Zn/Ag micro-galvanic couples formed on titanium and osseointegration effects in the presence of S. aureus

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1. Introduction

Titanium implants and its alloys have many clinical applications because of their favorable properties [1,2] but implant-associated infections caused by the adhesion and colonization of bacteria [3,4] and inadequate osseointegration may lead to osteolysis, implant loosening, and eventual failure [5]. Therefore, new titanium implants with the desirable osseointegration and antibacterial ability are highly desirable [6]. Our previous studies demonstrate that zinc (Zn) ion implantation into titanium can significantly enhance the osteogenic activity in vitro and stimulate bone growth in vivo [7–9], whereas silver (Ag) ion-implanted titanium exhibits excellent antibacterial ability both in vitro and in vivo [3,10,11]. Moreover, Zn/Ag co-implantation enhances the initial adhesion, proliferation, differentiation, and gene expressions of rat bone mesenchymal stem cells (rBMSCs) on titanium while excellent antimicrobial properties are also observed both in vitro and in vivo [12]. The difference among the three Zn/Ag micro-galvanic couples can be ascribed to the contact between the Ag NPs and Zn film, which affects the corrosion process. Our results indicate that the biological behavior can be controlled by the corrosion process of the Zn/Ag micro-galvanic couples.

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involving two or more different metallic ions has attracted high attention because of the increased versatility with regard to the surface treatment of knee joints, dental implants, artificial hips, and so on [19,20]. For instance, Krupa et al. [19] have demonstrated that sequential Ca and P ion implantation improves the biocompatibility as well as corrosion resistance of titanium and Xie et al. [20] have found that titanium after water and hydrogen PIII exhibits better surface bioactivity and cytocompatibility. Different from single-metal ion implantation, sequential implantation and co-implantation offer excellent advantages in controlling the individual concentrations and depth distributions of multiple metal ions in the near-surface of titanium [21]. Therefore, it is possible to control the Zn/Ag micro-galvanic couples by adjusting the implantation sequence of Zn and Ag ions.

In this work, to investigate the effects of ion implantation sequence on the corrosion behavior of micro-galvanic couples, three newly designed micro-galvanic couples were fabricated using Zn and Ag simultaneously and sequentially implanted into titanium by PIII. The corrosion potentials and rates of the three micro-galvanic couples are measured. Although it has been reported that the biological actions can be controlled by the micro-galvanic effects [18], the osseointegration and antibacterial properties need to be investigated systematically both in vitro and in vivo. Therefore, a new animal model was applied in the present study to evaluate the osseointegration and antibacterial properties in vivo. The objective is to investigate the influence of the three different micro-galvanic couples on the osteogenic activity and antibacterial ability of titanium both in vitro and in vivo and to further elucidate the underlying mechanisms.

2. Methods

2.1. Zn and Ag PIII

Pure Ti plates with dimensions of 10 mm × 10 mm × 1 mm were carefully polished by abrasive paper to a mirror finish, ultrasonically cleaned with ethanol and ultrapure water. Before Zn and Ag PIII, the specimens were cleaned by a radio-frequency argon plasma for 15 min (sample bias is –550 V). Dual Zn and Ag PIII were conducted using pulsed zinc and silver cathodic arc sources. The implantation voltage, voltage pulse duration, and pulsing frequency used in PIII were 30 kV, 500 μs, and 5 Hz, respectively. Zn and Ag ions were implanted into titanium for 90 min each. Zn/Ag dual-ion co-implanted titanium, Zn prior to Ag implanted titanium, and Ag prior to Zn implanted titanium were represented as Zn/Ag-PIII, Zn-Ag-PIII, and Ag-Zn-PIII, respectively.

2.2. Surface characterization

Field-emission scanning electron microscopy (FE-SEM) was used to examine the surface topography of the specimens. The elemental depth profiles and their chemical states were detected by X-ray photoelectron spectroscopy (XPS). Besides, the dynamic potential polarization plots were measured in 0.9% NaCl solution (pH = 7) using a CHI760c electrochemical workstation (CHI Instruments).

