Biological and antibacterial properties of composite coatings on titanium surfaces modified by microarc oxidation and sol-gel processing

Beibei LI, Tao YANG, Rongxin SUN and Pan MA

Department of Dental Implant Center, Beijing Stomatological Hospital, School of Stomatology, Capital Medical University, No. 4 Tiantan Xili, Dongcheng District, Beijing, 100010, PR China
Corresponding author, Pan MA; E-mail: mapanxw@163.com

The aim of this study was to assess the biological and antibacterial properties of composite coatings on titanium surfaces modified by microarc oxidation and sol-gel processing. A layer of hydroxyapatite (HA) with different concentrations of zinc (Zn) ions, prepared by the sol-gel method, was coated on microarc oxidized Ti (MAO-Ti) substrates. Five groups of specimens were tested. The microstructures, elemental compositions, and chemical phases of the composite coatings were investigated, and the biological and antibacterial properties of specimens were evaluated in vitro. The EDS and XRD results confirmed the composite coatings contained HA and Zn ions which was formed on titanium surfaces. The proliferation and ALP activity of BMSCs was significantly higher in group MAO-Ti+HA and MAO-Ti+HA+Zn(High), but MAO-Ti+HA+Zn(High) showed better antibacterial performance. The MAO-Ti substrate coated with the higher Zn concentration in the HA coating exhibited not only favorable biocompatibility, but also antibacterial action against Gram-negative anaerobic bacteria.

**Keywords:** Titanium, Composite coatings, Zinc ions, Biocompatibility, Antibacterial activity

**INTRODUCTION**

Titanium (Ti) and Ti alloys are widely used in dental and orthopedic implants because of their outstanding mechanical strength, chemical stability, and biocompatibility\(^5\). Bacterial peri-implant infections are inflammatory and affect the function of soft and hard tissues around the implant, and are among the most frequent causes of dental implant failure\(^6\).

The biocompatibility of metallic implant materials depends on the surface oxide layer, and therefore hydroxyapatite (HA) has frequently been used as a coating material on Ti implants to improve cell responses because HA is chemically and crystallographically similar to the inorganic components of hard tissues\(^7\). Several HA coating techniques, such as plasma-spraying, anodizing, and hydrothermal treatments, have been used to prepare HA layers on Ti implants\(^8\)\(^-\)\(^10\). However, all of these treatments are of limited efficacy because Ti and Ti alloys form metal bonds, whereas HA coatings form covalent or ionic bonds, and the elastic moduli and thermal expansion coefficients of these materials are quite different. Therefore, the bonding strength between the two is poor, and the HA coating can easily fall off, limiting long-term stability\(^7\).

One promising approach to mitigating the bacterial infection problems is the application of an antimicrobial surface coating with nontoxic elements\(^9\). Several researchers have attempted to graft bioactive molecules (such as anti-adhesive polymers or antibacterial agents) onto the Ti surface to inhibit bacterial colonization; however, these coatings tend to be unstable and the fabrication process is usually time-consuming and costly\(^9\)\(^-\)\(^10\). Although inorganic bioactive elements, such as the antibacterial elements silver (Ag) and copper (Cu), have shown long-term antibacterial ability, their potential cytotoxicity is a concern\(^11\)\(^-\)\(^12\). Zinc (Zn), a trace element essential to many biological functions including bone metabolism, can reduce bacterial adhesion and growth\(^13\)\(^-\)\(^14\). However, because the effect of Zn is dose-dependent, some Zn concentrations may be cytotoxic\(^10\).

To address the bonding issue, micro-arc oxidation (MAO) is an electrochemical procedure that can cover Ti substrates with a porous Ti dioxide (TiO\(_2\)) ceramic coating, with a bond strength that is quite high\(^10\). Although certain bioactive elements can be incorporated into the coatings by adding them in the electrolyte during the MAO process\(^15\), increasing the concentration of calcium (Ca) or phosphorus (P) ions in the oxide layer requires a high voltage\(^16\), which often leads to cracks in the coating that weaken the bond strength between the coating and the base metal\(^18\)\(^-\)\(^19\). In the MAO process, simultaneously introducing the Ca and Zn concentrations desired in the surface coating is difficult because cations compete with each other in the electrolyte for points at which they can bond with anions. However, MAO could be combined with other methods to address this problem. The sol-gel technique, involving the immersion of the substrate into a liquid medium, is a simple industrial method that allows a dense and uniform HA coating to be generated on any rough and complexly shaped surface\(^19\).

