Synergistic effects of dual Zn/Ag ion implantation in osteogenic activity and antibacterial ability of titanium

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Keywords: Zinc (Zn) and silver (Ag) are co-implanted into titanium by plasma immersion ion implantation. A Zn containing film with Ag nanoparticles (Ag NPs) possessing a wide size distribution is formed on the surface and the corrosion resistance is improved due to the micro-galvanic couples formed by the implanted Zn and Ag. Not only are the initial adhesion, spreading, proliferation and osteogenic differentiation of rBMSCs observed from the Zn/Ag implanted Ti in vitro, but also bacteria killing is achieved both in vitro and in vivo. Electrochemical polarization and ion release measurements suggest that the excellent osteogenic activity and antibacterial ability of the Zn/Ag co-implanted titanium are related to the synergistic effect resulting from the long-range interactions of the released Zn ions and short-range interactions of the embedded Ag NPs. The Zn/Ag co-implanted titanium offers both excellent osteogenic activity and antibacterial ability and has large potential in orthopedic and dental implants.

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1. Introduction

Titanium and its alloys have been widely used in orthopedic and dental implants because of their low elastic modulus, excellent corrosion resistance, and superior biocompatibility [1]. However, implant failures still occur due to insufficient osseointegration and implant-associated bacterial infections [2] and biomaterials that possess both excellent osteogenic activity and antibacterial ability are thus urgently needed to overcome this drawback. Although various attempts have been made to achieve good osteogenic activity [3] or antibacterial ability [4,5] individually, it is important to develop a simple and effective surface modification technique which can enhance both these two effects simultaneously.

Our previous study demonstrates that zinc-implanted titanium can significantly stimulate the initial adhesion and spreading, proliferation, differentiation of rat bone mesenchymal stem cells [6]. Various researches have also confirmed that zinc (Zn) as a structural or functional component of a large number of proteins [7] can increase osteogenic functions [8] and stimulate bone formation [9] by enhancing cell proliferation, differentiation, and osteoblast marker gene expressions [10]. However, the antibacterial ability of the Zn-implanted titanium in our previous study is not good enough to prevent bacterial infection. As a broad-spectrum antibacterial agent, silver (Ag) has been extensively used to improve the antibacterial ability of implants [11]. Our previous work has also confirmed that Ag-implanted titanium exhibits excellent antibacterial ability as well as good compatibility with osteoblasts due to the micro-galvanic effects generated by the implanted silver nanoparticles (Ag NPs) and titanium substrate [4]. Furthermore, as one of the most well-known and effective antibacterial agents, Ag can interact with bacterial cell wall membranes, destroy the wall membrane, and eventually cause the death of bacteria [12]. Despite many investigations on the antibacterial properties of Ag NPs [13,14], the exact mechanism is still not well understood. Recently, the antibacterial ability of Ag NPs has attracted much attention and Zhang et al. [15] have found that Ag NPs incorporated into TiO₂ coatings possess good antibacterial ability because of the interaction between the Ag NPs and bacterial cell wall membrane.

It is generally believed that the interactions between Zn ions and cells (or bacteria) are long-range ones as the interactions are based on the uptake of Zn ions that are present in the...
microenvironment between the biomaterials and cells (or bacteria) [16]. On the other hand, the interactions between Ag and cells (or bacteria) are short-range ones since the embedded Ag NPs can interact with bacteria via the micro-galvanic effects without release of Ag ions [4,5]. The long-range interactions of Zn ions can stimulate both the osteogenic activity and antibacterial ability of biomaterials [17], whereas the short-range interactions of Ag NPs can efficiently kill bacteria. Therefore, simultaneous incorporation of Zn and Ag into titanium may produce favorable osteogenic activity and antibacterial ability. In this respect, the effects of Zn and Ag on the microenvironment have been studied extensively. A simple and effective technique that introduces both Zn and Ag into Ti to achieve osteogenic enhancement and antibacterial ability is also highly desirable. Plasma immersion ion implantation (PIII) is a suitable and versatile method that can introduce nearly any elements into different types of substrates and biomedical components without the line-of-sight limitation. Since it is a surface modification technique, the favorable bulk properties of the substrates are preserved [18]. In this work, Zn and Ag are co-implanted into titanium and the osteogenic activity and antibacterial ability are investigated systematically in vitro.

2. Materials and experimental details

2.1. Specimen preparation and characterization

2.1.1. Preparation of Zn/Ag co-implanted titanium (Zn/Ag-PIII)

Titanium plates (Cp Ti, Grade 2) measuring 10 mm × 10 mm × 1 mm and 20 mm × 10 mm × 1 mm were polished on one side by abrasive papers to a mirror finish and ultrasonically cleaned successively with acetone, ethanol, and distilled water. Before plasma immersion ion implantation, the titanium plates were cleaned by radio frequency (RF) argon ions for 15 min at a bias of −550 V. Zn and Ag ions were then implanted into the polished side at 30 keV for 90 min. Zn/Ag co-implantation was carried out simultaneously using pulsed Zn and Ag cathodic arc plasma sources and the important PIII parameters are listed in Table 1 [14].

2.1.2. Surface structure and chemistry

The surface morphology of the samples before and after PIII was examined by field-emission scanning electron microscopy (FE-SEM, HITACHI, S-4800, Japan) and the chemical states and elemental depth profiles were determined by X-ray photoelectron spectroscopy (XPS, Physical electronics PHI 5802). The dynamic potential polarization curves were acquired in a physiological saline solution (0.9% NaCl at a pH of 7) at room temperature on a CHI760c electrochemical workstation (Chenhua, Shanghai, China). The measurements were carried out on a three-electrode electrochemical cell with a saturated calomel electrode (SCE) as the reference electrode, a graphite rod as the counter electrode, and the sample as the working electrode. The tests were conducted at a scanning rate of 10 mV/s.

