2O, regardless of the loading methods. As implant coatings, Ag NP–loaded TiO_2 NTs have two major advantages. One is that the loaded amount can be easily adjusted by varying the preparation parameters, for instance, the dimensions of TiO_2 NTs, deposition time in physical methods and $AgNO_3$ concentrations in chemical ones. The other is that the release profiles can meet clinical requirements. In general, Ag NP–loaded TiO_2 NTs release a large amount of Ag^+ during the early days and the rate decreases gradually

thereafter. The initial phase after implantation is more susceptible to infection because of the compromised host defense after surgery. Hence, the early potent antibacterial ability derived from a large amount of Ag^+ can kill planktonic bacteria to prevent postoperation infection. After this stage, mild antibacterial ability through sustained and low-dose release of Ag^+ is sufficient to inhibit bacterial adhesion and formation of biofilms on the implants. Typical results concerning this topic reported by Zhao et al. are summarised in Figure 4.5.

Although the powerful bactericidal capability of Ag NP–loaded TiO_2 NTs has been demonstrated, the longest experimental period only covers the early stage of infection (<2 months after implantation) and whether the strategy can combat delayed (2–24 months after implantation) and late (>24 months) infection is

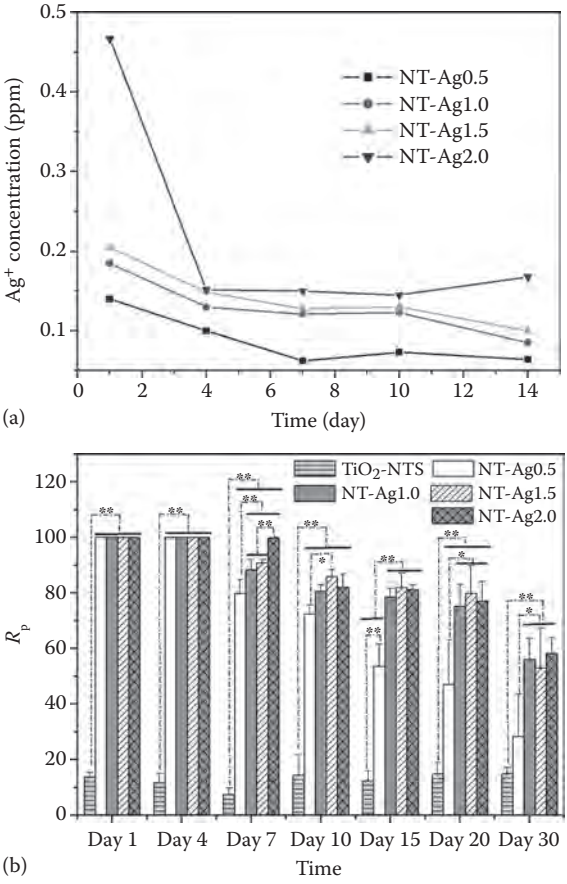

FIGURE 4.5
(a) Noncumulative Ag^+ release profiles from Ag NP–loaded TiO_2 NTs into PBS, (b) antibacterial rates against planktonic bacteria in the medium (R_p). (*Continued*)

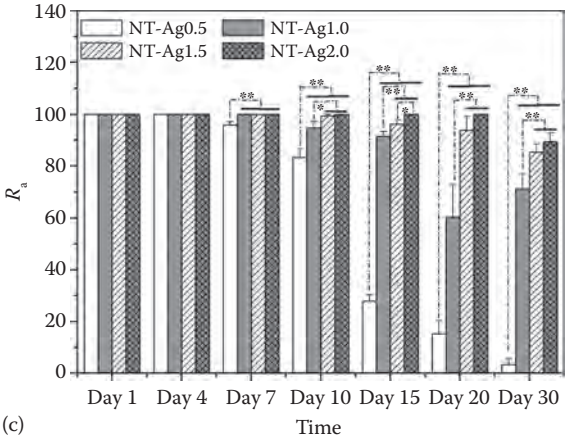

FIGURE 4.5 (CONTINUED)
(c) Antibacterial rates against adherent bacteria on the specimen (R_a). The antibacterial assay data are expressed as means ± standard deviations ($n = 3$). The one-way ANOVA followed by SNK post hoc test is utilised to determine the level of significance (*$p < 0.05$ and **$p < 0.01$). (Reprinted with permission from Zhao, Lingzhou, Hairong Wang, Kaifu Huo, Lingyun Cui, Wenrui Zhang, Hongwei Ni, Yumei Zhang, Zhifen Wu, and Paul K Chu. 2011. Antibacterial nano-structured titania coating incorporated with silver nanoparticles. *Biomaterials* 32 (24): 5706–5716.)