Therefore, the study presented here applied the HA coating, prepared by a sol-gel method, to Ti substrates with the MAO-applied TiO\(_2\) coating, which thus formed an intermediate layer that could mitigate the property
differences between the coatings and substrates. The composite coatings contained HA and different concentrations of Zn ions, controlled by regulating the content of Zn ions in the sol-gel solutions. The properties of the composite coatings, including their in vitro biological and antibacterial properties were investigated. The research hypothesis was that the composite coatings containing HA and Zn ions would promote biocompatibility and reduce infection.

MATERIALS AND METHODS

Specimen surface preparations
Commerically available pure Ti (Grade 2, Northwest Institute of Nonferrous Metals, Xian, China) was machined into disks with dimensions of 20 mm (diameter) ×1 mm (thickness) and ground using 600-, 800-, and 1000-grit SiC sandpapers. The surface treatments of the specimens were processed at the College of Chemical Engineering, Harbin Institute of Technology as follows.

For the MAO process (Fig. 1a), the electrolyte formula consisted of 8 g/L Na3PO4, 1 g/L NaOH, and 0.5 g/L NaF. Using Ti as the anode and stainless steel as the cathode, the treatment was applied with a constant current density of 20 A/dm² at a frequency of 300 Hz. The duty cycle was 15% and the duration was 15 min.

For the sol-gel process, the precursor solution (sol) was prepared with Ca(NO₃)₂·4H₂O as the Ca source, P₂O₅ as the P source, Zn(NO₃)₂·6H₂O as the Zn source, and absolute ethanol as the solvent (Figs. 1b, c). In order to produce pure HA, the Ca(NO₃)₂·4H₂O and P₂O₅ were added with a 1.67 Ca/P atomic ratio, the same ratio as that of in human bone tissue. Each MAO-treated Ti specimen (MAO-Ti) was spin-coated with the precursor sol at a spin rate of 3000 rpm for 20 s. The sol was then decomposed and the coating was rendered amorphous by sintering in a flat furnace. The HA coating was obtained by repeating the spin coating and sintering processes 10 times. Finally, the amorphous HA was completely crystallized by a rapid annealing process where in the temperature was raised to 650°C within 7 s, maintained there for 180 s, and reduced to 30°C within 30 min. This technique promoted crystallization and strengthened the adhesion between the coating and Ti substrate.

The specimens were ultrasonically cleaned by acetone, absolute ethanol, and deionized water, applying each cleaner for 5 min, and then sterilized under a high temperature (134°C) and a high pressure (202.8 kPa) for 4 min. The specimens were divided into the following five groups: Ti; MAO-Ti; MAO-Ti+HA; MAO-Ti+HA+Zn(Low); MAO-Ti+HA+Zn(High).

Surface characterization
The surface morphologies of different groups were evaluated by scanning electron microscopy (SEM; S-4800, Hitachi, Tokyo, Japan). The phase and composition of the surface layer were analyzed by X-ray diffraction (XRD; Bruker D8 Advance, Bruker, Karlsruhe, Germany) and energy dispersive spectroscopy (EDS) which was incorporated in the SEM, respectively; a roughness tester (JB-4C, Shanghai Precision Instrument, China) measured the surface roughness of the specimens.

In vitro cell test
The biological properties of the specimens were evaluated by in vitro cell tests. The ilia of 4-week-old male rabbits were used as the source of bone marrow mesenchymal stem cells (BMSCs). Dulbecco’s modified Eagle’s medium (DMEM, Life Technologies, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS, Life Technologies), was used as the culturing medium. The cells were cultured with DMEM in incubators containing 5% CO₂ at 37°C. Cell passage numbers 3 and 4 were used to characterize the proliferation and differentiation of the cells. Six specimens from each of the five groups were tested for cell proliferation and Alkaline phosphatase (ALP) activity. The protocol was approved.
Cell morphology: The BMSCs were seeded in 12-well culture dishes, each specimen in the five groups at a seeding density of 1×10⁶ cells per well and then cultured at 37°C in a humidified 5% CO₂ atmosphere for 24 h. The morphologies of the proliferated cells were observed by SEM after fixation with 2.5% glutaraldehyde, dehydration with graded ethanol (70%, 90% and 100%), and critical point drying using CO₂.

Cell proliferation: The proliferation of BMSCs on each specimen in the five groups was determined by the CCK-8 assay. BMSCs were seeded on each specimen at a density of 2×10⁵ cells per well and 500 μL of DMEM were then added to each well in the 12-well culture dishes, the culturing medium was changed every two days. On the first, third, and seventh days after inoculation, 50 μL of CCK-8 assay (CK04, Dojindo, Kumamoto, Japan) were then added to each well and maintained at 37°C for 4 h in a humidified atmosphere of 5% CO₂ in air. 100 μL of the solution in each well was transferred to a 96-well culture plate, and the optical density of the solution was evaluated using a micro-plate spectrophotometer at a wavelength of 450 nm in accordance with the protocol of the manufacturer.

