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Research Article

Antibacterial TiO_2 Coating Incorporating Silver Nanoparticles by Microarc Oxidation and Ion Implantation

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Infection associated with titanium implants remains the most common serious complication in hard tissue replacement surgery. Since such postoperative infections are usually difficult to cure, it is critical to find optimal strategies for preventing infections. In this study, TiO_2 coating incorporating silver (Ag) nanoparticles were fabricated on pure titanium by microarc oxidation and ion implantation. The antibacterial activity was evaluated by exposing the specimens to *Staphylococcus aureus* and comparing the reaction of the pathogens to Ti-MAO-Ag with Ti-MAO controls. Ti-MAO-Ag clearly inhibited bacterial colonization more than the control specimen. The coating's antibacterial ability was enhanced by increasing the dose of silver ion implantation, and Ti-MAO-Ag20.0 had the best antibacterial ability. In addition, cytocompatibility was assessed by culturing cell colonies on the specimens. The cells grew well on both specimens. These findings indicate that surface modification by means of this process combining MAO and silver ion implantation is useful in providing antibacterial activity and exhibits cytocompatibility with titanium implants.

1. Introduction

Titanium- (Ti-) based implants are widely used in the surgical domain as hard tissue replacements due to their relatively low elastic modulus, good fatigue strength, formability, corrosion resistance, and high biocompatibility [1]. However, the maintenance of bioinertness and the prevention of toxic metal ion release are becoming key challenges to the safety of implants in the human body. For this reason, numerous strategies have been developed to produce favorable surfaces on the implants which not only help to retain the excellent bulk properties of titanium during a long life cycle but also favor the prevention or delay of toxic metal ion release [2–6].

Nowadays, stricter antibacterial requirements of implants have been urgently demanded for clinical use. Bacterial infection associated with implants has become a growing threat to human health, as it may cause various complications such as swelling, tenderness, erythema, extensive soft tissue trauma, warmth, and diminished range of joint motion, leaving patients suffering from prolonged morbidity, extended rehabilitation, and repeated debridement and surgery [7–11]. Moreover, such infections are usually difficult to treat, which motivates people to develop various methods to prevent infections rather than treat them. However, in spite of the great efforts made to mitigate bacterial contamination such as thorough disinfection and strict aseptic surgical operation schemes, bacterial invasion and complications still occur in the nearby tissues after implant surgery [12, 13]. On the other hand, several biomaterial surface modification approaches have been attempted to improve the antibacterial ability of the implant and have demonstrated great progresses in reducing the incidence of implant-associated infections [12].

The infections associated with implants are characterized by bacterial colonization and biofilm formation on the implanted device as well as infection of the adjacent tissues. It is generally accepted that the most effective way to prevent biofilm buildup on implants is to prohibit the initial bacterial adhesion, as the biofilms are quite difficult to remove after formation. Therefore, postoperative infection rates may be greatly reduced through improving the antimicrobial properties of the implant surface by means of surface modifications. Antibacterial surfaces were originally produced by direct

impregnation with antibiotics, with the purpose of preventing the initial adhesion of bacteria onto the implant surface [7–10, 14–17]. However, although these antibiotic-loaded surfaces demonstrated superior curative effects, potential toxicity and increased bacterial drug resistance due to the slow-release doses have become new increased risks in surgery [8]. In addition to antibiotic-releasing coatings, research interest has also focused on developing covalently bonded drugs to realize long-lasting antibacterial ability. However, the susceptibility of bacteria to drugs in the vicinity of implants presents a problem, and drug resistance of bacteria isolated from implants has been reported [8, 18].

Recently, silver-containing coatings have attracted increasing attention due to the nontoxicity of the active Ag^+ to human cells and its antimicrobial activity [8, 19, 20]. Ag^+ is a strong bactericide with satisfactory stability and presents significant broad spectrum antimicrobial effects on both gram-positive and gram-negative bacteria [20]. The antibacterial effect can last for a long time and can be less prone to producing antibacterial resistance [20, 21]. Moreover, silver-containing coatings could inhibit bacterial attachment onto the implants [21]. *In vitro* studies demonstrate excellent biocompatibility of silver-containing coatings without genotoxicity or cytotoxicity [22]. *In vivo* studies, on the other hand, indicate that silver-containing coatings have no local or systemic side-effects [20–22].