2.1.3. Water contact angles

The water contact angles on the various samples were measured on a contact angle instrument (SL2008B, Solon, China) in accordance with the method used in the literature [19].

2.1.4. Surface zeta potentials

The surface zeta potentials of the various samples (20 mm × 10 mm × 1 mm) were determined using a Surpass electrokinetic analyzer (Anton Parr, Austria). The tests were carried out on a three-electrode electrochemical cell with a saturated calomel electrode (SCE) as the reference electrode, a graphite rod as the counter electrode, and the sample as the working electrode. The tests were conducted at a scanning rate of 10 mV/s.

2.1.5. Release of zinc and silver ions

The ion implanted titanium samples were immersed in 10 ml 0.01 M NaCl (at a pH of 7) for various periods of time at 37 °C without stirring. The amounts of released Zn and Ag at 7, 14, 21, and 28 days were determined by analyzing the resulting solutions using an inductively-coupled plasma atomic emission spectroscopy (ICP-AES).

2.2. In vitro cytocompatibility evaluation

2.2.1. Cell cultures

The rat bone mesenchymal stem cells (rBMSCs) (Stem Cell Bank, Chinese Academy of Sciences, Shanghai, China) were seeded on the various surfaces to evaluate the cytocompatibility. The cells were cultured at 37 °C in a 5% CO2 incubator in 75 cm2 flasks (Corning Incorporated, USA) containing 8 ml of the minimum essential medium (α-MEM) (Minimum Essential Medium alpha-Medium, Gibco, Invitrogen, Inc) supplemented with 10% fetal bovine serum (FBS) (HyClone, USA) and 1% antimicrobial of penicillin and streptomycin (Antibiotic-Antimycotic, Hyclone, USA). The cells were subcultured every 3 days and only cells of passages 3–5 were used in the experiments.

2.2.2. Cell adhesion and spreading assay

The Rat BMSCs were seeded on the various surfaces at a cell density of 5.0 × 104 cells/ml. After culturing for 1, 4, and 24 h, the cells were washed thrice with the phosphate buffer saline (PBS) (pH = 7.4, HyClone, USA), fixed with 4% paraformaldehyde (PFA) solution (Sigma, USA) for 10 min at room temperature, and permeabilized with 0.1% (v/v) Triton X-100 (Amresco, USA) for 2 min. Afterwards, the cells were stained with FITC-Phallodin (Sigma, USA) at room temperature in darkness for 1 h and further stained with DAPI (Sigma, USA) for 5 min. The F-actin and cell nuclei were examined on a fluorescence microscopy (Olympus, Japan).

2.2.3. Cell proliferation

A 1 ml cell suspension with a density of 1.0 × 10^5 cells/ml was seeded onto each sample. After culturing for 1, 4, and 7 days, cell proliferation was evaluated using the alamarBlue™ assay (AbD Serotec Ltd, UK). Specifically, the samples were transferred onto a new 24-well plate and gently rinsed with PBS twice. 0.5 ml of the fresh medium with 5% alamarBlue™ was added and the samples were incubated for another 4 h. Afterwards, 100 μl of the medium were transferred to a 96-well plate (Nunc, USA). Accumulation of reduced alamarBlue™ in the medium was examined by an enzyme labeling instrument (BIO TEK, ELX 800) at extinction wavelengths of 570 and 600 nm. Calculation of cell proliferation followed the instruction of the alamarBlue™ assay.

2.2.4. Cell morphology

After each incubation time point, the samples with the rBMSCs were rinsed with PBS twice and fixed with 2.5% glutaraldehyde. Before SEM examination, the samples were dehydrated in gradient ethanol solutions (30, 50, 75, 90, 95 and 100 v/v%) for 10 min each sequentially, followed by drying in the hexamethyldiisazane ethanol solution series. The samples with cells were coated with platinum before SEM was performed at 5 kV.

2.2.5. Alkaline phosphatase activity assay

The Rat BMSCs were seeded on the surfaces of the samples at a cell density of 1.0 × 10^4 cells/ml (7 days) and 0.5 × 10^4 cells/ml (14 days) to evaluate the alkaline phosphatase (ALP) activity. After each time point, the cells were fixed with 4% PFA and incubated in a mixture containing fast blue RR salt and naphthol AS-MX phosphate for ALP staining. For the quantitative assay, the cells were dissociated from the surface and further incubated with p-nitrophenyl phosphate (pNPP) (Sigma, USA) at 37 °C for 30 min. The ALP activity of rBMSCs was determined by measuring the optical density (absorbance) at a wavelength of 405 nm, while the total protein content was determined using the BCA protein assay. The ALP levels were normalized to the total protein content and the experiments were carried out in triplicates.

2.2.6. Extracellular matrix mineralization

Extracellular matrix (ECM) mineralization on the various surfaces was quantified by Alizarin Red staining. The Rat BMSCs with densities of 1.0 × 10^4 cells/ml (7 days) and 0.5 × 10^4 cells/ml (14 days) were cultured for 7 and 14 days, washed with PBS thrice, fixed in 75% ethanol for 1 h, and staining with 40 μM Alizarin Red for

\[ z = \frac{d}{dp} \left( n \frac{l}{\varepsilon_0} \right) \]

in which, \( z \) is the zeta potential, \( d/dp \) represents the slope of streaming current versus pressure difference, \( n \), \( l \), and \( \varepsilon_0 \) denote the electrolyte viscosity, dielectric constant, and vacuum permittivity of the electrolyte, and \( l \) and \( A \) are the length and cross section of the streaming channel, respectively. During the measurement, the aforementioned parameters were collected by SurPASS control and evaluation software and the final zeta potentials were calculated from data collected after taking into account the parameters \( n \), \( l \), \( \varepsilon_0 \), and \( A \).
10 min. Afterwards, the cells were washed with distilled water until no red color was observed and images were taken. To conduct the quantitative analysis, the stain was dissolved in 10% cetyltrimethyl ammonium chloride in 10 mM sodium phosphate. The OD values of absorbance at 600 nm were measured and the experiments were conducted in triplicates.