ALP activity and staining: ALP activity is regarded as a marker for the early stages of cell differentiation, which is sensitive to chemical composition. We examined the effects of the specimens on the osteogenic differentiation of BMSCs by quantitative ALP and ALP staining. BMSCs with a density of 2×10⁵ cells per well in the 12-well culture dishes were seeded on six specimens from each of the five groups, and BMSCs were cultured with osteogenic induction medium after two days later. The ALP activity was quantified at three and seven days after BMSC inoculation to evaluate early osteoblastic differentiation using an ALP assay kit (Nanjing Jiangcheng, Nanjing, China), following the manufacturer’s protocol. ALP activity was tested by detecting the optical density values at 520 nm. Ten days after BMSC inoculation, each group of cells was washed with PBS (Gibco, NY, USA), fixed by 75% ethanol for 1 h at 4°C and stained by an ALP kit (NobleRyder, Beijing, China) according to the manufacturer’s protocol.

Antimicrobial assay
Gram-negative anaerobic bacteria (Porphyromonas gingivalis) were selected as the primary pathogenic microbe for testing the anti-adhesion performance of the specimen surfaces, as observed by the SEM. The specimens were cultured in 1×10⁶ CFU/mL bacterial suspensions for 24 h. The surfaces were then rinsed with PBS three times, fixed with 2.5% glutaraldehyde solution at 4°C, dehydrated in the graded ethanol series for 10 min at each grade, freeze-dried, coated with gold, and observed by SEM.

Plate-counting schemes were used to investigate the antimicrobial activity of the specimens. A 200 μL suspension with a concentration of 1×10⁶ CFU/mL was dripped on the sterilized specimen surface and incubated at 37°C for 24 h in an anaerobic tank. After adding 5.0 mL of the sterilized PBS solution to each dish, the dish was vortexed for 3 min and then the solution in the dish was diluted 10 times. Next, 100 μL of the diluted bacterial suspension from the specimen surface was sprayed over Petri dishes filled with solid culture medium, and then the Petri dishes were incubated at 37°C for 7 days in an anaerobic tank. Finally, the Porphyromonas gingivalis colonies were counted. Five specimens from group Ti; MAO-Ti; MAO-Ti+HA; MAO-Ti+HA+Zn(Low); MAO-Ti+HA+Zn(High) were tested, and the mean colony count was calculated for each group.

Statistical analysis
The experimental data from this study were presented as the mean±standard deviation and analyzed in SPSS 20.0 software. Statistical analyses were conducted via a one-way analysis of variance (ANOVA) among the five groups. The non-parametric test was used in antimicrobial activity test. The differences were considered to be significant at p<0.05.

RESULTS
Coating characterization
Figure 2 shows the surface morphologies of the five groups. In group Ti, only the machining grooves were observed on the surface. After the MAO treatment (group MAO-Ti), the surface was rough and porous, with a structure similar to that of trabecular bone. In groups MAO-Ti+HA and MAO-Ti+HA+Zn(Low), the surface layer was uniformly porous, and some of the large pores were slightly smaller, possibly because the larger pores were filled with gel. In group MAO-Ti+HA+Zn(High), the surface morphology was even more uniform. The surface roughnesses of 15 specimens from each group were measured, and the results are shown in Table 1. The surface treatments increased the roughness, with the greatest roughness measured from the MAO-Ti+HA group. With increased Zn in the gel, the roughness decreased, and the results were statistically significant (p<0.05).