The required Ag dose in the implants is typically low, which makes it possible to introduce Ag into biocompatible coatings. In fact, various well-established techniques such as plasma ion implantation have been proposed to produce silver-containing coatings [23]. By incorporating a sufficient amount of Ag to enhance the antibacterial ability of porous coatings, a surface that retains biocompatibility and relatively long-term antibacterial ability can be produced. In this study, the silver was introduced by an ion implantation method into TiO_2 coatings produced by microarc oxidation (MAO). MAO on the surface of titanium implants can provide a porous biofunctional TiO_2 coating with good adhesion to the substrate and high apatite-forming ability [6, 24–29]. The incorporation of Ag was expected to improve the antibacterial ability of the TiO_2 coatings. The microstructure, the antimicrobial properties, and the biocompatibility of the TiO_2 coatings in which Ag was implanted were investigated in detail.

2. Materials and Methods

2.1. Coating Specimen Preparation. Commercially available pure titanium alloys (TA2) were used as specimens. The titanium specimens were cut into plates with dimensions of $15 \times 10 \times 2$ mm. The surfaces of the plates were abraded with emery papers of 200, 400, 600, 800, and 1200 grit in turn and washed in an ultrasonic bath for 20 min with acetone, ethanol, and deionized water, respectively. The titanium plates were then dried in an oven at room temperature.

MAO was carried out on the Ti plates with an AC-type high power supply (PN-III). The plates served as the anode electrode and a stainless steel plate was used as the counter electrode. The electrolyte was 0.2 M calcium acetate monohydrate ($(CH_3COO)_2Ca \cdot H_2O$, CA) and 0.02 M β-glycerophosphoric acid disodium salt pentahydrate ($C_3H_7Na_2O_6P \cdot 5H_2O$, β-GP). After treatment at 350 V for 5 minutes, porous TiO_2 coatings formed on the plate surface. The thickness of the oxide coating ranged from 12 μm to 20 μm and the average diameter of the pore size was 5 to 10 μm.

2.2. Silver Ion Implantation. Silver ion implantation was performed using an ion implantation machine equipped with a metal vapor vacuum arc (MEVVA) ion source. The initial gas pressure in the ion implantation chamber was under 2×10^{-3} Pa. Silver was implanted into the TiO_2 coatings with an acceleration energy of 65 keV. The silver implantation doses were 1.0, 5.0, 10.0, and 20.0×10^{17} ions/cm^2, respectively. The corresponding samples were denoted as Ti-MAO-Ag1.0, Ti-MAO-Ag5.0, Ti-MAO-Ag10.0, and Ti-MAO-Ag20.0, respectively.

2.3. In Vitro Antimicrobial Properties. The antibacterial activities of the silver-containing coatings were characterized based on the American Society for Testing and Materials (ASTM) G21-1996 ("Standard Practice for Determining Resistance of Synthetic Polymeric Materials to Fungi"). Gram-positive *Staphylococcus aureus* (ATCC 6538) was used to evaluate the antibacterial ability of the silver-containing coatings since staphylococci mainly account for infections of both temporarily and permanently implanted orthopedic devices. All the specimens and petri dishes were sterilized by autoclave at 121°C for 1 h. Afterwards, bacteria solution with a concentration of 5.6×10^6 CFU/mL was dripped onto the sample surface. The specimens were covered by plastic film in a sterile dish and incubated at 37°C for 24 h. Each specimen and its covered film were washed with 5 mL of stroke-physiological saline solution. The wash eluate was collected and diluted to 1 : 50 with saline solution. Then 100 μL of the diluted solution was incubated on a standard culture plate containing LB broth (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl). After further incubation at 37°C for 24 h, the active bacteria were counted and the antibacterial rate was calculated using the following formula:

$$R = \frac{(B - A)}{B} \times 100\%, \quad (1)$$

where R is the antibacterial rate (%), B is the average number of viable bacteria on the corresponding MAO titanium specimen without Ag ion implantation which is consistent with the control group, and A is the average number of viable bacteria on the Ag-ion-implanted MAO specimen. The measurement was repeated three times for *S. aureus* culture.