2.3. Zn and Ag release

The titanium samples before and after Zn/Ag dual-ion implantation were immersed in 10 ml of 0.9% NaCl solution for 7, 14, 21, and 28 days at 37 °C. The solutions were refreshed every 7 days. The concentrations of released Zn and Ag were detected by inductively-coupled plasma atomic emission spectroscopy (ICP-AES).

2.4. In vitro osteogenic activity

2.4.1. Cell proliferation and viability

Cell proliferation and viability was determined by alamarBlue™ method. The rBMSCs were seeded on the modified surfaces at a density of 2.0 × 10^4 cells/ml. At each incubation period, the specimens (with the cells) were rinsed twice with PBS and 0.5 ml of fresh medium with 5% alamarBlue™ was added. After incubation for another 4 h, the absorbance values of 100 μl of medium at 570, and 600 nm were recorded. Calculation of cell proliferation and viability followed the instruction of alamarBlue™ assay.

2.4.2. ALP activity assay

The rBMSCs were seeded on the specimens (three replicates) at a cell density of 1.0 × 10^4 cells/ml (for 7 days) or 0.5 × 10^4 cell/ml (for 14 days). After 7 and 14 days’ incubation, a commercially available kit was employed for ALP staining. After culturing with p-nitrophenyl phosphate at 37 °C for 30 min, the ALP activity was determined by measuring the optical density at a wavelength of 405 nm for the quantitative assay. Finally, the ALP activity was normalized to total protein contents determined by a BCA protein assay.

2.4.3. Collagen secretion

Collagen secretion on the samples (three replicates) was evaluated by Sirius Red staining. The rBMSCs were seeded on the surfaces at a density of 1.0 × 10^4 cells/ml (for 7 days) or 0.5 × 10^4 cells/ml (for 14 days). On day 7 and 14, the cells were washed thrice with PBS, fixed in 4% PFA for 20 min, rinsed with PBS three times, and stained with 0.1% Sirius Red for 18 h. The cells were washed with 0.1 M acetic acid until no color appeared. In the quantitative analysis, the stain was dissolved in 0.5 ml of the solution (0.2 M NaOH: methanol = 1:1) and OD values were determined based on the absorbance at 492 nm.

2.4.4. Matrix mineralization

Extracellular matrix (ECM) mineralization was investigated by Alizarin Red. The rBMSCs (the cell density was the same as that used in collagen secretion) were cultured on the various specimens for 7 and 14 days, washed thrice with PBS, then fixed in 75% ethanol for 1 h, finally they were stained with 40 mM Alizarin Red for 10 min. To take the images, the cells were gently rinsed with ultrapure water until red color disappeared. The stain was dissolved in 10% cetylpyridinium chloride, and OD value at wavelength of 600 nm was measured.

2.4.5. Quantitative real-time PCR assay

Real-time PCR was employed to analysis the expressions of osteogenesis-related genes. The rBMSCs were seeded on the specimens at densities of 1.0 × 10^4 cell/well (for 7 days) or 0.5 × 10^4 cell/well (for 14 days). The total RNA was extracted using TRIzol reagent and the cDNA was reverse transcribed from 1.0 μg of the RNA. RT-PCR was conducted on the Roche LightCycler480 system using a SYBR Green I maternix. The relative expressions of osteogenesis-related genes (ALP, Col-I, Runx2, and OCN) were normalized to that of the reference gene F-actin, the primers for RT-PCR are listed in Table S1.

2.5. In vitro antibacterial tests

The in vitro antimicrobial properties of the specimens were determined by bacteria counting method and Live/Dead staining.
using both *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*). Before the experiments, the specimens were sterilized in 75% ethanol for 2 h. The bacterial suspension (concentration of $10^7$ cfu/ml) was dripped onto the surfaces to a density of 60 μl/cm². After 24 h incubation at 37 °C, the dissociated bacteria were collected, introduced to a standard agar culture plate and for another 24 h incubation. The bacterial colonies were counted and the antibacterial ratio was calculated using the formula below

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

in which A is average number of the bacteria on Ti (CFU/specimen), B is average number of the bacteria on testing specimens (CFU/specimen). For SEM examination, the bacteria were fixed with 2.5% glutaraldehyde solution, dehydrated in gradient ethanol solutions (30, 50, 75, 90, 95 and 100 v/v %), and finally dried in hexamethyl disilazane ethanol solution series.