The chemical compositions of the coatings were determined by EDS analysis, as shown in Fig. 3. The MAO-Ti group had Ti, O, and P elements in the coating, indicating that O and P were incorporated into the TiO₂ layer during the MAO process. In the group MAO-Ti+HA, the Ca/P mass ratio and the atomic mass ratio was 1.61 and 1.25 in the coating, respectively; in the group MAO-Ti+HA+Zn(Low), the mass percentage and atomic percentage of Zn was 1.82% and 0.73%, respectively, and the Ca/P mass ratio was 1.52 with the atomic ratio of 1.18. In the group MAO-Ti+HA+Zn(High), the mass percentage and atomic percentage of Zn was 7.45% and 3.03%, respectively, and the Ca/P mass ratio was 1.21 with the atomic ratio of 0.92. The coating phases were characterized by XRD analysis, as shown in Fig. 4. A large amount of anatase phase TiO₂ and a small amount of rutile phase TiO₂ were detected in group Ti-MAO. In
groups MAO-Ti+HA, MAO-Ti+HA+Zn(Low), and MAO-
Ti+HA+Zn(High), HA phase and CA₆(PO₄)₂ phase were
observed; however, the Zn compounds were not detected.
The XRD semi-quantitative (S-Q) analysis results were,
namely: Ti 100% in the Ti group; Ti 62.9%, TiO₂ (anatase)
34.7%, TiO₂ (rutile) 2.4% in the MAO-Ti group; Ti 61.4%,
TiO₂ (anatase) 23.7%, HA 13.2%, CA₆(PO₄)₂ 1.7% in the
MAO-Ti+HA group; Ti 58.5%, TiO₂ (anatase) 30.6%, HA
9.0%, CA₆(PO₄)₂ 1.9% in the MAO-Ti+HA+Zn(Low) group;
Ti 50.9%, TiO₂ (anatase) 34.4%, HA 9.7%, CA₆(PO₄)₂ 5.0%
in the MAO-Ti+HA+Zn(High) group.

Cell responses
The morphologies of the BMSCs, which proliferated on
the specimens during culturing for 24 h, are shown in
Fig. 5. The SEM images show that the BMSCs on group
Ti spread relatively poorly with an oblate shape. The cells
on groups MAO-Ti and MAO-Ti+HA+Zn(Low) showed
slightly less extended cell membranes, and the filopodia
of the cells were short and rare. On groups MAO-Ti+HA
and MAO-Ti+HA+Zn(High), the cells spread out more
actively, and the cells’ stretched filopodia attached
tightly to the specimen surfaces.

Proliferation of BMSCs: Fig. 6 shows the
proliferative abilities of BMSCs cultured with specimens
of each group. These results no significant differences
in the proliferation effects of the different surfaces
after culturing for one or three days. However, at seven
days, the absorbances of groups MAO-Ti+HA and MAO-
Ti+HA+Zn(High) were higher than those of groups
Ti, MAO-Ti and MAO-Ti+HA+Zn(Low), although the
difference between groups MAO-Ti+HA and MAO-
Ti+HA+Zn(High) was not statistically significant
(p=0.116).

ALP activity and expression: ALP chemical staining
was performed on specimens from each of the five
groups after ten days of cultivation with BMSCs, and
the results were recorded using a camera (A7R3, Sony,
Japan), as shown in Figs. 7(a–e). The ALP staining
showed significantly more positive areas in groups
MAO-Ti+HA and MAO-Ti+HA+Zn(High) than in groups
Ti, MAO-Ti, and MAO-Ti+HA+Zn(Low). Group MAO-
Ti+HA exhibited particularly large and densely nodular
positive areas. Fig. 7(f) compares the ALP activities
of the BMSCs cultured for three and seven days on the
different specimens. In groups MAO-Ti+HA or MAO-
Ti+HA+Zn(High), the ALP quantitative detection was
significantly higher compared than in groups Ti, MAO-Ti
and MAO-Ti+HA+Zn(Low) at two time points (p<0.05),
although the difference between groups MAO-Ti+HA and
MAO-Ti+HA+Zn(High) was not statistically significant
(p=0.754 and p=0.465 at two time points). These results
showed that the ionic components and concentration
of MAO-Ti+HA and MAO-Ti+HA+Zn(High) promoted the
osteogenic differentiation of BMSCs, however, we should
pay attention that the group MAO-Ti+HA+Zn(Low)
contained the same ionic components, even could be
considered an intermediate between these two groups,
and yet did not show the same results.

Table 1 Specimen roughness in the five groups (±s)*

|          | Ti     | MAO-Ti | MAO-Ti+HA | MAO-Ti+HA+Zn (Low) | MAO-Ti+HA+Zn (High) |
|----------|--------|--------|-----------|--------------------|---------------------|
| Ra       | 0.13±0.02 | 0.98±0.07 | 1.17±0.10 | 0.76±0.10          | 0.59±0.05           |

* All results were statistically significant according to one-way ANOVA (p<0.05).
Fig. 3  Surface chemical composition of group: a) Ti, b) MAO-Ti, c) MAO-Ti+HA, d) MAO-Ti+HA+Zn(Low), and e) MAO-Ti+HA+Zn(High).

Fig. 4  The XRD patterns of group: a) Ti, b) MAO-Ti, c) MAO-Ti+HA, d) MAO-Ti+HA+Zn(Low), and e) MAO-Ti+HA+Zn(High).
Fig. 5 SEM images (left column: ×1,000; right column: ×2,000) of BMSC morphologies after culturing for 24 h on specimens of group a) Ti, b) MAO-Ti, c) MAO-Ti+HA, d) MAO-Ti+HA+Zn(Low), e) MAO-Ti+HA+Zn(High).