2.4. Cell Culture. The 3T3 cell line (Chinese hamster fibroblasts) was used for cytotoxicity tests. The culture medium consisted of alpha-minimum essential medium (α-MEM) supplemented with 10% fetal calf serum (FBS), 100 U mL^{-1} of penicillin, and 100 μg mL^{-1} of streptomycin sulfate. Experiments were conducted in an incubator at 37°C with

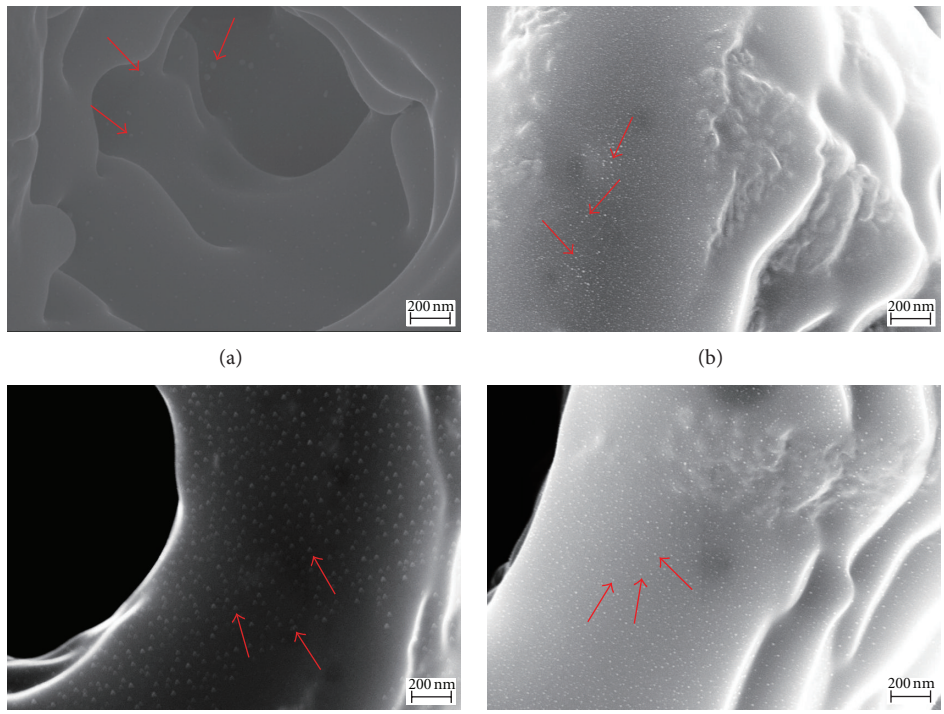


Figure 1: Surface morphologies for different specimens with various implanted Ag doses on MAO coating surfaces: (a) Ti-MAO-Ag1.0, (b) Ti-MAO-Ag5.0, (c) Ti-MAO-Ag10.0, and (d) Ti-MAO-Ag20.0.