For Live/Dead staining, the specimens were rinsed with PBS twice after culturing for 24 h, and stained with 0.5 ml of the Live/Dead BacLight reagent in dark for 15 min.

2.6. In vivo osteogenic and antibacterial activity

2.6.1. Surgical implantation

The experiments were approved by the Animal Care and Experiment Committee of Sixth People’s Hospital Affiliated with the School of Medicine of Shanghai Jiaotong University. Using aseptic techniques, 4 holes perpendicular to the centerline of the tibia were sequentially drilled using a Kirschner wire (2 mm diameter) of the left tibia of male rabbits (pitches of 1 cm). Four implants were randomly implanted into each drilled hole. For bacterial inoculation, 20 μl of the bacterial suspension with a density of $10^4$ cfu/ml *S. aureus* (ATCC 43300) was injected into the medullary cavity with a microsyringe. After bacterial inoculation, the fascia and skin were sutured. Following surgery, the rabbits were housed in the separate cages and allowed to eat and drink ad libitum.

2.6.2. Radiographic and micro-computed tomography evaluation

Radiography of days 3, 14, 28, and 42 was performed while the rabbits were under 3% pentobarbital (1 ml/kg) anesthesia. The X-ray results were assessed in line with the literature [22]. The operated tibia were dissected, harvested, and fixed in 10% buffered formalin. After fixation for 48 h, the specimens were scanned by high-resolution micro-computed tomography (micro-CT; Skyscan 1176, Skyscan, Belgium) at an image resolution of 18.0 μm (55kVp and 181 μA radiation source with 0.5 mm aluminum filter). The 2D and 3D high-resolution reconstructed images were obtained by the software provided by the manufacturer.

2.6.3. Histopathological analysis

After 6 weeks and microCT scanning, the samples were decalcified using 10% EDTA solution for 28 days, followed by washing with running tap water for about 4 h, and then transferred to a 75% ethanol solution and embedded in paraffin. 5 μm thick shaft sections were collected. Masson’s Trichrome staining, hematoxylin-eosin (HE) staining, and Giemsa staining were used to assess the morphology and bacterial contamination [23].

2.7. Statistical analysis

The data were expressed as means ± standard deviations. The statistical analysis was performed using the two-way analysis using a GraphPad Prism statistical software package, and the p values <0.05 were considered to be statistically significant.

3. Results

3.1. Characterization of the Zn/Ag dual-ion implanted titanium

The surface views of the specimens are shown in Fig. 1. The Ti

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*Fig. 1. Surface views: (a) Pure Ti, (b) Zn/Ag-PIII, (c) Zn-Ag-PIII, and (d) Ag-Zn-PIII.*
surface shows a flat and smooth topography. However, a large number of Ag nanoparticles (Ag NPs) with a wide size distribution can be observed from Zn/Ag-PIII (Fig. 1b). It was also evident that the clarity of the Ag NPs is not so good and most of them appear to be wrapped by a Zn film. This may be ascribed to simultaneous nucleation of the implanted Zn and Ag and the results are in agreement with our previous work [12]. Larger Ag NPs can be found from Zn-Ag-PIII (Fig. 1c) which looks clearer on the surface of the Zn film produced by the previously implanted Zn ions. Ag NPs can hardly be detected from Ag-Zn-PIII (red arrows in Fig. 1d) possibly because some have been sputtered from the surface and some are covered by the Zn film formed afterwards. The difference in the embedding of Ag NPs in the various specimens arises from the change in the implantation sequence.