still unclear. Similar to other drugs, release of Ag^+ is too rapid in the early stage and too slow in the later stage. From the chemical viewpoint, in order to achieve sustained release of a moderate amount of Ag^+, controlling the rates of the chemical reactions (Equations 4.4 and 4.5) by regulating the supply of O_2 and H^+ may be effective. However, this strategy is difficult to implement *in vivo*. A proposed alternative is illustrated in Figure 4.6. The NTs doped with small Ag NPs release Ag^+ swiftly in the initial stage because of the large specific surface area. The consumption is relatively fast, and after a short period, the released Ag^+ concentration may be lower than the minimum inhibitory concentration (MIC). In contrast, for small + big Ag NP–loaded TiO_2 NTs with the same dose, the small ones contribute to initial fast release of Ag^+ to ensure that the implant survives the susceptible stage, whereas the big ones provide sustained release of Ag^+ above the MIC to achieve long-term antibacterial ability.

Alternatively, Ag NPs may detach from the NT walls and contribute to the antibacterial ability in several ways (Chernousova and Epple 2013; Rai et al. 2009). The first is that they may attach to sulfur-containing cell walls and membranes to disturb functions and properties such as permeability and respiration. In addition, they may penetrate into the bacteria binding to and inactivating phosphorus-containing DNA. Ag NPs lead to the formation of reactive oxygen species (ROS), which may induce oxidative damage of proteins and DNA. Nevertheless, detachment and direct release of Ag NPs from TiO_2 NTs may compromise the long-term antibacterial activity and should be avoided.

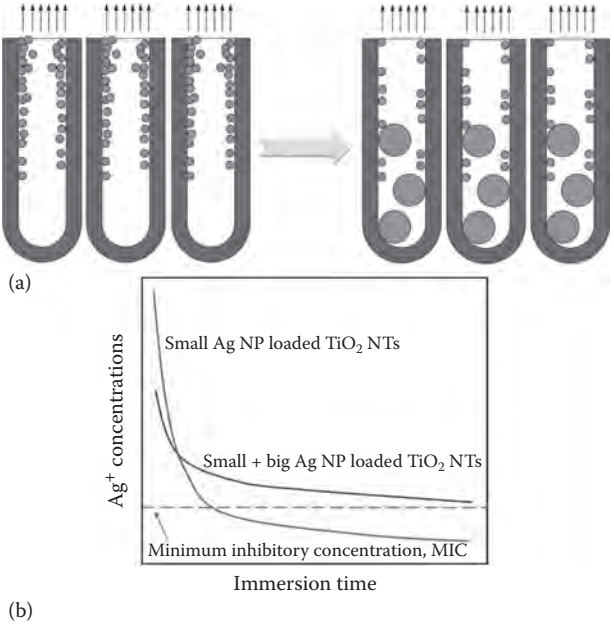

FIGURE 4.6
Schematic diagrams showing incorporation of small and big Ag NPs instead of only small ones into the TiO_2 NTs (a) to achieve long-term release of Ag^+ above MIC (b).

4.4.3 Biocompatibility of the Ag NP–Loaded NTs

The use of Ag as a bactericide may raise safety concerns and so the biocompatibility of Ag NP–loaded TiO_2 NTs should be fully understood before clinical use. Recent research has shown that the cytotoxicity of this structure is closely related to the amount of released Ag^+ (Zhao et al. 2011). Excessive release of Ag^+ leads to elevated lactate dehydrogenase activity as well as suppressed proliferation, intracellular total protein synthesis and ALP activity of osteoblasts, while insufficient supply compromises the antibacterial activity. Therefore, considering the safety window of Ag^+ concentrations to balance the antibacterial activity and biocompatibility is of importance. Much research has been devoted to the exploration of this window, and it has been shown that the MIC of Ag^+ is between 0.1 and 20 mg/L, and for mammalian cells, the toxic concentration is 1–10 mg/L (Chernousova and Epple 2013). Although a definite conclusion has not yet been drawn because of different experimental conditions and bacteria/cell types, the safe range of Ag^+ concentration for treatment of implant-associated infection is expected to be between 0.1 and 1 mg/L. It has been shown that by tailoring the NT dimensions and loading manners of Ag NPs, good antibacterial ability (Figure 4.7) and biocompatibility (Figure 4.8) can be simultaneously achieved (Lan et al. 2013). However, good biocompatibility is usually linked to a small

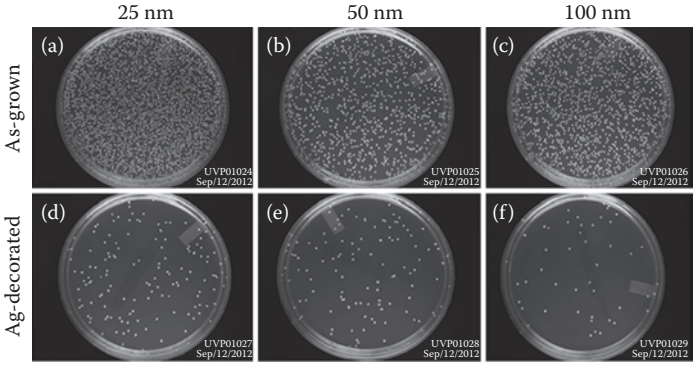

FIGURE 4.7
Photographs of bacteria cultures. Plates with *S. aureus* were grown in the presence of as-grown (a through c) and Ag-decorated (d through f) TiO_2 NTs with different diameters.