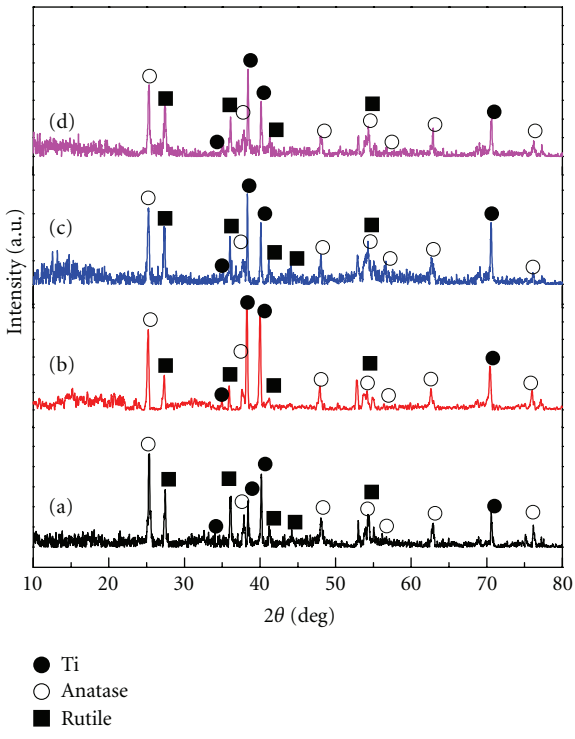


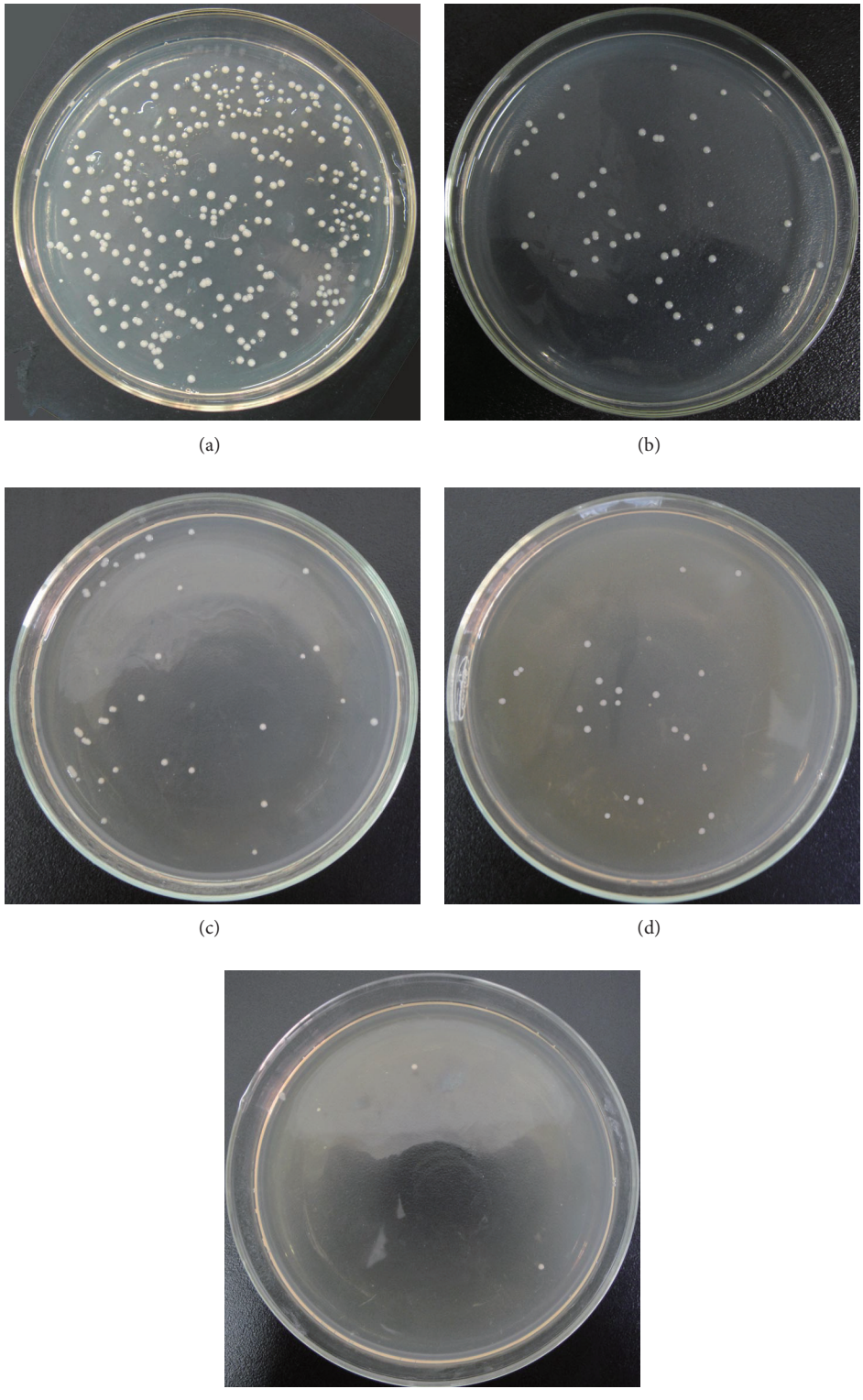
Figure 2: XRD patterns of samples with different implanted Ag doses: (a) Ti-MAO-Ag1.0, (b) Ti-MAO-Ag5.0, (c) Ti-MAO-Ag10.0, and (d) Ti-MAO-Ag20.0.