The depth profile of Zn (Fig. 2a) resembles a Gaussian distribution from 10 to 140 nm but significantly more Zn can be observed from the surface (0–10 nm) in the following order of Ag-Zn-PIII > Zn/Ag-PIII > Zn-Ag-PIII. The depth profiles of Ag (Fig. 2b) also resemble Gaussian distributions (from 20 to 140 nm) and the surface contents of Ag arising from deposition follow the order of Zn-Ag-PIII > Zn/Ag-PIII > Ag-Zn-PIII, which is opposite to that of Zn. As mentioned earlier, few Ag NPs are observed from Ag-Zn-PIII. Considering that the surface Ag content in Ag-Zn-PIII (1.57%) is almost half of that in Zn/Ag-PIII which is 2.89%, it can be concluded that some of the Ag NPs are covered by the Zn film formed later despite the sputtered Ag NPs. The high-resolution spectra of Zn 2p and Ag 3d detected from the various surfaces (Fig. S1) indicate that the implanted Zn exist as ZnO (1021.9 eV) in the near surface and metallic Zn (1021.0 eV) underneath [24,25], while the Ag NPs have the metallic state in lieu of the oxidized one [26], consistent with our previous study [12].

To investigate the corrosion resistance of the Zn/Ag dual-ion implanted titanium and corrosion rate of the micro-galvanic couples, potentiodynamic polarization is conducted in 0.9% NaCl solution. The tafel plots of the specimens are presented in Fig. 3a. The corrosion potentials of all the specimens shift positively as opposed to pure Ti, especially Zn/Ag-PIII and Zn-Ag-PIII, in agreement with our previous study [12]. The corresponding corrosion currents of Ti, Zn/Ag-PIII, Zn-Ag-PIII, and Ag-Zn-PIII are 8.98 × 10⁻², 6.71 × 10⁻², 4.99 × 10⁻², and 1.81 × 10⁻², respectively. The larger corrosion potential and smaller corrosion current suggest better corrosion resistance on the sequentially implanted titanium [27].

To elucidate the relationship between the release of Zn and Ag from the Zn/Ag dual-ion implanted titanium and the micro-galvanic couples, the amounts of released Zn and Ag are measured by ICP-AES. The concentration of Zn²⁺ released from Zn/Ag-PIII is larger than that from Zn-Ag-PIII (Fig. 3b). However, the Zn²⁺ concentration of Ag-Zn-PIII is significantly larger than those of Zn/Ag-PIII and Zn-Ag-PIII possibly due to the bigger Zn surface content (Fig. 2a). The concentration of released Ag is quite small (Fig. 3c) and not more than 0.0695 µg/ml after soaking in 0.9% NaCl solution for 28 days. The positively-shifted corrosion potential and difference in the corrosion currents as well as Zn and Ag released from the Zn/Ag dual-ion implanted titanium likely stems from the difference in the corrosion rates of the micro-galvanic couples and this will be discussed in more details in the discussion section.

3.2. In vitro osteogenic activity

Fig. 4a shows the proliferation of rBMSCs cultured on the surface for various times. There is no obvious difference among the four groups at the first day. And cell proliferation on Zn/Ag-PIII and Ag-Zn-PIII is more substantial than that on pure Ti and Zn-Ag-PIII at day 4. The cells proliferate better on the implanted samples compared to Ti at day 7, suggesting that Zn/Ag dual-ion implantation spurs proliferation of rBMSCs.

The ALP activity of rBMSCs is presented in Fig. 4b. The ALP expressions on the Zn/Ag dual-ion implanted titanium are notably
improved at day 7 and 14, especially on Zn/Ag-PIII specimen. The results are further confirmed by the ALP staining assay, as shown in Fig. S2, indicating the enhancement of Zn/Ag dual-ion implantation on rBMSCs differentiation.

Collagen secretion from rBMSCs measured by Sirius Red staining is displayed in Fig. 4c. More collagen is secreted at day 7, especially Zn/Ag-PIII and again, the trend becomes more significant at day 14, further confirming the results by staining analysis (Fig. S3). ECM mineralization assayed by Alizarin Red staining is presented in Fig. 4d. Matrix mineralization on the Zn/Ag dual-ion implanted titanium was up-regulated as opposed to Ti at day 7, especially Zn/Ag-PIII. At day 14, matrix mineralization on Zn/Ag-PIII is still higher than that on Ti, which was consistence with the staining results (Fig. S4).