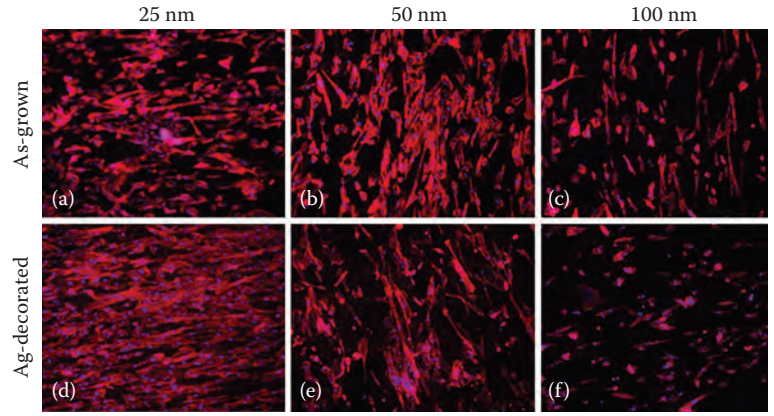

FIGURE 4.8
(See colour insert.) Fluorescence microscopy images of human fibroblasts attached to the as-grown (a through c) and Ag-decorated (d through f) TiO_2 NTs with different diameters. The red fluorescence indicates cytoskeletal actin and the blue fluorescence indicates cell nuclei. For both as-grown and Ag-decorated TiO_2 NTs, longer and better-defined actin cytoskeleton and a higher density of fibroblasts are observed from NTs with a smaller diameter.

concentration of Ag NPs, which may compromise the long-term antibacterial ability. Accordingly, TiO_2 NTs with a large concentration of Ag NPs that can continuously release Ag^+ within the safety window is highly desirable. To this end, several approaches can be adopted, for instance, increasing the ratio of length to diameter (Peng et al. 2009) and narrowing the openings of the NTs by depositing biocompatible species (Tsuchiya et al. 2006). Ideally, the NTs should release a moderate amount of Ag^+ only when bacteria invasion occurs in order to minimise the side effects of sustained Ag^+ release and guarantee long-term antibacterial activity. Since the occurrence

of infection leads to variations in the local microenvironment in the vicinity of the implant, coating the NTs with environmentally sensitive materials to control the release of Ag^+ may be promising (Qiu and Park 2012; Winter et al. 2007).

Besides Ag^+, Ag NPs may detach from the NT walls and be taken up readily by cells through receptor-mediated endocytosis and micropinocytosis. It is deemed that the size, shape, solubility, surface area and physicochemical and structural properties of the NPs influence their biokinetics, which may in turn determine the toxicity. AshaRani et al. (2008) have shown that exposure of mammalian cells to Ag NPs with a small size (6–20 nm in diameter) can result in mitochondrial dysfunction, DNA damage, chromosomal aberrations and cell cycle arrest. In contrast, as shown by some recent papers (Carlson et al. 2008; Kim et al. 2012; Park et al. 2011), large Ag NPs are more biocompatible than small ones. Therefore, TiO_2 NTs loaded with relatively big Ag NPs may reduce cytotoxicity. It is necessary to explore novel approaches to avoid detachment of Ag NPs from the NT walls to overcome the side effects.

4.5 TiO_2 NTs Embedded with Silver Oxide NPs

4.5.1 Preparation Method

As aforementioned, Ag NPs can be readily loaded into TiO_2 NTs by various techniques to improve the antibacterial capability. However, the Ag NPs introduced by these methods adsorb physically onto the NT walls and there are concerns about either the failure of delivering sustained antibacterial effects or serious cytotoxicity attributed to the detachment of loose Ag NPs in conjunction with burst release of Ag^+ (schematically shown in Figure 4.9a). It is thus necessary to develop new Ag doping strategies to achieve

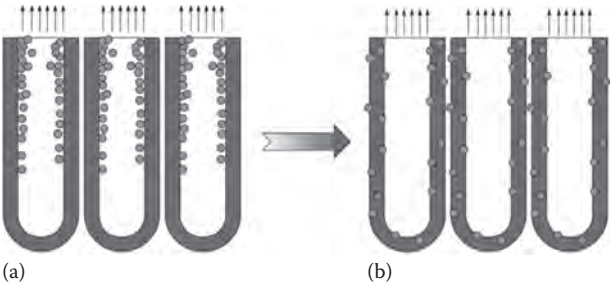

FIGURE 4.9
Schematic illustration of the difference between (a) physically adsorbed Ag NPs and (b) embedded Ag_2O NPs in the TiO_2 NTs.

the antibacterial capability without compromising the benefits in promoting osteoblast functions. Our recent work has confirmed the feasibility to dope Ag during the formation of TiO_2 NTs by anodising the TiAg composite coatings deposited by magnetron sputtering (Gao et al. 2014). Using the 'deposition–anodisation' technique, Ag can be *in situ* incorporated into the TiO_2 nanotubular walls and released in a sustained and controllable manner (schematically shown in Figure 4.9b).