2.2.7. Quantitative real-time PCR (RT-PCR) analysis

The expressions of osteogenesis related genes by rBMSCs were investigated using RT-PCR. The rat BMSCs at densities of 1.0 × 10^5 cell/well (7 days) and 0.5 × 10^5 cell/well (20 days) were cultured in 24 and 14 days. After each time point, the cells were washed with PBS three times and the total RNA was extracted by 1 ml of the TRIzol reagent. After adding 0.2 ml of chloroform, shaking vigorously for 15 s, and centrifuging at 12,000 rpm for 15 min at 4 °C, the aqueous phase was collected and put in a new 1.5 ml tube. 0.5 ml of isopropanol (100%) was added to the collected aqueous phase and centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant was removed and the RNA pellet was washed with 1 ml of 75% ethanol for 5 min at 4 °C. The purity of the RNA was assessed using A260/280 nm. Afterwards, 1.0 μg of the RNA was reverse transcribed into cDNA using the Transcriptor First Strand cDNA Synthesis Kit for RT-PCR (Roche). RT-PCR was performed on the Roche LightCycler480 system using an SYBR Green I PCR Kit. Expressions of the osteogenesis related genes included ALP, type I collagen (Col-I), osteocalcin (OCN), and runt-related transcription factor 2 (Runx2). The relative expressions for the target genes were normalized to that of the reference gene F-actin and the primers for RT-PCR are listed in Table 2.

2.3. In vitro antibacterial assessment

The antibacterial activity of the pure Ti, Zn-PiII, Ag-PiII, and Zn/Ag-PiII samples was determined by both Live/Dead staining and bacteria counting using Escherichia coli (E. coli, ATCC 25922) and Staphylococcus aureus (S. aureus, ATCC 25922). The samples were sterilized in 75% ethanol for 2 h at room temperature. A drop of bacterial suspension with a concentration of 10^5 CFU/ml was placed on the sample. After incubation at 37 °C for 24 h, the bacteria were stained based on the instruction of the Live/Dead BacLight® Bacterial Viability Kits (L13152). Briefly, the samples were rinsed twice with a physiological saline solution and 500 μl of the Live/Dead BacLight staining reagent mixture were added. After incubation without light for 10 min at room temperature, the samples with the bacteria were transferred to a cover-slip using a forceps and observed by a fluorescence microscope.

The samples with the bacterial suspension were cultured at 37 °C for 24 h, and put into each test tube with 5 ml of sterile physiological saline. Afterwards, the test tube was vortexed for 1 min using a vortex mixer to dissociate the bacteria from the sample surface, the dissociated bacterial suspension was then serially diluted in 10-fold steps with physiological saline and 200 μl of the diluted bacterial suspension was introduced to a standard agar culture plate for further incubation for 24 h. The active bacteria were counted according to the National Standard of China GB/T 4789.2 protocol and the antibacterial ratio was calculated as follows:

\[
\frac{(A - B)}{A} \times 100% 
\]

where A is the average number of bacteria on the control sample (CFU/sample) and B is the average number of bacteria on the testing samples (CFU/sample).

In the SEM observation, a drop of bacterial suspension with a concentration of 10^3 CFU/ml was placed on the sample. After incubation at 37 °C for 24 h, the bacteria were fixed with 2.5% glutaraldehyde solution and dehydrated using the same procedures described in Section 2.2.4.

2.4. In vivo experiments

2.4.1. Surgical procedures

All the animal 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. A total of 20 male SD rats, aged 4 weeks old, were used in the work. The rats were divided into four groups of 5 as follows: Ti, Zn-PiII, Ag-PiII, and Zn/Ag-PiII. The rats were anesthetized with 4% chloral hydrate (0.9 ml/100 g) for prior to surgery. The left leg was shaved, depilated, and disinfected with iodine. The rat was placed on a sterile drape to provide sterile conditions during surgery. After the left femoral condyle was exposed by skin incision, a hole was drilled through the cortical and cancellous bone with a Kirschner wire (1.5 mm diameter) in order to access the medullary cavity, and was gradually reamed with a Kirschner wire 2.0 mm in diameter. 20 μl of the bacterial suspension with a concentration of 10^6 CFU/ml S. aureus were injected into the femoral medullary cavity with a micro-syringe and the sterile implants (2 mm in diameter and 7 mm in length) were inserted in afterwards. The wound was closed carefully. After surgery, rats were housed in ventilated rooms and given access to water and food. X-rays of the rats were taken on the day of surgery and every two weeks.

2.4.2. Sample preparation

The rats were sacrificed at 6 weeks after surgery and the left femurs with the implants were harvested. Two randomly chosen femurs were prepared for microbiological evaluation and 2 more for histological evaluation.

2.4.3. Microbiological evaluation

Radiography was performed on days 7, 14, 28, and 42. For X-rays, digital films and a Mobilitec Plus X-ray unit were used. All the radiographic results were assessed in a blind manner according to the literature [20], including periosteal reaction and general impression. In the microbiological evaluation, the specimens were explaned, rolled over on nutrient agar plates, and placed in 2.5 ml of TSB. The agar plates and specimens containing TSB were further incubated at 37 °C for 24 h and turbidity of TSB was judged (turbidity represents positive growth the clear sample shows no growth).