Antibacterial activity
Figure 8 shows the bacterial adhesion on the surface of specimens in each group Figs. 8(a–e). A large number of bacteria adhered to the surfaces of specimens in groups MAO-Ti, and these more porous surfaces may have provided a better environment for bacteria retention. Fewer bacteria adhered to the surfaces of specimens in groups Ti, MAO-Ti+HA, and MAO-Ti+HA+Zn(Low); this was probably due to the different surface morphologies of the specimens. The surfaces of specimens in group MAO-Ti+HA+Zn(High) show the least number of bacteria Fig. 8(e).

Figure 9 shows the number of bacterial colonies generated by incubation at 37°C for 24 h in anaerobic environments with specimens from group Ti, MAO-

**Fig. 6** Proliferative abilities of BMSCs cultured with specimens of group: a) Ti, b) MAO-Ti, c) MAO-Ti+HA, d) MAO-Ti+HA+Zn(Low), e) MAO-Ti+HA+Zn(High), detected by CCK-8 assays at 450 nm; *: the absorbances of groups c and e were higher than those of groups a, b and d, but no statistical difference between groups c and e (p=0.116).

**Fig. 7** ALP chemical staining of specimens in group: a) Ti, b) MAO-Ti, c) MAO-Ti+HA, d) MAO-Ti+HA+Zn(Low), and e) MAO-Ti+HA+Zn(High). f) Comparison of ALP activity in the five groups of BMSCs detected after three and seven days of cultivation.

Ti, MAO-Ti+HA, MAO-Ti+HA+Zn(Low) and MAO-Ti+HA+Zn(High). A large number of bacterial colonies were generated by groups Ti, MAO-Ti, less bacterial colonies were generated by groups MAO-Ti+HA and MAO-Ti+HA+Zn(Low), group MAO-Ti+HA+Zn(High) showed the fewest bacterial colonies. The median of
DISCUSSION

Although Ti and Ti alloys are widely used clinically, failures still occur mainly because of deficient osseointegration or implant-associated infections\(^{20}\). Next-generation implant materials with biologically favorable coatings that support osteogenesis and antibacterial actions are of scientific and clinical significance\(^{21}\). Sato et al.\(^{22}\) tried to form yttrium-doped nanocrystalline HA coatings on Ti to promote osteoblast functions, and that work motivated the combination of HA and Zn used here to support biological and antimicrobial activities.

Zn stimulates osteoblast proliferation and mineralization, promotes osteoblast marker gene expression and calcium deposition, inhibits bone resorption by reducing osteoclast formation and adsorbing ability, and has anti-inflammatory properties\(^{13,14}\). However, Zn effects are dose dependent, and excessive or insufficient doses can induce toxicity\(^{23}\). Adding the ideal Zn content in the oxide layer is difficult to achieve with a single technique. However, combining MAO with sol-gel deposition is a simple, controllable, and cost-effective method of adding the desired content in the surface layer.

Through the MAO treatment, the newly TiO\(_2\) ceramic coating was formed on the Ti substrate. As an intermediate layer, TiO\(_2\) decreased the elastic modulus and the stress concentration caused by the mismatched thermal expansion coefficients of the HA coating and the Ti substrate, thereby improving their bonding strength and stability\(^{24}\). The EDS results showed that combining the MAO and sol-gel processes resulted in surfaces containing Ca, P, and Zn. The incorporation of Ca or P ions into the surface layer can improve osteoblast cell responses and support osseointegration, as has been confirmed by several previous researchers\(^{25,26}\), and anatase TiO\(_2\) supports important biological activities such as mineralization and protein adsorption\(^{27}\). Characteristic HA peaks and Ca\(_3\)(PO\(_4\))\(_2\) peaks were observed in groups MAO-Ti+HA, MAO-Ti+HA+Zn(Low), and MAO-Ti+HA+Zn(High). The formation of the Ca\(_3\)(PO\(_4\))\(_2\) peak may occur because some of the HA could have lost one or more hydroxyl groups during the rapid annealing process. However, the XRD results did not show the presence of Zn compounds, possibly because the Zn content was below the detection level, or because the Zn was present in an amorphous form and entered the coating in the form of ions without being detected.