The osteogenesis-related genes expressions on the specimens are displayed in Fig. 4e, f, g, and h. Although the expressions of ALP on the Zn/Ag dual-ion implanted titanium are only slightly up-regulated at day 7, the expressions at day 14 is more significant compared to Ti. As an early marker of osteoblastic differentiation, Col-I is statistically up-regulated on the modified specimens at day 7, especially Zn/Ag-PIII and Ag-Zn-PIII. The expressions at day 14 are still higher than that on Ti. The Runx2 (a key bone-specific transcription factor) expression shows statistically difference on Zn/Ag-PIII at day 7 compared to the other three groups, whereas those on the Zn/Ag dual-ion implanted titanium are all up-regulated at day 14 compared to Ti. The up-regulated expression of OCN (a later marker of osteoblastic differentiation) indicates that osteoblastic differentiation occurs in the later stage on the modified specimens.

3.3. In vitro antibacterial tests

Biomaterials often face a wide variety of Gram positive and Gram negative bacteria in the human body [28]. In the present work, E. coli and S. aureus are utilized to evaluate the antimicrobial properties by employing the bacteria counting method and Live/Dead staining. It can be easily seen from the bacteria counting results (Fig. S5), the reduction rate of E. coli on Zn/Ag-PIII is

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Fig. 4. Cell proliferation (a), Quantitative ALP activity (b), Collagen secretion (c), ECM mineralization (d) of rBMSCs cultured on the various surfaces, and osteogenesis-related gene: (e) ALP, (f) Col-I, (g) Runx2 and (h) OCN expressions by rBMSCs on various surfaces; *P < 0.05, **P < 0.01, ***P < 0.001.
approximately 98%, whereas these on Zn-Ag-PIII and Ag-Zn-PIII are 87% and 52%, respectively. The larger reduction rate on Zn/Ag-PIII and Zn-Ag-PIII is likely due to the embedded Ag NPs which are believed to disrupt the bacteria membrane through short-range interactions rather than release of Ag ions as reported earlier [10,11,18]. The reduction rates of *S. aureus* on Zn/Ag-PIII, Zn-Ag-PIII, and Ag-Zn-PIII are approximately 99%, 98%, and 68%, respectively, presenting a similar tendency.

Fig. 5a shows the fluorescent images of *E. coli* and *S. aureus* on the various surfaces. Red spots can hardly be detected on Ti whereas there are many green spots, indicating the existence of a large number of live *E. coli* cells. However, little live bacteria and a few dead bacteria can be observed from the Zn/Ag dual-ion implanted titanium, suggesting that the implanted samples, especially Zn/Ag-PIII and Zn-Ag-PIII, are more resistant against *E. coli*. Similar results are observed from *S. aureus*.

The morphology of *E. coli* and *S. aureus* are displayed in Fig. 5b. The *E. coli* cells on Ti have a rod shape, while complete lysis and cytosolic content leakage are observed from Zn/Ag-PIII and Zn-Ag-PIII, very few intact cells are detected from Ag-Zn-PIII. This is in agreement with the Live/Dead staining (Fig. 5a) and bacteria counting results (Fig. S5). *S. aureus* on Ti have the normal spherical shape without apparent leakage (Fig. 5b), but complete lysis is observed on Zn/Ag-PIII and Zn-Ag-PIII and it occurs occasionally on Ag-Zn-PIII.