2.4.4. Histological evaluation

In histological staining, the specimens were dehydrated in gradient ethanol solutions (75%, 90, 95 and 100 v/v %) and cut into 5 μm thick transverse sections. The transverse histological sections were put on slides and coated with 3-aminopropyl triethoxysilane. The sections from each specimen were stained with hematoxylin–eosin (HE) and Giemsa. Histological determination was conducted on a fluorescence microscope. The HE stained transverse sections were analyzed for bone histology, whereas the Giemsa stained sections were analyzed for evidence of inflammation [21] and presence of neutrophil.

2.5. Statistical analysis

All data were expressed as means ± standard deviations. The statistical analysis was done by using a GraphPad Prism statistical software package. The statistical significance of the difference was measured using two-way analysis of variance and P values < 0.05 were considered statistically significant.

3. Results

3.1. Surface characterization

Fig. 1 shows the surface morphology of the pure titanium and ion implantation treated samples designated as Zn-PiII, Ag-PiII, and Zn/Ag-PiII. The pure titanium surface has a flat topography whereas a micro-rough surface with a Zn containing film is observed from Zn-PiII (Fig. 1b) and the results are consistent with our previous study [6]. Homogeneously distributed nanoparticles about 6 nm in size exist on the Ag-PiII surface (Fig. 1c) and more Ag nanoparticles (Ag NPs) with a wider size distribution are observed from Zn/Ag-PiII (Fig. 1d). The difference in the Ag NPs between Zn/Ag-PiII and Ag-PiII is mainly ascribed to knock-on collisions and interactions between the energetic Zn and Ag ions during the co-implantation process consequently enhancing nucleation of the Ag NPs [22].

To determine the chemical states and elemental depth profiles, Zn/Ag-PiII is analyzed by XPS and the results are presented in Fig. 2. The Ti 2p doublet at 455.6 eV (2p1/2) and 458.6 eV (2p3/2) corresponds to titanium dioxide (TiO2) [23,24] as shown in Fig. 2a. According to the Zn 2p spectra obtained from the surface and interior, the peak at 1021.9eV corresponds to zinc oxide (ZnO) whereas the peak near 1021.0 eV is associated with metallic zinc (Fig. 2b) [25,26]. The Ag 3d doublet near 345.0 eV (3d3/2) and 368.0 eV (3d5/2) corresponds to metallic silver (Fig. 2c) [27] and there is no evidence of Ag oxidation indicating that the Ag NPs have the metallic state.

The elemental depth profiles in Fig. 2d resemble Gaussian distributions between 20 and 120 nm. The large content of Zn from the
surface to about 10 nm arises from deposition during PIII, indicating that a Zn containing film was formed on the surface of Zn/Ag-PIII. The peak concentration of Zn in Zn-PIII is twice that of Ag in Ag-PIII mainly because of easier ionization of Zn. The ionization energy $E_Q$ can be defined as the amount of energy required to remove an electron from an isolated atom or an ion of charge state $Q$ and can be expressed as follows:

$$E_{Q}^{sum} = \sum_{Q=0}^{Q-1} E_{Q}$$  \hspace{1cm} (1)
The ionization energy of Ag (21.5 eV) is nearly twice that of Zn which is 9.39 eV [28]. In this case, the amount of ionized Zn ions is nearly two times that of Ag ions under the same implantation voltage, thus resulting in the amount of implanted Zn being nearly twice that of Ag. However, the peak concentrations of Zn and Ag in Zn/Ag-PIII are smaller than those of Zn-PII and Ag-PII. It may be due to collisions between the Zn and Ag ions during co-implantation and subsequent energy loss.

The water contact angles on Zn-PII, Ag-PII, and Zn/Ag-PII are 104.42 ± 0.55°, 101.21 ± 2.04°, 104.80 ± 1.46°, respectively, which are comparable to that of pure Ti, 107.36 ± 2.88°, suggesting that ion implantation almost does not change the surface wettability of titanium.

In order to investigate the electrical charges on the sample surface, the zeta potentials are detected from pure Ti and ion implanted samples. The zeta potential represents the surface electrical charges when ions in the solution adsorb on the surface implanted samples. The zeta potentials are detected from pure Ti and ion implanted samples. The difference in the zeta potentials is likely attributed to the microstructure and the electrical properties of the ion implanted samples. And the more negative zeta potential of Ag-PIII than Zn-PIII is because the divalent Zn2⁺ ions are more positive than that of pure Ti at a pH of 7.4, and the zeta potential of Zn/Ag-PIII is between those of Zn-PII and Ag-PII. It can be further confirmed by the gap in the zeta potentials between pure Ti and the ion implanted samples. The difference in the zeta potentials is likely attributed to the microstructure and the electrochemical properties of the ion implanted samples. And the more negative zeta potential of Ag-PIII than Zn-PIII is because the divalent Zn²⁺ ions are more effective than the monovalent Ag⁺ ions, in reducing the absolute value of the zeta potential.

As revealed in our previous study, micro-galvanic couples may be formed on the Ag-implanted titanium [4]. Potentiodynamic polarization is performed in 0.9% NaCl to investigate the micro-galvanic efficiency and the Tafel plots are shown in Fig. 3b. The corrosion potentials (Ecorr) of Zn-PII and Ag-PII shift negatively compared to pure Ti and the data are in good agreement with our previous studies. However, Zn/Ag-PII shows a more positive corrosion potential than pure Ti, indicating better corrosion resistance and potential existence of micro-galvanic couples formed by the implanted Zn and Ag.

To further understand the relationship between the Zn and Ag ions in the ion implanted samples and their biological effects, the amount of ions released from the ion implanted samples are measured by ICP-AES. The concentrations of released Zn ions are presented in Fig. 3c. The cumulative Zn²⁺ concentration leached from Zn/Ag-PIII is higher than that from Zn-PII as the immersion time is extended from 7 to 28 days. However, the concentration of released Ag after immersion of Zn/Ag-PII for 7 days is only 0.0072 μg/ml, which is lower than that from Ag-PII (0.0144 μg/ml). Furthermore, the cumulative Ag⁺ concentration is not more than 0.0396 μg/ml after immersion for 28 days and is quite small. The positively shifted corrosion potential, higher cumulative Zn²⁺ concentration, and smaller Ag⁺ concentration observed from Zn/Ag-PIII compared to Zn-PII and Ag-PII indicate the formation of micro-galvanic couples by Zn and Ag implantation and this issue will be discussed further in the discussion section.