The beneficial effects of the composite coating were reflected in the in vitro cell responses, where the proliferation and ALP activity of groups MAO-Ti+HA and MAO-Ti+HA+Zn(High) were significantly improved. In these groups, the BMSCs were well-distributed in osteoblast-type cell shapes, demonstrating that the chemical composition and concentration of the surface can promote cell spread. Although Huo et al.\(^{21}\) reported that Zn incorporation in the surface could promote spread, the underlying mechanisms remained unclear. However, in this study, the cell spread promotion was

![Fig. 9](image)

**Fig. 9** Bacterial colonies incubated at 37°C for seven days on the surfaces of specimens in group: a) Ti, b) MAO-Ti, c) MAO-Ti+HA, d) MAO-Ti+HA+Zn(Low), and e) MAO-Ti+HA+Zn(High); the box plot of bacterial colonies for this five groups was showed in f); *: \(p<0.05\), **: \(p<0.01\), ***: \(p<0.001\) (non-parametric test).

Bacterial colonies incubated at 37°C for seven days on the surfaces of specimens in group: a) Ti, b) MAO-Ti, c) MAO-Ti+HA, d) MAO-Ti+HA+Zn(Low), and e) MAO-Ti+HA+Zn(High); the box plot of bacterial colonies for this five groups was showed in f); *: \(p<0.05\), **: \(p<0.01\), ***: \(p<0.001\) (non-parametric test).

![Fig. 8](image)

**Fig. 8** Bacterial adhesion on specimens in group: a) Ti, b) MAO-Ti, c) MAO-Ti+HA, d) MAO-Ti+HA+Zn(Low), and e) MAO-Ti+HA+Zn(High) incubated at 37°C for 24 h. In (e), at ×5,000 magnification, the surfaces of specimens in group MAO-Ti+HA+Zn(High) show the least number of bacteria. (f) The bacterial cell morphology at ×20,000 magnification.

Bacterial adhesion on specimens in group: a) Ti, b) MAO-Ti, c) MAO-Ti+HA, d) MAO-Ti+HA+Zn(Low), and e) MAO-Ti+HA+Zn(High) incubated at 37°C for 24 h. In (e), at ×5,000 magnification, the surfaces of specimens in group MAO-Ti+HA+Zn(High) show the least number of bacteria. (f) The bacterial cell morphology at ×20,000 magnification.
detected in group MAO-Ti+HA+Zn(High) but not in MAO-Ti+HA+Zn(Low), and therefore the dose dependency of Zn may also affect its support of cell spread. In this study, the ALP activity significantly increased in groups MAO-Ti+HA and MAO-Ti+HA+Zn(High), and these results were considered to be closely related to the HA and the amount of Zn ions contained in the oxide layer. However, the group MAO-Ti+HA+Zn(Low) contained the same ionic components but showed a decreased cell response activity, this may be attributed to the excessive low concentration of Zn in the coatings, which suggested that the content of ionic also play an important role in biocompatibility, the cytocompatibility can be obtained only with optimal Zn release\textsuperscript{28}. Therefore, although the Zn content in group MAO-Ti+HA+Zn(High) was higher, biocompatibility was controlled by a safe rate of Zn release. However, the amount of Zn released from the specimens of this study was unclear, and this should be investigated in our future work.

Peri-implant diseases are a common clinical complication, with high incidences of peri-implant mucositis (in approximately 80\% of subjects) and peri-implantitis (in 28\% to 56\% of subjects)\textsuperscript{29}. In most cases, the composition of the flora is similar to the subgingival flora of chronic periodontitis, which is dominated by Gram-negative bacteria. *Porphyromonas gingivalis* is a common periodontal pathogen that has been correlated with peri-implantitis, and this risk is increased by high concentrations of bacteria\textsuperscript{29}. The specimens of different groups in this study exhibited different adhesive and antibacterial abilities. Bacteria tended to colonize the rough and porous surfaces in groups MAO-Ti, whereas groups Ti, MAO-Ti+HA+Zn(Low) showed fewer bacteria. MAO-Ti+HA+Zn(High) shows the least number of bacteria and the fewest bacterial colonies. Although there is no statistical difference between groups MAO-Ti+HA+Zn(High) and MAO-Ti+HA, for the rank sum test which was used in antimicrobial activity test, the estimation of the difference is conservative, especially when there is a large difference in the number of bacterial colonies between groups. Within the limitations of this study, we infer that the difference between the MAO-Ti+HA and MAO-Ti+HA+Zn(High) has clinical significance. These results allow us to partially support the hypothesis that composite coatings containing HA and Zn ions could promote biocompatibility and reduce infection. Although anatase TiO\textsubscript{2} has been reported to reduce the adhesion of *Streptococcus oris*\textsuperscript{30}, the anatase TiO\textsubscript{2} detected in group MAO-Ti resulted in more *Porphyromonas gingivalis* adhered to the coatings. Therefore, both the type of bacteria and the surface morphology may play important roles in bacteria adhesion. The incorporated Zn inhibited bacterial colonization by generating reactive oxygen species, or because of synergistic effects of both the contained and released Zn\textsuperscript{14}. The antibacterial mechanism may also be related to the surface charges of the implants. When implants are immersed in a physiological liquid, surface ions (Zn\textsuperscript{2+}, Ca\textsuperscript{2+}) interact with negatively charged bacterial cell membranes, altering bacterial cell permeability and damaging the integrity of cell membranes, eventually leading to cytosolic leakage and bacterial cell death, although the mechanism requires further exploration\textsuperscript{31,32}. The high mass percentages of Zn in the layer are not necessarily released immediately, and a larger Zn loading capacity may be released gradually over time. However, further research is necessary to determine the Zn loading and release capacities in the specimens and also to identify changes in antibacterial ability over time. More detailed biological evaluations of the composite coating are in progress, and those results will be reported separately.