The fabrication flow chart of silver oxide (Ag_2O) NP–embedded TiO_2 NTs (denoted as NT-Ag_2O) is schematically illustrated in Figure 4.10. Succinctly speaking, the TiAg coating is deposited on the Ti substrate by pulsed DC magnetron sputtering and then the coating is anodised in an F-containing electrolyte to fabricate the NTs. The SEM images of anodised samples in Figure 4.11 confirm the fabrication of self-organised homogeneous and

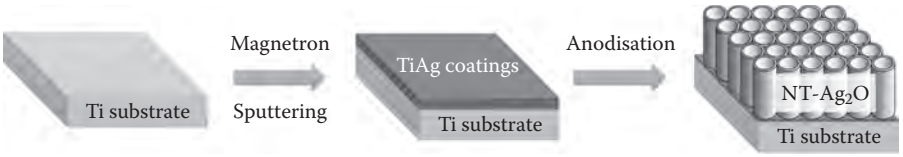

FIGURE 4.10
Schematic diagram of the preparation process of Ag_2O NP–embedded TiO_2 NTs.

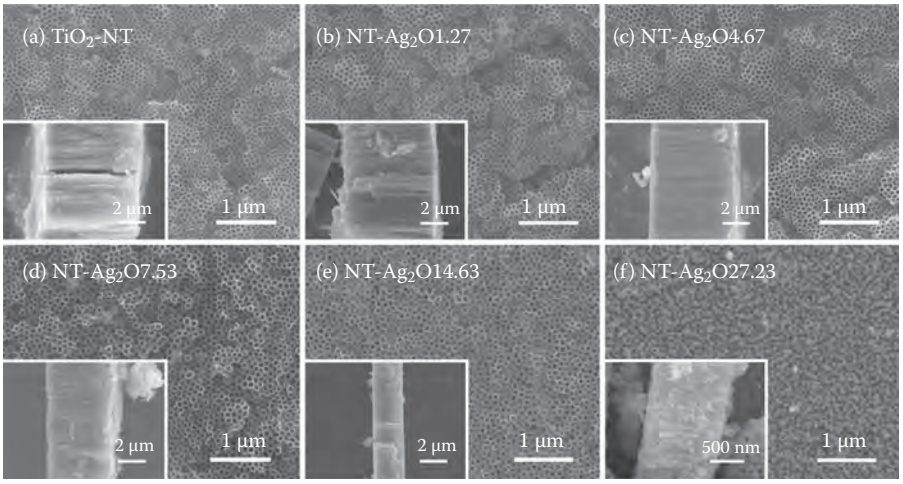

FIGURE 4.11
Surface and cross-sectional SEM images of the (a) anodised pure Ti and (b–f) TiAg coatings. The numbers behind NT-Ag_2O labels represent the atomic concentration of Ag in the corresponding TiAg coatings. The samples are anodised at 30 V and 30°C in an ethylene glycol solution containing 0.3 wt% NH_4F and 2.0 vol% H_2O for 4 h. (Reprinted with permission from Gao, Ang, Ruiqiang Hang, Xiaobo Huang, Lingzhou Zhao, Xiangyu Zhang, Lin Wang, Bin Tang, Shengli Ma, and Paul K Chu. 2014. The effects of titania nanotubes with embedded silver oxide nanoparticles on bacteria and osteoblasts. *Biomaterials* 35 (13):4223–4235.)

uniform NTs by anodising the TiAg coating. However, excessive Ag in the TiAg coating gives rise to a nonuniform nanoporous structure under the given anodic conditions (Figure 4.11f). To fabricate a well-defined nanotubular structure on the coating with a large Ag concentration, optimisation of the anodic parameters is necessary.