3.2. Response of rBMSCs

The initial cell adhesion and spreading activity are assayed by staining with FITC and DAPI to visualize the F-actin and nuclei, respectively, and the results are shown in Fig. 4. The rBMSCs on the pure Ti sample exhibit a spherical morphology in the first hour with the lack of filopodia extensions, while the expressions of F-actin on Zn-PII, Ag-PII, and Zn/Ag-PII are significantly better than that on pure Ti. Moreover, mitosis phase cells are found on the ion implanted samples within 1 h [white arrows in Fig. 4], whereas mitosis phase cells can hardly be observed from pure Ti until 24 h, indicating that the ion implanted samples are more favorable to initial adhesion and spreading of rBMSCs. This may be ascribed to that the relatively more positive zeta potentials of the ion implanted specimens (Fig. 3a) is beneficial to the initial stage of cell adhesion [30]. After culturing for 4 h, the expressions of F-actin on the various surfaces are better than those after 1 h. In addition, the expressions on the ion implanted samples are better than that on pure Ti, especially on Zn/Ag-PII and a lot of filopodia can be detected. However, the difference in the expressions of F-actin after culturing for 24 h is not significant and the rBMSCs on all the surfaces exhibit a polygonal shape with a large number of filopodia and lamellipodia. The results indicate that Zn/Ag co-implantation can enhance the initial adhesion and spreading activity of rBMSCs and all the samples are favorable to the growth of rBMSCs with no cytotoxicity.

The cell morphology on the various surfaces after culturing for 1, 4, and 7 days is splayed in Fig. 5a. After 1 day, all the cells spread well with a large number of filopodia and clustered cells cover most of the surface with connections among them through filopodia after 4 days, especially Zn/Ag-PII on which the cell connection is conducive to cell proliferation [31]. After 7 days, the cells with good connections completely cover the various surfaces, and layers of cells can be detected from Zn/Ag-PII. The proliferation and vitality of rBMSCs are evaluated by the alamarBlue™ assay and the results are presented in Fig. 5b. Cell proliferation on the four groups of samples shows no statistically difference after 1 day. However, after 4 days, the cells on Zn-PII and Ag-PII show slightly higher proliferation than pure Ti and among the implanted sample. Zn/Ag-PII exhibits the highest cell proliferation. This trend becomes more obvious after 7 days, indicating that ion implanted samples are favorable to the proliferation and vitality of rBMSCs with no cytotoxicity with the best results observed from Zn/Ag-PII.

The rBMSCs cultured on the pure Ti and ion implanted samples for 7 and 14 days are stained by the ALP staining kit, and the ALP-
positive areas are displayed in Fig. 6a. The ALP-positive areas on the ion implanted samples are larger than that on pure Ti after 7 days and Zn/Ag-PIII has the largest ALP-positive area. After 14 days, more ALP is generated and this trend becomes more significant. The results are corroborated by the quantitative analysis in Fig. 6b, suggesting that Zn/Ag-PIII can stimulate the ALP activity of rBMSCs and the synergistic effect.

Extracellular matrix (ECM) mineralization of rBMSCs assayed by Alizarin Red staining is shown in Fig. 7. The ECM mineralization of rBMSCs after culturing for 7 days on Zn-PIII is slightly enhanced compared to pure Ti, while Ag-PIII and Zn/Ag-PIII exhibit significantly up-regulated ECM mineralization. After 14 days, ECM mineralization on Zn-PIII and Ag-PIII is similar and enhanced compared to pure Ti and Zn/Ag-PIII shows the most significant ECM mineralization.

The expressions of osteogenesis related genes including ALP, Col-I, Runx2, and OCN on the various surfaces are quantified by Real-time PCR and the results are shown in Fig. 8. RT-PCR analysis shows that the ion implanted samples enhance the gene expressions of rBMSCs to various degrees. After culturing for 7 days, the gene expressions of ALP and Col-I on Zn/Ag-PIII are the highest among the four groups, followed by Ag-PIII, Zn-PIII, and pure Ti in this order. In particular, the expressions of Runx2 and OCN on Zn/Ag-PIII and Ag-PIII are similar but higher than those on Zn-PIII and pure Ti. After 14 days, the rBMSCs cultured on Zn/Ag-PIII still show the most enhanced gene expressions of ALP, Col-I, Runx2, and OCN. The gene expressions on Zn-PIII are higher than those on pure Ti except Col-I, whereas the gene expressions on Ag-PIII are significantly higher than those on Zn-PIII and pure Ti. The results indicate that ion implanted samples can stimulate the gene expressions of rBMSCs and Zn/Ag co-implanted titanium exhibits a synergistic effect.

These results indicate that the ion implanted samples exhibit improved initial adhesion and spreading activity, proliferation and vitality, ALP activity, ECM mineralization, and osteogenesis related gene expressions. The enhanced osteogenic activity on Zn-PIII is mainly ascribed to the properly released Zn$^{2+}$ ions (Fig. 3c) and it is consistent with our previous observation [6]. Accordingly, the larger the amount of Zn$^{2+}$ released from the ion implanted samples, the better is the osteogenic activity. Herein, the amount of Zn$^{2+}$ ions released from Zn/Ag-PIII is approximately twice that from Zn-PIII (Fig. 3c) and so the osteogenic activity on Zn/Ag-PIII is better than that on Zn-PIII. Ag-PIII also shows enhanced proliferation, differentiation, and osteogenesis related gene expressions of rBMSCs with no cytotoxicity. Our previous work [4] and Fiedler et al. [32] have also shown that Ag-implanted titanium exhibits improved proliferation of human osteoblasts with no cytotoxicity. The excellent osteogenic activity on Ag-PIII may be related to the embedded Ag NPs positively altering the microenvironment between the biomaterials and cells to benefit the behavior of rBMSCs and more in-depth investigations are necessary to explore the exact mechanism of Ag-implanted titanium from the perspective of osteogenic activity.