### CONCLUSIONS

In this paper, surface layers containing HA and different contents of Zn were coated by the sol-gel method on microarc-oxidized Ti (MAO-Ti) substrates, with the aim of improving biocompatibility and antibacterial activity. Within the limitations of this study, the following conclusions were derived: Group MAO-Ti+HA+Zn(High) showed the most optimal balance between bone promotion and antibacterial ability and has immense potential in orthopedics and other biomedical applications. However, the most optimum Zn concentration to support biological activity and the Zn loading mode of this composite coatings still remains unclear and needs to further research.

### FUNDINGS

The study was supported by the Discipline Construction Fund of Beijing Stomatological Hospital [grant number18-09-12], Beijing Natural Science Foundation [grant number 7172088] and National Natural Science Foundation of China [grant number 81974153].

### ACKNOWLEDGMENTS

The authors thank Xiyuan Wang for his assistance with the specimens’ surface treatments.

### CONFLICTS OF INTEREST

The authors declare no conflict of interest.

### REFERENCES

1. Ekelund JA, Lindquist LW, Carlsson GE, Jenet T. Implant treatment in the edentulous mandible: a prospective study on Branemark system implants over more than 20 years. Int J Prosthodont 2003; 16: 602-608.
2. Lindhe J, Meyle J. Peri-implant diseases: Consensus Report of the Sixth European Workshop on Periodontology. J Clin Periodontol 2008; 35: 282-285.
3. Kim HW, Koh YH, Li LH, Lee S, Kim HE. Hydroxyapatite coating on titanium substrate with titania buffer layer processed by sol-gel method. Biomaterials 2004; 25: 2533-2538.
4. de Groot K, Geesink R, Klein CPAT, Serekian P. Plasma-sprayed coatings of hydroxylapatite. J Biomed Mater Res 1987; 21: 1375-1381.
5) Son WW, Zhu X, Shin HJ, Ong JL, Kim KH. In vivo histological response to anodized and anodized/hydrothermally treated titanium implants. J Biomed Mater Res Part B Appl Biomater 2003; 66: 520-525.

6) Ishizawa H, Fujino M, Ogino M. Histomorphometric evaluation of the thin hydroxyapatite layer formed through anodization followed by hydrothermal treatment. J Biomed Mater Res 1997; 35: 199-206.

7) Mori S, Burr DB. Increased intracortical remodeling following fatigue damage. Bone 1995;14: 103-109.

8) Pulso D, Nanci A. Understanding and controlling the bone-implant interface. Biomaterials 1999; 20: 2311-2321.

9) Zhang F, Zhang Z, Zhu X, Kang ET, Neoh KG. Silk-functionalized titanium surfaces for enhancing osteoblast functions and reducing bacterial adhesion. Biomaterials 2008; 29: 4751-4759.

10) Hu X, Neoh KG, Shi Z, Kang ET, Poh C, Wang W. An in vitro assessment of titanium functionalized with polysaccharides conjugated with vascular endothelial growth factor for enhanced osseointegration and inhibition of bacterial adhesion. Biomaterials 2010; 31: 8854-8863.

11) Zhao L, Wang H, Huo K, Cui L, Zhang W, Ni H. Antibacterial nano-structured titania coating incorporated with silver nanoparticles. Biomaterials 2011; 32: 5706-5716.

12) Wu H, Zhang X, Geng Z, Yin Y, Hang R. Preparation, antibacterial effects and corrosion resistant of porous Cu–TiO2 coatings. Appl Surf Sci 2014; 308: 43-49.