The TEM images in Figure 4.12 reveal that after anodisation, the crystallised NPs are uniformly distributed along the amorphous nanotubular wall. While some are totally enwrapped by the tubular wall, others are partially embedded. If the Ag concentration in the TiAg coating is large, the density of NPs after anodisation increases as expected. The high-resolution TEM

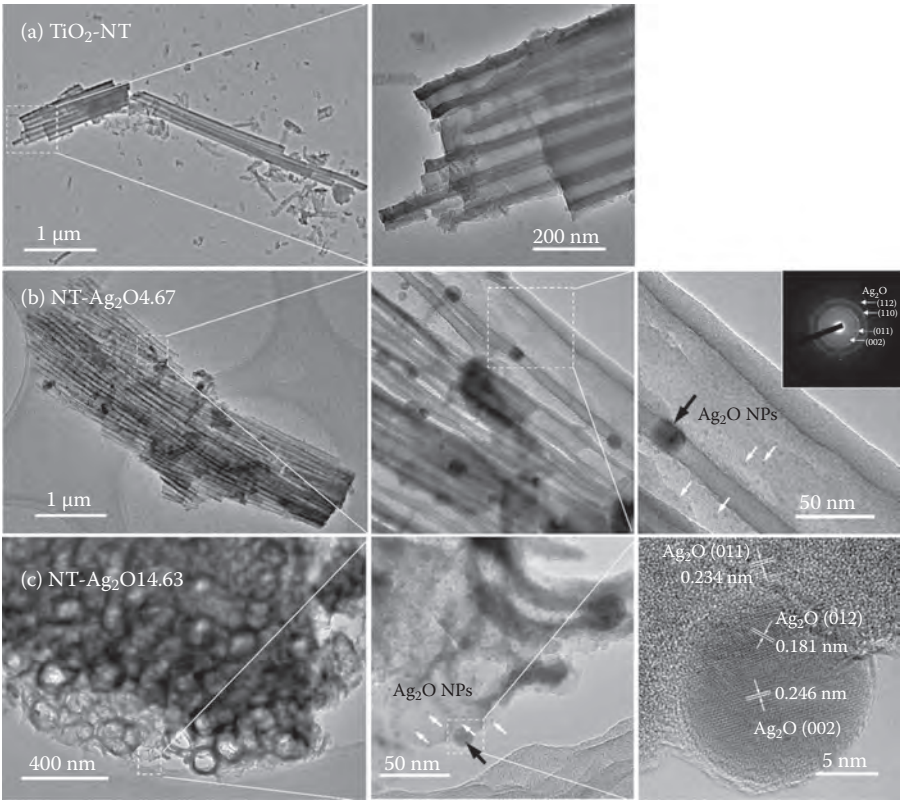

FIGURE 4.12
Typical TEM images of (a) as-anodised TiO_2 NTs, (b) NT-Ag_2O4.67, and (c) NT-Ag_2O14.63 together with the corresponding high magnification or HR-TEM images taken from the area enclosed by a square. The number after NT-Ag_2O represents the atomic concentration of Ag in the corresponding TiAg coatings. The inset in (b) is the SAED pattern obtained from the corresponding region. (Reprinted with permission from Gao, Ang, Ruiqiang Hang, Xiaobo Huang, Lingzhou Zhao, Xiangyu Zhang, Lin Wang, Bin Tang, Shengli Ma, and Paul K Chu. 2014. The effects of titania nanotubes with embedded silver oxide nanoparticles on bacteria and osteoblasts. *Biomaterials* 35 (13):4223–4235.)

images disclose lattice spacings of 1.81, 2.34 and 2.46 Å, corresponding to the (012), (011) and (002) crystallographic planes of hexagonal Ag_2O, respectively. The selected-area electron diffraction (SAED) shows four diffraction rings representative of the (002), (011), (110) and (112) crystallographic planes of Ag_2O as well. The results show that during anodisation of the TiAg coatings, Ag is oxidised to Ag_2O NPs and embedded into the TiO_2 NTs during growth.

As aforementioned, growth of TiO_2 NTs is the result of the competition between electrochemical anodisation and chemical dissolution of the oxide layer in the F-containing electrolyte. The similarity in the current density versus time plots obtained during anodisation of pure Ti and TiAg4.67 coating (Figure 4.13) indicates that the presence of Ag has little influence on the formation of the NTs. Four stages are illustrated in the insets in Figure 4.13. Addition of Ag accelerates dissolution of the NTs (manifested as a large current density), presumably because of the high solubility of Ag–F compounds in the electrolyte.

Magnetron sputtering is a well-developed and low-cost industrial technique for the deposition of uniform and adherent coatings (Kelly and Arnell 2000). Because of the low processing temperature, the Ag intrinsically doped TiO_2 NTs can be fabricated on a variety of substrate materials such as ceramics, polymers, metals and natural products. This technique can also be extended to introduce other elements such as silicon, strontium, zinc and copper to yield more diverse biofunctionalities. The amount of incorporated agents