3.3. In vitro antibacterial ability

To evaluate the antibacterial ability, both Live/Dead staining and bacteria counting are used. The Live/Dead assay is an effective method to visualize the living and dead bacteria based on the color change during the experiment [33]. SYTO 9 is known to penetrate...
to the cell membrane of both living and dead bacteria to stain nucleic acid producing green fluorescence. In comparison, PI is able to penetrate the disrupted cell membrane of only dead bacteria to stain the nucleic acid and emit red fluorescence. Fluorescent images of the *E. coli* treated samples are shown in Fig. 9. The red spots (in web version) on pure Ti are negligible and there are many green spots (in web version), indicating the inability of pure Ti to kill bacteria. On the contrary, more red spots (in web version) are observed from Zn-PIII and there are less green spots (in web version) indicating improved antibacterial ability of Zn-PIII against *E. coli*. Significantly more dead bacteria and less living bacteria are observed from Ag-PIII revealing a synergistic antibacterial ability of Zn/Ag PIII and the results are in good agreement with the Live/Dead staining results in Fig. 9. The reduction of *S. aureus* on Zn-PIII, Ag-PIII, and Zn/Ag-PIII is approximately 45%, 96%, and 99%, respectively disclosing a similar trend.

SEM is utilized to examine the morphology and membrane integrity and the results are displayed in Fig. 12 (E. coli) and Fig. 13 (*S. aureus*). The typical morphology of *E. coli* is shown in Fig. 12a and the bacterial on pure Ti have mostly the rod shape whereas those on Zn-PIII are corrugated with a distorted shape. Apparent cell debris are observed from Ag-PIII and cells with a normal shape can be hardly found. The bacteria on Zn/Ag-PIII are completely lysed indicating that Zn/Ag-PIII has the best bacterial resistance against *E. coli* in vitro. Similarly, the *S. aureus* cells have a spherical shape with a smooth surface on pure Ti but irregular shape and rougher surface on Zn-PIII samples. Although the shape of some bacteria can be observed from Ag-PIII, the membrane is not as sharp as that on the pure Ti and bacteria lysis is observed. Completely lysed bacteria are observed from Zn/Ag-PIII. These results imply that the death of both *E. coli* and *S. aureus* is partly due to the disruption of the membrane integrity and lysis of bacteria. The antibacterial ability is mainly ascribed to the short-range interactions between the implanted Ag and bacteria rather than released Ag ions [4].

### 3.4. In vivo antibacterial ability

#### 3.4.1. Microbiological evaluation

The X-ray images of the four groups of animals are depicted in Fig. 14. No radiographic signs of osseous destruction are detected at 1 week but radiographic signs of low-density areas surrounding the pure Ti implant and expansion of medullary cavity are observed from the animals at 2 weeks (red arrows in Fig. 14), indicating potential bacterial infection similar to other reports [20,34]. There is no significant difference among the ion implanted samples and no osseous destruction is detected. At 6 weeks after surgery, bacterial infection on the pure Ti group does not increase with time.
To further investigate bacterial infection among the four groups in vivo, roll-over cultures are conducted and the results are shown in Fig. 15. Massive bacterial growth is observed from the agar plate of the pure Ti group (Fig. 15a) and it can be further confirmed by the cultures of the implant immersed in sterile TSB (inset in Fig. 15a). The positive TSB culture (cloudy appearance) indicates the existence of bacterial infection [34, 35]. The bacterial growth of the Zn-PIII group on agar plates is less than that of the pure Ti group, and the TSB culture is also slightly less cloudy (Fig. 15b), implying the partial antibacterial ability. In comparison, the bacterial growth on the Ag-PIII and Zn/Ag-PIII groups on agar plates is significantly reduced and the corresponding TSB cultures are negative (clear appearance) as shown in Fig. 15c and d, disclosing that both Ag-PIII and Zn/Ag-PIII have excellent antibacterial ability in vivo.

3.4.2. Histological evaluation

Giemsa staining of the four groups is presented in Fig. 16. Many lobulated neutrophils (red arrow in Fig. 16a) are observed from the femoral medullary cavity of the pure Ti group to indicate the presence of bacterial infection [21, 36]. As one of the first cells recruited to infected tissues, neutrophils migrate rapidly from circulating blood to infected sites in response to infection stimuli and efficiently inactivate invading bacteria by various ways. Hence, observation of a large number of neutrophils suggests bacterial infection. The number of neutrophils observed from the Zn-PIII group is less than that from the pure Ti group and the majority of the cells are normal ones within the medullary cavity (Fig. 16b), indicating relatively minor infection and partial antibacterial ability of Zn-PIII. In comparison, no neutrophils are found from the medullary cavity of the Ag-PIII and Zn/Ag-PIII groups, as shown in Fig. 16c and d, suggesting the absence of bacterial infection and excellent antibacterial ability.

The fluorescent images acquired from the transverse section of the bone 6 weeks after implantation are displayed in Fig. 17. The bone around the implant and inside the medullary cavity is destroyed due to bacterial infection, which is confirmed by the large number of neutrophils between the implant and bone (red

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**Fig. 7.** (a) Matrix mineralization of rMSCs cultured on various surfaces for 7 and 14 days and (b) Corresponding colorimetrically qualitative results; **P < 0.01, ***P < 0.001.