a humidified atmosphere of 95% air and 5% CO_2 for 24 h. The specimens were sterilized by heating at 180°C for 1 h. The cells were fixed with 5 mL of 10% formalin for 30 min, stained with 8 mL of 0.15% methylene blue for an additional 30 min, washed thoroughly with different concentration alcohol strictly, and dried [30].

2.5. Microstructure and Surface Analyses. The surface morphologies of the coatings were examined by scanning electron microscopy (SEM, Zeiss SUPRA 40, Germany). The crystalline structure was characterized by X-ray diffraction (XRD, D/Max 2400 V, Rigaku, Tokyo, Japan) using Cu K_α radiation in the 2θ range of 10–80° with an accelerating voltage of 36 kV and a current of 100 mA. The chemical composition of the surface layer was analyzed by energy dispersive spectrometry (EDS).

3. Results and Discussion

The SEM top-morphology images of the coatings incorporating different doses of silver are shown in Figure 1. The typical porous surfaces on MAO-treated pure titanium can be clearly seen, indicating that the implantation of Ag had little effect on the surface morphology of the TiO_2 coating formed. The implanted Ag presented in the form of nanoparticles distributed homogeneously on the surface and even inside the open pores. Inspection of the porous coating surfaces



(a) (b) (c) (d) (e)

Figure 3: Photos of culture plates after antibacterial tests against *Staphylococcus aureus*: (a) control specimen Ti-MAO, (b) Ti-MAO-Ag1.0, (c) Ti-MAO-Ag5.0, (d) Ti-MAO-Ag10.0, and (e) Ti-MAO-Ag20.0.

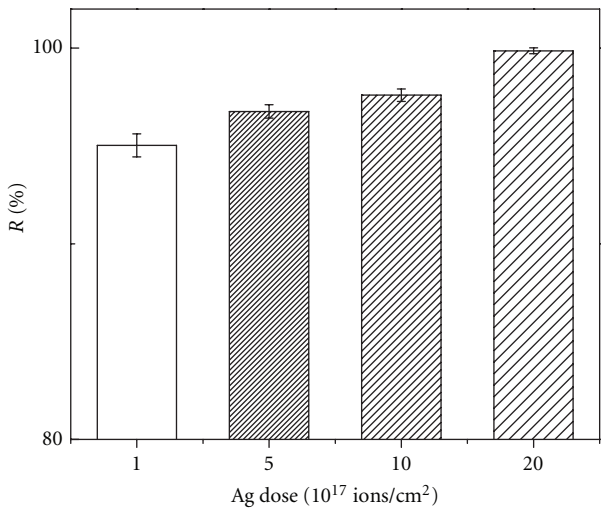


FIGURE 4: Antibacterial rate (R) evolution with the incorporated Ag doses.

with different implanted doses shows that the amount of Ag nanoparticles on the porous coating surfaces increased with increases in the dose implanted.

Figure 2 exhibits the XRD patterns of the specimens with different doses of implanted silver. Only the peaks related to the anatase and rutile phases and the phase from the Ti substrate can be indexed for all the specimens. The peaks of Ag or Ag alloys do not appear, confirming that the Ag was present as nanoparticles. The results are contrary to those reported by other research groups [19, 31], who found that the weak Ag diffraction peaks could be identified although the Ag particles were in the nanocrystalline form. The difference could be attributed to the amounts of silver incorporated and the methods of incorporation. The ion implantation technique demonstrated the unique advantage of providing a superfine nanoparticle in contrast to the other methods of incorporation.

EDS composition analysis indicated that the incorporated Ag content on the sample surface increases with increasing the ion implantation doses of Ag, as shown in Table 1, which is consistent with the SEM observations.