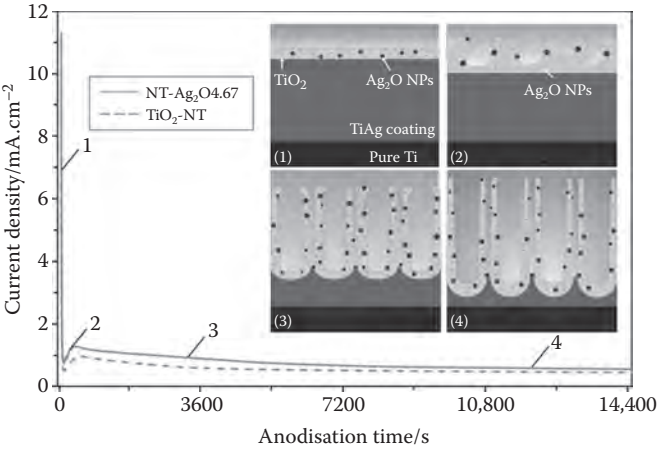

FIGURE 4.13
Typical current density–time curves during anodisation of the as-deposited pure Ti and TiAg4.67 film with the inset showing the formation mechanism of NT-Ag_2O. (Reprinted with permission from Gao, Ang, Ruiqiang Hang, Xiaobo Huang, Lingzhou Zhao, Xiangyu Zhang, Lin Wang, Bin Tang, Shengli Ma, and Paul K Chu. 2014. The effects of titania nanotubes with embedded silver oxide nanoparticles on bacteria and osteoblasts. *Biomaterials* 35 (13):4223–4235.)

and subsequent release patterns can be controlled by varying the dopant concentration in the coatings and morphology of the NTs. This 'deposition–anodisation' technique provides a versatile and effective approach to prepare biomedical coatings with different functions.

4.5.2 Antibacterial Activity of Ag_2O NP–Embedded NTs

The antibacterial activities against Gram-positive *S. aureus* and Gram-negative *E. coli* have been accessed for as long as 28 days under conditions that are harsher than those *in vivo*. Figure 4.14b and c indicates that during the first 14 days, NT-Ag_2O inactivates almost all the bacteria. Despite a slight decrease after repeating for 28 days, high antibacterial rates higher than 97% against both types of bacteria are maintained, thus indicating excellent long-term, broad-spectrum antibacterial activity of NT-Ag_2O.

Since Ag_2O NPs are embedded in the NTs, the contribution of detached particles to the bactericidal effect may be excluded. Therefore, the structure may exert its long-term antibacterial activity through sustained and controllable release of Ag^+. The noncumulative Ag^+ release profiles (Figure 4.14a) acquired from the NT-Ag_2O show initial rapid release followed by the sustained slower release believed to be beneficial to clinical applications (Zhao et al. 2011). During the 28 days, the amounts of released Ag^+ drop from approximately 40 parts per billion (ppb) to less than 10 ppb, but the antibacterial rate is maintained at greater than 97%, indicating that released Ag^+ may not be the only reason for the good antibacterial ability of NT-Ag_2O. It has been indicated that direct contact between bacteria and Ag NPs can induce structural changes and functional damage of the plasma membranes of bacteria (Chernousova and Epple 2013; Marambio-Jones and Hoek 2010; Morones et al. 2005). The SEM images in Figure 4.14d show the morphology of *S. aureus* cultured on NT-Ag_2O. They have a cracked and ruptured morphology and are surrounded by cell fragments indicating leakage of cytoplasmic contents. It may be concluded that the good long-term antibacterial activity of the NT-Ag_2O is ascribed to the synergistic effects of released Ag^+ and direct contact between the bacteria and Ag_2O NPs. Since the two antibacterial mechanisms complement each other, NT-Ag_2O may create a sustained antibacterial environment adjacent to the implant surface.

4.5.3 Biocompatibility of Ag_2O NP–Embedded NTs

Ag_2O NPs themselves and Ag^+ released together may exert toxicity to cells in a similar mechanism to bacteria. The loosely adhered NPs diffuse and are taken up by cells to impair cellular functions by generating ROS, by releasing Ag^+ and by other mechanisms (Greulich et al. 2011). However, the Ag_2O NPs are immobilised on the nanotubular wall in NT-Ag_2O, thus minimising the risk of cell uptake. Moreover, Ag^+ can show cytotoxicity in a dose-dependent manner (Chen and Schluesener 2008; Park et al. 2010). Release of