**Fig. 8.** Real-time PCR analysis: (a) ALP, (b) Col-I, (c) Runx2 and (d) OCN expressions by rMSCs on various surfaces after incubation for 7 and 14 days; *P < 0.05, **P < 0.01, ***P < 0.001.
arrow in Fig. 17e) [36] consistent with the Giemsa staining results in Fig. 16a. Although the cells in the medullary cavity of the Zn-PIII group are normal ones since no neutrophils are observed and almost all the cells are monocytes (Fig. 17b), the unusual presence of a large number of cells may be ascribed to the infiltration of slight bacterial infection. A continuous layer of fibrous tissue is observed between the implant and bone (green arrow in Fig. 17b and f), indicating that infection is repaired gradually [37]. Consistent with
the Giemsa staining results, no neutrophils are detected from the Ag-PIII group and the presence of spindle-shaped fibroblast cells (green arrow in Fig. 17c and g) also indicates the bacterial infection repair, which may also be proven by the presence of lots of osteocyte (black arrow in Fig. 17). The absence of neutrophils and continuous bone tissue between the implant and bone shown in Fig. 17d and h indicates that the Zn/Ag co-implanted titanium exhibits excellent antibacterial ability and the ability to stimulate

Fig. 11. SEM morphology: (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 < 0.01, ***P < 0.001.

Fig. 12. SEM morphology of E. coli seeded on various surfaces: (a) Pure Ti, (b) Zn-PIII, (c) Ag-PIII, and (d) Zn/Ag-PIII with the bacteria concentration being 10^7 cfu/ml.
bone formation based on periosteal reaction consistent with the X-ray results in Fig. 14.

There is evidence that bacterial infection occurs on the pure Ti group in vivo. First of all, low-density areas surrounding the implant and expansion of medullary cavity are detected from the animals after 2 weeks (Fig. 14). Secondly, substantial bacterial growth is observed from the agar plates (Fig. 15a). Thirdly, many neutrophils are detected from the medullary cavity between the implant and

![Fig. 13. SEM morphology of S. aureus seeded on various surfaces: (a) Pure Ti, (b) Zn-PIII, (c) Ag-PIII, and (d) Zn/Ag-PIII with the bacteria concentration being 10^7 cfu/ml.](image)

**Fig. 13.** SEM morphology of *S. aureus* seeded on various surfaces: (a) Pure Ti, (b) Zn-PIII, (c) Ag-PIII, and (d) Zn/Ag-PIII with the bacteria concentration being 10^7 cfu/ml.

![Fig. 14. X-ray images of rats implanted with the four kinds of rod implants at 1, 2, and 6 weeks post surgery. No radiographic signs of osseous destruction are detected at 1 week; radiographic signs of low-density areas surrounding the pure Ti group and expansion of medullary cavity are observed from the animals at 2 weeks, no significant difference among the ion implanted samples and no osseous destruction is detected; bacterial infection on the pure Ti group does not increase with time (red arrows) and there is no evidence of further development of osseous destruction at 6 week. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)](image)

**Fig. 14.** X-ray images of rats implanted with the four kinds of rod implants at 1, 2, and 6 weeks post surgery. No radiographic signs of osseous destruction are detected at 1 week; radiographic signs of low-density areas surrounding the pure Ti group and expansion of medullary cavity are observed from the animals at 2 weeks, no significant difference among the ion implanted samples and no osseous destruction is detected; bacterial infection on the pure Ti group does not increase with time (red arrows) and there is no evidence of further development of osseous destruction at 6 week. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
bone as a sign of bacterial infection (Figs. 16a and 17a). All in all, the results reveal bacterial infection in the pure Ti group. On the contrary, stimulation of bone formation is observed from the ion implanted groups, especially the Zn/Ag PIII one. Although a few neutrophils are detectable from the Zn-PIII group (Fig. 16b), the presence of fibrous tissues indicates repair of infection and stimulation of new bone formation (Fig. 17b and f)[37]. The small degree of bacterial growth on the roll-over cultures (Fig. 15c), absence of neutrophils (Fig. 16c), and presence of spindle-shaped fibroblast cells (Fig. 17c and g) indicate the excellent antibacterial ability of the Ag-PIII group in vivo.

4. Discussion

Ag NPs with a wide size distribution are embedded into titanium by dual Zn/Ag PIII due to enhanced nucleation during co-implantation. The Zn/Ag co-implanted titanium can significantly enhance the osteogenic activity in vitro and antibacterial ability both in vitro and in vivo possibly due to the existence of the micro-galvanic couples formed by the implanted Zn and Ag. A native oxide film forms spontaneously on the titanium surface when exposed to air [1] as confirmed by the XPS Ti 2p high-resolution spectra in Fig. 2a. The standard electrode potential of TiO2 (E_{TiO2}) is −0.502 V while the standard electrode potentials of Zn (E_{Zn}) and Ag (E_{Ag}) are −0.7618 V and 0.7996 V, respectively [4,38]. Therefore, the micro-galvanic couples can be easily triggered when the Zn/Ag co-implanted titanium is immersed in a physiological liquid without other stabilizers, as shown in Fig. 18. The implanted Zn serves as the anode of the micro-galvanic couple and releases Zn^{2+} ions whereas Ag NPs serve as the cathode and the substrate serves as an electronic pathway according to the principle of corrosion electrochemistry. As a result, the corrosion resistance of is improved (Fig. 3b).