The antibacterial ability of MAO coatings in which Ag was implanted was investigated by growing *S. aureus* colonies on LB plates. The MAO coatings incorporating Ag present strong inhibition of *S. aureus* colony formation compared to the MAO coating without incorporated Ag, where significant amounts of colonies were found (see Figure 3(a)). In contrast, nearly no viable bacteria could be seen on Ti-MAO-Ag20.0 (Figure 3(e)). Moreover, the number of bacteria that could be seen decreased as the dose of Ag incorporated increased, suggesting that the antibacterial ability increased with the content of incorporated Ag. The detailed calculation of the antibacterial rate shown in Figure 4 demonstrates that the antibacterial rate increases with increases in the dose of Ag incorporated from 95.8% to nearly 100%. The results are in good accordance with a previous report [19], where TiO_2 nanotubes incorporating Ag demonstrated superior antibacterial ability by killing all the planktonic bacteria in the suspension during the first few days.

However, the mechanism of inhibitory action of silver ions on microorganisms remains controversial. It is proposed that DNA loses its replication ability and cellular proteins become inactivated by the reaction with Ag [32, 33]. Other research results showed that Ag^+ bound to functional groups of proteins, resulting in protein denaturation [32]. Concerning the mechanism of how the silver nanoparticles act as biocidal material against *S. aureus*, some reports have suggested that electrostatic attraction between negatively charged bacterial cells and positively charged nanoparticles is crucial for the activity of nanoparticles as bactericidal materials [34]. However, there are also contradictory reports revealing that, despite the negative charge on the silver nanoparticle surfaces, silver nanoparticles present good antibacterial activity by interacting directly with "building elements" of the bacterial membrane structure, resulting in structural change in the bacterial membrane, degradation, and finally cell death [32]. This mechanism has been verified in a series of studies [32, 35–37], which would explain the good antibacterial ability observed in this study.

The cytotoxicity of the specimens was evaluated by observing the morphology of the 3T3 cell colony formation. The formation of normal fibroblasts with a spindle-like shape was observed on the surfaces of all samples (Figure 5). The morphologies of the fibroblasts show little difference when comparing the samples with and without Ag implantation, indicating that the Ag-containing coatings demonstrated similar biocompatibility to the titanium treated directly by MAO. The latter has been reported to demonstrate superior biocompatibility and no cytotoxicity [38, 39].

Toxicity from silver has been observed in the form of argyria only when there are large open wounds and large amounts of silver ions have been used for dressing. Reports of silver allergy are scarce in the literature. On the contrary, most studies propose that silver nanoparticles are nontoxic. However, with respect of their small size and variable properties, it is suggested that they are hazardous to the environment of the human body [21, 40]. Hussain et al. [41] studied the toxicity of silver nanoparticles of different sizes in a rat liver cell line. They reported that abnormal size, cellular shrinkage, and irregular shape of the cells increased as the concentration of silver nanoparticles increased, suggesting that the cytotoxicity of silver nanoparticles to mitochondrial activity increased. In this study, the cells spread well and displayed a normal shape in the coatings incorporating silver, indicating that these implanted doses of Ag^+ in the coatings are nontoxic to the colony cells.

4. Conclusion

Ag was incorporated in MAO TiO_2 coatings on pure titanium by an ion implantation method. The ion implantation technique demonstrated the unique advantage of providing a superfine silver nanoparticle. With increases in the implanted doses, the Ag content in the coatings increased. The in vitro antimicrobial tests indicated that the