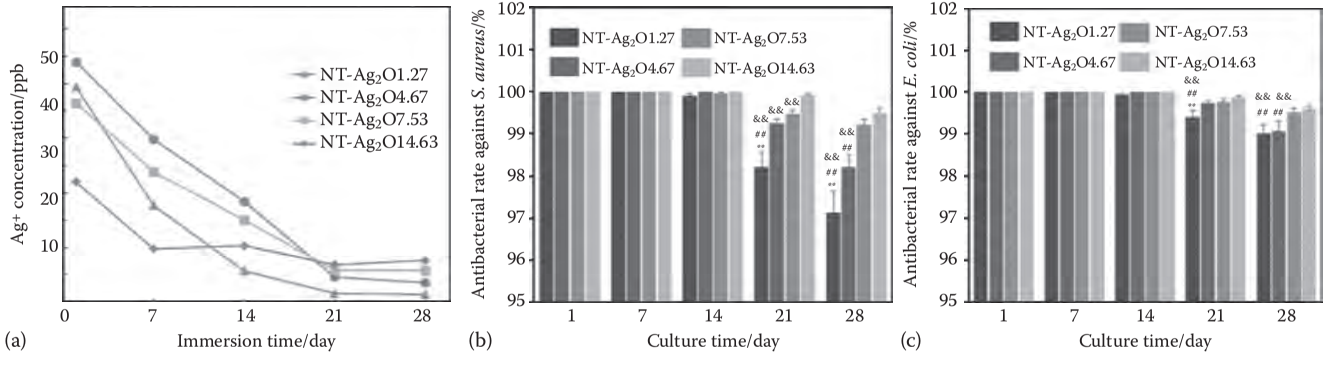

FIGURE 4.14

(a) Noncumulative Ag^+ release profiles of NT-Ag_2O; (b) antibacterial ability of NT-Ag_2O against *S. aureus*; (c) antibacterial ability of NT-Ag_2O against *E. coli*. (*Continued*)

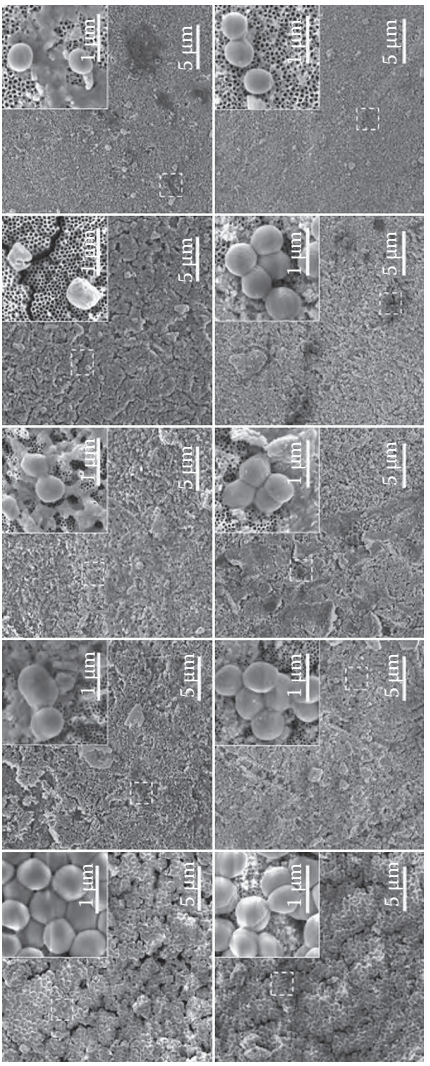

FIGURE 4.14 (CONTINUED)
(d) SEM images of *S. aureus* incubated on the TiO_2 NTAs and NT-Ag_2O, which have been immersed in PBS for 1 and 28 days. $^{**}p < 0.01$ compared to the NT-Ag_2O4.67, $^{\#\#}p < 0.01$ compared to the NT-Ag_2O7.53, $^{\&\&}p < 0.01$ compared to the NT-Ag_2O14.63. (Reprinted with permission from Gao, Ang, Ruiqiang Hang, Xiaobo Huang, Lingzhou Zhao, Xiangyu Zhang, Lin Wang, Bin Tang, Shengli Ma, and Paul K Chu. 2014. The effects of titania nanotubes with embedded silver oxide nanoparticles on bacteria and osteoblasts. *Biomaterials* 35 (13):4223–4235.)

Ag^+ from the Ag_2O NPs is related to the local acidic environment (Equation 4.5). However, as for NT-Ag_2O, because of the barrier effect of the surrounding TiO_2, noncumulative Ag^+ release from NT-Ag_2O is maximum of approximately 50 ppb, which contributes to the antibacterial activity but is lower than the generally accepted threshold of toxic concentrations for human cells (Chernousova and Epple 2013; Lewinski et al. 2008). This can explain the results obtained from cell proliferation and viability assay that no appreciable deleterious effects are detected on cells cultured on NT-Ag_2O.

TiO_2 NTs are known for their effectiveness in promoting osteoblast functions (Popat et al. 2007b; von Wilmowsky et al. 2009; Wang et al. 2011b). After incorporation with Ag_2O NPs, no obvious side effects are observed concerning the activity of ALP (Figure 4.15a), which is the early differentiation marker of osteoblasts. Although excessive Ag in the NTs show some hindering effects on collagen synthesis and extracellular matrix mineralisation