### 3.4. In vivo osseointegration and antibacterial ability

#### 3.4.1. X-ray and micro-CT analysis

Implant-related infection and new bone formation after surgery are evaluated by X-ray and micro-CT. Fig. 6a shows the X-ray radiographical images of the implants at prescribed time points. No evident signs of osseous destruction are observed within 3 days. However, small-density areas are evident in the tibia with the Ti implant after 2 weeks’ implantation. The presence of cortical bone thinning and disruption as well as osteolysis indicates bacterial infection and inflammation and the observation is in accordance with septic osteomyelitis [22,29]. The small-density areas, which often lead to osteolysis, become much more obvious after 4 weeks’ implantation. After 6 weeks, significant osseous destruction and soft tissue swelling can be detected. It is also evident that Ti implant loses contact with the host bone, indicating that bacterial infection loosens the implant and contributes to bone resorption in the cortical bone region around the implant. In contrast, osseous destruction is not observed by X-ray from the tibias in the Zn/Ag dual-ion implanted titanium groups within 6 weeks, despite the presence of sparse signs of tissue swelling in Ag-Zn-PIII. The results demonstrate that Zn/Ag dual-ion implantation produces good antibacterial effects in vivo.

Micro-CT scans are obtained from the tibia 6 weeks after surgery and the results are presented in Fig. 6b. The formation of abscess, osteolysis, and resorption of cortical bone around the Ti implant are consistent with the radiographic results in Fig. 6a. Moreover, necrotic bone sequestra (red arrow) can be found from the Ti implant indicating bacterial infection and inflammation. Sequestra is a complication of osteomyelitis and present when bacterial infection occurs around the implant due to the scarce vascularity of bones [30,31]. On the contrary, no sign of inflammation can be observed from Zn/Ag-PIII and Zn-Ag-PIII and there is also evidence of new bone formation around the implant near the cortical bone, indicating that these two groups exhibit excellent osseointegration at the presence of *S. aureus* in vivo. New bone formation is also observed from Ag-Zn-PIII, but the presence of osteolysis suggests that Ag-Zn-PIII only has partial

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**Fig. 5.** Fluorescent images of live and dead bacteria cultured on the various surfaces (a) and SEM morphology (b) of *E. coli* and *S. aureus* seeded on various surfaces.
antibacterial ability. The trend is consistent with the antibacterial tests in vitro.

3.4.2. Histological evaluation

To evaluate bacterial infection and osseointegration on the rabbit tibia, Masson’s trichrome staining, HE, and Giemsa staining are employed. The histological slice after Masson’s trichrome staining shows the typical signs of bone infection (Fig. S6) as illustrated by the formation of abscess and destruction of cortical bone around the Ti implant [32]. It is consistent with the radiographic and micro-CT results in Fig. 6. Bone resorption is accompanied by a large number of inflammatory cells in the medullary cavity. There is no sign of bacterial infection on Zn/Ag-PIII and Zn-Ag-PIII and evidence of new bone formation on the surface in the medullary cavity indicative of osseointegration and antibacterial ability in vivo. Relatively slight abscess, minor bone destruction, and less new bone formation are detected from Ag-Zn-PIII and the results are confirmed by HE and Giemsa staining.

The high-magnification histological images are displayed in Fig. 7. Tissues infiltrated by inflammatory cells can easily be observed from Ti after Masson’s trichrome staining and multinucleated osteoclasts (yellow arrows) are detected near the trabecular bone. As the principal resorptive cells of bone, osteoclasts appear at sites of active bone resorption and develop specialized cell membranes further dissolving bone minerals by active \( \text{H}^+ \) secretion in the microenvironment in between them and bone [33,34]. Therefore, the observation of osteoclasts suggests the presence of bacterial infection and bone resorption. No sign of bacterial infection or bone resorption can be detected from Zn/Ag-PIII and Zn-Ag-PIII.

Formation of new bone extending to the medullary cavity is observed from the implant surface and it is consistent with the micro-CT analysis in Fig. 6b. The newly formed bone grows vertically to the cortical bone at the interface between the implant and host bone. Similar results are obtained by HE and Giemsa staining as shown in Fig. 7, indicating that Zn/Ag dual-ion implantation produces good osseointegration and antibacterial effects in vivo.

4. Discussion

Three different micro-galvanic couples are fabricated on titanium using PIII in our experiments and they exhibit different surface morphology. Specifically, the Ag NPs on Zn/Ag-PIII are covered by a simultaneously formed Zn film, leaving only the top to be visible on the titanium surface. In comparison, they are on the surface of the Zn film on Zn-Ag-PIII and the majority of the Ag NPs are underneath the Zn film on Ag-Zn-PIII (Fig. 1). According to the three different structures, the 3D Max software is employed to create the surface models of the Zn/Ag dual-ion implanted titanium as shown in Fig. 8.