13) Nagata M, Lonnerdal B. Role of zinc in cellular zinc trafficking and mineralization in a murine osteoblast-like cell line. J Nutr Biochem 2011; 22: 172-178.

14) Hu H, Zhang W, Qiao Y, Jiang X, Liu X, Ding C. Antibacterial activity and increased bone marrow stem cell functions of Zn-incorporated TiO2 coatings on titanium. Acta Biomater 2012; 8: 904-915.

15) Seil JT, Webster TJ. Reduced Staphylococcus aureus proliferation and biofilm formation on zinc oxide nanoparticle PVC composite surfaces. Acta Biomater 2011; 7: 2579-2584.

16) Li LH, Kong YM, Kim HW, Kim YW, Kim HE. Improved biological performance of Ti implants due to surface modification by micro-arc oxidation. Biomaterials 2004; 25: 2867-2875.

17) Krzakala A, Kazek-Kesik A, Simka W. Application of plasma electrolytic oxidation to bioactive surface formation on titanium and its alloys. RSC Adv 2013; 3: 19725-19743.

18) Guo HF, An MZ, Huo HB, Xu Ld. Microstructure characteristic of ceramic coatings fabricated on magnesium alloys by micro-arc oxidation in alkaline silicate solutions. Appl Surf Sci 2006; 252: 7911-7916.

19) Li LH, Kim HW, Lee SH, Kong YM, Kim HE. Biocompatibility of titanium implants modified by microarc oxidation and hydroxyapatite coating. J Biomed Mater Res A 2005; 73: 48-54.

20) Zhang W, Li Z, Liu Y, Ye D, Li J, Xu L. Biofunctionalization of a titanium surface with a nano-sawtooth structure regulates the behavior of rat bone marrow mesenchymal stem cells. Int J Nanomedicine 2012; 7: 4459-4472.

21) Hoo K, Zhang X, Wang H, Zhao, Liu X. Osteogenic activity and antibacterial effects on titanium surfaces modified with Zn-incorporated nanotube arrays. Biomaterials 2013; 34:3467-3478.

22) Sato M, Sambito MA, Aalani A, Kalkhoran NM, Slamovich EB. Increased osteoblast functions on undoped and yttrium-doped nanocrystalline hydroxyapatite coatings on titanium. Biomaterials 2006; 27: 2358-2369.

23) Saino E, Grandi S, Quartarone E, Mialiardi V, Galli D, Bloise N. In vitro calcified matrix deposition by human osteoblasts onto a zinc-containing bioactive glass. Eur Cell Mater 2011; 21: 59-72.

24) Xiu P, Jia Z, Lv J, Cheng Z. Tailored surface treatment of 3d printed porous Ti6Al4V by microarc oxidation for enhanced osseointegration via optimized bone in-growth patterns and interlocked bone/implant interface. ACS Appl Mater Interfaces 2016; 8: 17964-17975.

25) Hanawa T, Kamiura Y, Yamamoto S, Kohgo T, Amemiya A, Ukai H, et al. Early bone formation around calcium-ion-implanted titanium inserted into rat tibia. J Biomed Mater Res 1997; 36: 131-136.

26) Ishizawa H, Ogino M. Formation and characterization of anodic titanium oxide films containing Ca and P. J Biomed Mater Res 1995; 29: 65-72.

27) Heng Y, Yu M, Liu J. Surface hydroxyl groups direct cellular response on amorphous and anatase TiO2 nanodots. Colloids Surf B Biointerfaces 2014; 123: 68-74.

28) Tenenbaum H, Bogen O, Sèverac F, Elkaim R, Davideau JL. Long-term prospective cohort study on dental implants: clinical and microbiological parameters. Clin Oral Implants Res 2016; 28: 86-94.

29) Persson GR, Revnert S. Cluster of bacteria associated with peri-implantitis. Clin Implant Dent Relat Res 2014; 16: 783-789.

30) Dizaj SM, Lotfipour F, Barzegar-Jalali M, Zarrintan MH, Adibkia K. Antimicrobial activity of the metals and metal oxide nanoparticles. Mater Sci Eng C Mater Biol Appl 2014; 44: 278-284.

31) Rabea EI, Badawy ET, Stevens CV, Smaghe G, Steurbaut W. Chitosan as antimicrobial agent: Applications and mode of action. Biomacromolecules 2003; 4: 1457-1465.

32) Qin H, Zhao Y, An Z, Cheng M, Wang Q. Enhanced antibacterial properties, biocompatibility, and corrosion resistance of degradable Mg-Nd-Zn-Zr alloy. Biomaterials 2015; 53: 211-220.