The anodes and cathodes are in the conducting state when the Zn/Ag co-implanted titanium is immersed in the physiological liquid. In this case, the anodic reaction occurs in accordance with the follow equation:

$$\text{Zn} \rightarrow \text{Zn}^{2+} + 2e^-.$$  \hspace{1cm} (2)

Zn^{2+} ions are released to the surrounding liquid. On the other hand, electrons are transported to the implanted Ag NPs through the electronic pathway (pink arrow (in web version) in Fig. 18) where they are consumed by the cathodic hydrogen evolution reaction. The hydrogen evolution reaction occurs because the electrode potential of Zn which is $E_{\text{Zn/Zn}^{2+}} = -0.7618$ V is lower than that.

![Fig. 15. Roll-over cultures obtained from explanted implants after incubation for 24 h: (a) Pure Ti group, (b) Zn-PIII group, (c) Ag-PIII group, and (d) Zn/Ag-PIII group. The inserts are implants immersed in TSB.](image)

![Fig. 16. Giemsa staining of the transverse sections of the implants at distal femoral cortical bone at 6 weeks after surgery: (a) Pure Ti group, (b) Zn-PIII group, (c) Ag-PIII group, and (d) Zn/Ag-PIII group. Red arrows, lobulated neutrophils. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)](image)
of H of $E_{\text{H}^+/\text{H}} = 0 \text{ V}$ [38]. The cathodic reaction occurs according to equation (3):

$$2\text{H}^+ + 2e^- \rightarrow \text{H}_2.$$  \hspace{1cm} (3)

$\text{H}^+$ ions are thus consumed. Furthermore, since the Zn and Ag surface concentrations are relatively high (Fig. 2d), the efficiency of the micro-galvanic couples is high, thus resulting in rapid decrease of $\text{H}^+$ ions and increase of $\text{Zn}^{2+}$ ions in the surrounding environment. The phenomenon explains why the amount of $\text{Zn}^{2+}$ ions from Zn/Ag-PIII is larger than that from Zn-PIII (Fig. 3c).

The good osteogenic activity and antibacterial ability of $\text{Zn}^{2+}$ are due to long-range interactions. In our experiments, appropriate amounts of $\text{Zn}^{2+}$ are released from Zn-PIII and Zn/Ag-PIII (Fig. 3c) and they enter the microenvironment between the biomaterials and cells and are absorbed by cells thus playing a vital role in the osteogenic activity. With regard to the antibacterial ability, the long-range interactions between $\text{Zn}^{2+}$ and bacteria are shown in Fig. 18. The released $\text{Zn}^{2+}$ ions are transported to the bacteria cytosol via the ion channels at the expense of energy consumption [39] and hence, the living condition of the bacteria becomes worse as the bacteria transport more $\text{Zn}^{2+}$ ions. In addition, a high concentration of $\text{Zn}^{2+}$ in the cytosol is detrimental to the bacteria thus producing the partial antibacterial ability on Zn-PIII.

Although high concentrations of Ag$^{+}$ can induce cytotoxicity [40], it is generally accepted that Ag is safe and cytocompatible at low concentrations [4,41]. In this work, the amount of released Ag$^{+}$ ions is negligible and the excellent antibacterial ability of Ag-PIII samples is due to the short-range interactions. At the same time, the Ag ions released to the microenvironment play a long-range role in enhancing the antibacterial activity while the embedded Ag NPs play a short-range role synergistically in vitro and in vivo, as illustrated in Fig. 18. An appropriate amount of released $\text{Zn}^{2+}$ is transported from the Zn/Ag co-implanted titanium to the microenvironment between the biomaterials and cells. The released $\text{Zn}^{2+}$ ions can induce cytolysis of bacteria and consume protons at the same time on the Zn/Ag co-implanted titanium. The $\text{Zn}^{2+}$ ions released to the microenvironment change the electrochemical environment of the implants, alter the electrochemical gradient, and cause bacteria death. However, the structure and size of rBMSCs are different from those of bacteria cells and the proton depleted regions are not large enough to affect the proton electrochemical gradient of the rBMSCs. Therefore, the synthesis of ATP cannot be interfered. The enhanced osteogenic activity stems from the positively altered microenvironment being beneficial to rBMSCs. These are the reasons for the excellent antibacterial ability and good osteogenic activity observed from Ag-PIII.

The release of $\text{Zn}^{2+}$ and consumption of protons occur at the same time on the Zn/Ag co-implanted titanium due to the existence of the micro-galvanic couples. The $\text{Zn}^{2+}$ ions released to the microenvironment play a long-range role in enhancing the antibacterial activity while the embedded Ag NPs play a short-range role synergistically in vitro and in vivo, as illustrated in Fig. 18. An appropriate amount of released $\text{Zn}^{2+}$ from the Zn/Ag co-implanted titanium can significantly improve the osteogenic activity of rBMSCs via the long-range interactions. At the same time, the Ag NPs embedded in the Zn/Ag co-implanted titanium create the microenvironment between the biomaterials and cells beneficial to rBMSCs via short-range interactions. All in all, the synergism of the long-range and short-range interactions improves the osteogenic activity of the Zn/Ag co-implanted titanium. Even though the hypothesis in Fig. 18 explains the relevant mechanism to a certain degree, more work is needed to fathom it in details but nevertheless, this work provides new insights to the use of dual Zn and Ag plasma immersion ion implantation to enhance the osteogenic activity and antibacterial ability or biomedical Ti materials.

5. Conclusion

Ag NPs with a wide size distribution are embedded in titanium surface by a dual Zn/Ag plasma immersion ion implantation. The corrosion resistance of the co-implanted samples is improved due to the micro-galvanic couples formed by the implanted Zn and Ag. The co-implanted samples are effective in promoting initial adhesion and spreading, proliferation, differentiation, and osteogenesis related gene expressions of rBMSCs in addition to being highly...
effective in killing bacteria both in vitro and in vivo. The excellent osteogenic activity and antibacterial ability can be attributed to the synergistic effects of the long-range interactions rendered by Zn and short-range interactions of Ag stemming from the micro-galvanic couples in the Zn/Ag co-implanted titanium.

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