It has been demonstrated that micro-galvanic couples are formed by Zn and Ag PIII with Zn serving as the anode and Ag as the cathode [12]. In the present study, we further investigate the corrosion process of the three different micro-galvanic couples to demonstrate the relationship between the enhanced biological behaviors and the micro-galvanic couples. The corrosion potentials of the Zn/Ag dual-ion implanted titanium shift positively compared to pure Ti (shown in Fig. 3a) and the corrosion currents are especially important when corrosion occurs. The corrosion currents
increase in the following order: Ag-Zn-PIII < Zn-Ag-PIII < Zn/Ag-PIII. This may be because the Ag NPs on Ag-Zn-PIII are almost covered by the Zn film and it is relatively difficult for the micro-galvanic couples to reach the conducting state thereby resulting in the smaller corrosion current and less consumption of H⁺ according to the following cathodic reaction:

\[ 2H^+ + 2e^- \rightarrow H_2 \]  

(1)

Compared to Ag-Zn-PIII, it is easier for Zn/Ag-PIII and Zn-Ag-PIII to reach the conducting state as the embedded Ag NPs are in direct contact with the Zn film. Hence, the micro-galvanic couples are triggered when the samples are immersed in a physiological medium. Nevertheless, the difference in the corrosion currents is likely due to the difference in the contact between the Ag NPs and Zn film in Zn/Ag-PIII and Zn-Ag-PIII. According to the surface morphology and model (Fig. 8), the Ag NPs on Zn/Ag-PIII are embedded on the titanium surface and wrapped by a Zn film whereas those on Zn-Ag-PIII are immobilized on the top of Zn film. The contact area between the Ag NPs and Zn on Zn/Ag-PIII is larger than that on Zn-Ag-PIII. The micro-galvanic couples on Zn/Ag-PIII are still triggered until Zn, which is in contact with the Ag NPs, is consumed thereby exposing the Ag NPs in the microenvironment between the titanium and cell (or bacteria). However, triggering of the micro-galvanic couples on Zn-Ag-PIII ends when Ag NPs leave the titanium surface and are released into the microenvironment. Therefore, the corrosion rate of Zn/Ag-PIII is faster than that of Zn-Ag-PIII as confirmed by the corrosion currents.

A larger corrosion current implies a faster reaction and more substantial release of Zn²⁺ and consumption of H⁺. According to the 3D Max model in Fig. 8, the amount of Zn²⁺ released from Zn/Ag-PIII is larger than that from Zn-Ag-PIII (confirmed by the ion release results in Fig. 3b). The larger amount of released Zn²⁺ ions is due to the significantly larger surface Zn content on Ag-Zn-PIII (Fig. 3b). Zn²⁺ in the microenvironment between the titanium and cells can be adjusted by Zn transporters [35,36] further exerting a stimulatory effect on the metabolism of bones [37]. Moreover, the synergistic effects of the long-range imposed by Zn²⁺ and short-range interactions produced by the Ag NPs enhance the osteogenic activity and antibacterial properties both in vitro and

Fig. 7. High magnification histological images of masson's trichrome staining, HE staining, and giemsa staining of transverse sections at 6 weeks after surgery. The images below are higher magnifications of the areas within the yellow boxes. Multinucleated osteoclasts (yellow arrows) are detected near the trabecular bone around Ti implant, indicating the presence of bacterial infection and bone resorption. No signs of bacterial infection or bone resorption but new bone formation are detected from the Zn/Ag-PIII, Zn-Ag-PIII, and Ag-PIII groups. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
The pH of the microenvironment between the osteoclasts and bone surface is crucial to cellular processes and should be maintained at 4.5 [33,38]. Consumption of H\(^+\) by the cathodic hydrogen evolution reaction of the micro-galvanic couples inhibits bone resorption by osteoclasts. According to the corrosion currents, the consumption of H\(^+\) increases in the following order: Ag-Zn-PIII < Zn-Ag-PIII < Zn/Ag-PIII and therefore, bone resorption on Ag-Zn-PIII is more serious and Zn/Ag-PIII exhibits the best osseointegration in vivo (Fig. S6). The present work demonstrates that the biological properties can be controlled by the corrosion process on the designed micro-galvanic couples.