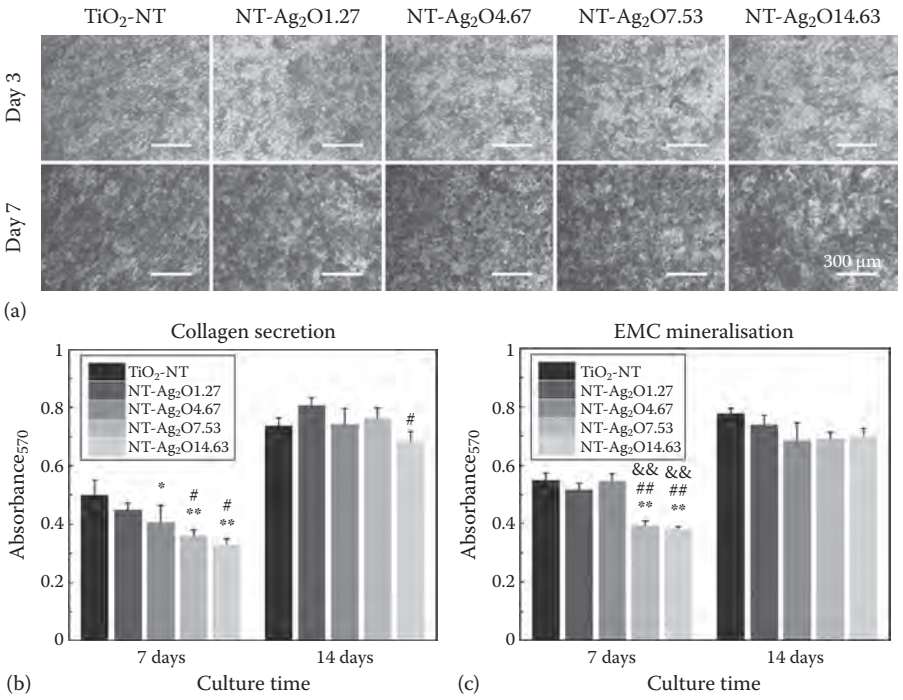

FIGURE 4.15

(a) Microscopic images of ALP product; (b) collagen secretion and (c) extracellular matrix mineralisation by MC3T3-E1 cells cultured on TiO_2 NTAs and NT-Ag_2O after osteogenic induction for 7 and 14 days. $^*p < 0.05$ and $^{**}p < 0.01$ compared to the TiO_2 NTAs, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared to the NT-Ag_2O1.27, $^{\&\&}p < 0.01$ compared to the NT-Ag_2O4.67. (Reprinted with permission from Gao, Ang, Ruiqiang Hang, Xiaobo Huang, Lingzhou Zhao, Xiangyu Zhang, Lin Wang, Bin Tang, Shengli Ma, and Paul K Chu. 2014. The effects of titania nanotubes with embedded silver oxide nanoparticles on bacteria and osteoblasts. *Biomaterials* 35 (13):4223–4235.)

compared to TiO_2 NTs during the early stage of culture, nearly no influence can be observed from prolonged culturing (Figure 4.15b and c) and the initial side effects may be easily eliminated by depositing a thin coating such as hydroxyapatite or chitosan. Generally, Ag_2O NP–embedded TiO_2 NTs have excellent long-term antibacterial capability without appreciable cytotoxicity and can accelerate osseointegration.

4.6 Critical Concerns

The biological properties of TiO_2 NTs have been extensively studied in the past several years. However, their mechanical and chemical stability is frequently overlooked although it is crucial to clinical applications. In particular, their poor adhesion on metallic substrates because of the presence of an F-rich layer (FRL) is a concern. Annealing has been reported to alter the interfacial chemistry and improve adhesion (Schmidt-Stein et al. 2010; Xiong et al. 2011). As previously reported, burying the FRL by additional anodisation in an F-free electrolyte may be promising (Yu et al. 2014; Zhang et al. 2012). Another concern is the poor mechanical strength that may manifest as brittle fracture of the tube apexes during storage, transportation or surgical operation. There is still no good solution to overcome the hurdle. Wang et al. (2011a) have reported that after the as-anodised amorphous TiO_2 NTs are immersed in water at room temperature, they spontaneously transform to anatase mesoporous nanowires. Since the human body is a water-rich environment, the use of amorphous TiO_2 NTs as implant coatings should be carefully considered. Nevertheless, another study has shown that the amorphous TiO_2 NTs can preserve the geometry even after long-term *in vivo* implantation (von Wilmowsky et al. 2009), suggesting that organic and inorganic species in the aqueous medium may affect the transformation.

4.7 Conclusion

TiO_2 NTs can serve as Ag NP carriers to prevent implant-associated infection via active release of Ag^+. By tailoring the NT dimensions, loading methods and incorporated amounts, the antibacterial activity and biocompatibility can be balanced. Compared to TiO_2 NTs loaded with Ag NPs, Ag_2O NP–embedded TiO_2 NTs may be a safer choice because of the controllable release of Ag^+ and small risk of NP detachment. Although the structure is very promising as implant coatings, their long-term effectiveness and mechanical and chemical stability need more in-depth studies before clinical use.

Acknowledgements

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