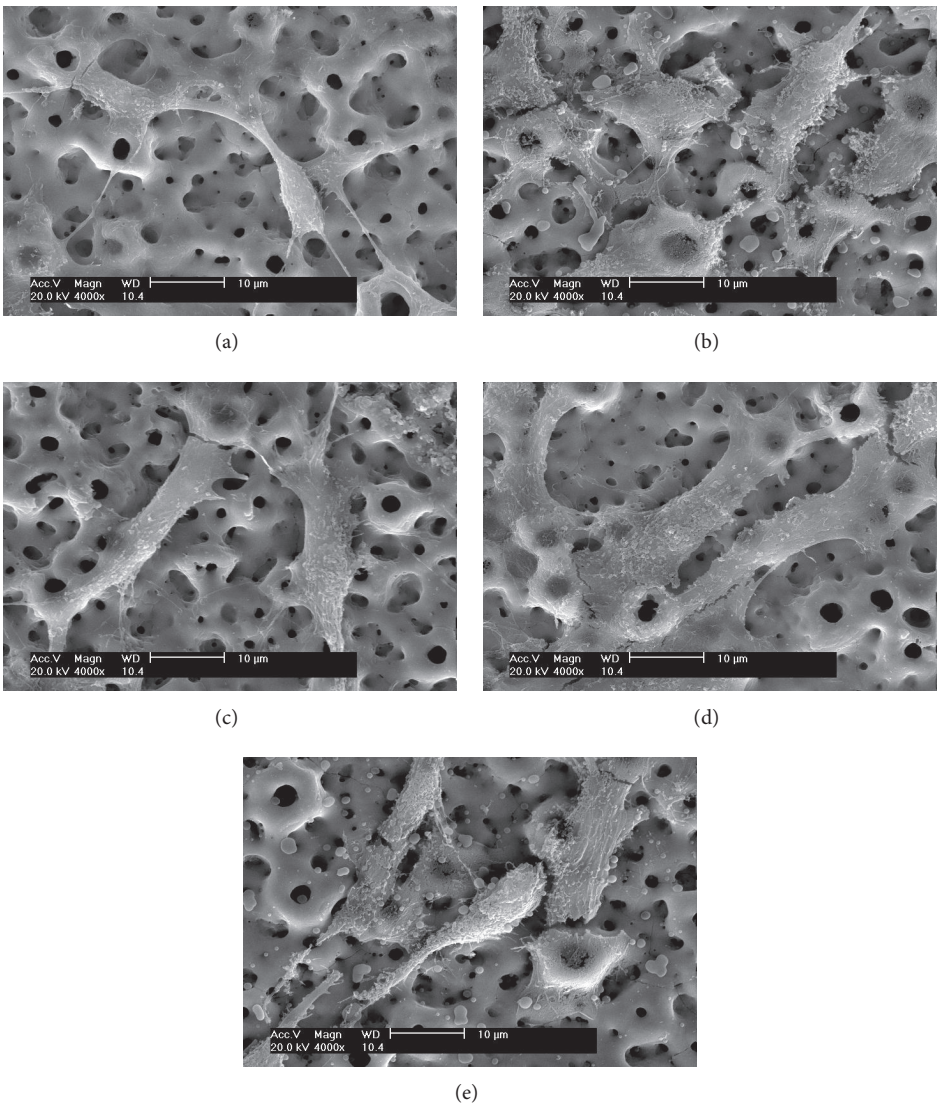


Figure 5: The surface morphology of the cells cultured on the surface of the Ag incorporated coatings by SEM: (a) the referred MAO-treated titanium, (b) Ti-MAO-Ag1.0, (c) Ti-MAO-Ag5.0, (d) Ti-MAO-Ag10.0, and (e) Ti-MAO-Ag20.0.

Table 1: Compositions on the Ag^+ implanted surfaces from EDS analysis.

No.	Specimen	Dose (10^{17} ions/cm^2)	Element (wt. %)				
			Ti	O	Ca	P	Ag
1	Ti-MAO-Ag1.0	1.0	43.8	42.7	9.5	3.5	0.5
2	Ti-MAO-Ag5.0	5.0	55.5	34.5	6.8	2.5	0.8
3	Ti-MAO-Ag10.0	10	49.6	42.7	4.5	2.0	1.2
4	Ti-MAO-Ag20.0	20	51.0	34.3	9.5	3.9	1.3

Ag-containing coatings possessed good antibacterial ability, which may have resulted from the interaction between the nanocrystalline silver and the bacterial membrane. The superior biocompatibility and noncytotoxicity of the silver-containing porous structural coatings were confirmed by the cell culture tests. The combination of antibacterial ability and biocompatibility as well as noncytotoxicity indicates that the ion implantation method could provide a promising strategy for the fabrication of a long-term antibacterial surface and thus an attractive biomaterial.

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