5. Conclusion

Three types of Zn/Ag micro-galvanic couples are fabricated on titanium by plasma immersion ion implantation to investigate the osseointegration and antibacterial effects as well as the involved mechanisms. The micro-galvanic couples exhibit excellent osteogenic activity and antibacterial ability in vitro without producing cytotoxicity. The Zn/Ag micro-galvanic couple formed on Zn/Ag dual-ion co-implanted titanium shows the best osseointegration as well as good antibacterial properties in vivo obtained from a rabbit tibia model. The difference among the three structures in vitro and in vivo can be explained by the contact between the Ag NPs and Zn film, which further affects the corrosion rates of the micro-galvanic couples. The findings provide insights into the design of new orthopedic and dental implant implants with simultaneous osseointegration and antibacterial properties.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biomaterials.2015.06.040.

References

and biocompatibility of titanium surfaces with graded silver incorporation in titania nanotubes, Biomaterials 35 (2014) 4255–4265.


Supplementary materials for
Zn/Ag micro-galvanic couples formed on titanium and
osseointegration effects in the presence of S. aureus

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Supplemental Content:

1. Supplemental table,

2. Captions and descriptions of the supplemental figures.
1. Supplementary Table

**Table S1. Primers for RT-PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Prime sequence (F, forward; R, reverse; 5’ to 3’)</th>
<th>Product Size (bp)</th>
</tr>
</thead>
</table>
| F-actin | F: CACCCGCGAGTACAACCTTC  
R: CCCATACCCACCATCACACC | 207 |
| ALP | F: CGTCTCCATGGTGATGATTATGCT  
R: CCCAGGCACAGTGTTCAAG | 209 |
| Col-I | F: CTGCCACAAGGAAATATGTATCACC  
R: GAAGCAAAGTTTCCTCCAAGACC | 198 |
| OCN | F: GCCCTGACTGCATTCTGCCTCT  
R: TCACCACCTTACTGCCTCTCCTG | 103 |
| Runx2 | F: TCTTCCAAAAGCAGAGCG  
R: TGCCATTGCAGGTTGTCG | 154 |
2. Supplementary Figures

Supplementary Fig. S1. High resolution spectra detected from Zn/Ag dual-ion implanted titanium surfaces: (a) Zn 2p, (b) Ag 3d from Zn/Ag-PIII; (c) Zn 2p, (d) Ag 3d from Zn-Ag-PIII; (e) Zn 2p, (f) Ag 3d from Ag-Zn-PIII
**Supplementary Fig. S2.** ALP positive areas of rBMSCs cultured on various surfaces for 7 and 14 days

**Supplementary Fig. S3.** Collagen secretion of rMSCs cultured on various surfaces for 7 and 14 days
Supplementary Fig. S4. Matrix mineralization of rBMSCs cultured on various surfaces for 7 and 14 days.

Supplementary Fig. S5. Re-cultivated bacterial colonies on agar: (a) E. coli and (b) S. aureus seeded on various surfaces with the bacteria concentration being $10^7$ cfu/ml; Percentage reduction: (c) E. coli and (d) S. aureus re-cultured on agar after dissociation from the various surfaces with the re-cultivated bacteria concentration being $10^7$ cfu/ml; ***P < 0.001.
Supplementary Fig. S6. Low magnification histological images of masson’s trichrome staining, HE staining, and giemsa staining of transverse sections at 6 weeks after surgery. Formation of abscess, destruction of cortical bone accompanied by large number of inflammatory cells can be observed around Ti implant. Slight abscess, minor bone destruction and less new bone formation are detected from Ag-Zn-PIII. However, no sign of bacterial infection is detected from Zn/Ag-PIII and Zn-Ag-PIII groups.