Nano Ag/ZnO-Incorporated Hydroxyapatite Composite Coatings: Highly Effective Infection Prevention and Excellent Osteointegration

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* Supporting Information

ABSTRACT: Interfacial characteristics play an important role in infection prevention and osteointegration of artificial bone implants. In this work, both Ag nanoparticles (AgNPs) and ZnO NPs are incorporated into hydroxyapatite (HA) nanopowders and deposited onto Ti6Al4V (Ti6) implants by laser cladding. The composite coatings possess a hierarchical surface structure with homogeneous distributions of Ag and ZnO. The Ag and ZnO NPs that are immobilized by laser cladding ensure long-term and gradual release of Ag and Zn ions at low cumulative concentrations of 36.2 and 56.4 μg/L after immersion for 21 days. A large concentration of Ag released initially increases the cytotoxicity but the large initial ZnO content enhances the cell viability and osteogenetic ability. The nano Ag/ZnO-embedded HA coating (Ag/ZnO/HA = 7:3:90 wt %, namely Ag7ZnO3HA) exhibits optimal antibacterial efficacy and osteogenetic capability, as exemplified by the broad spectrum antibacterial efficacy of 96.5 and 85.8% against Escherichia coli (E. coli) and Staphylococcus aureus (S. aureus), respectively, together with enhanced osteoinductivity with higher alkaline phosphatase (ALP) activity of 134.60 U/g protein compared to 70.79 U/g protein for the untreated implants after culturing for 7 days. The rabbit femoral implant model further confirms that the optimized composite coating accelerates the formation of new bone tissues indicating 87.15% of the newly formed bone area and osteointegration showing 83.75% of the bone−implant contact area even in the presence of injected S. aureus. The laser-cladded Ag7ZnO3HA composite coatings are promising metallic implants with excellent intrinsic antibacterial activity and osteointegration ability.

KEYWORDS: antibacterial, implant, osteointegration, laser cladding, composite coating, infection prevention

1. INTRODUCTION

There is increasing demand for artificial orthopedic implants because of rapid development of orthomorphia, increasing incidents of fracture and joint degeneration, and bone defects due to aging population.1 For example, in the United States, the market for total hip implants is about 572,000 annually, and the demand for total knee arthroplasties is estimated to be 3.48 million by 2030.2 Because of the shortage of autograft bone and immunorejection of allograft bone, the development of artificial implant materials is important and necessary. Clinically, metallic biomaterials are commonly used in the fixation of bone fracture and implantation of joint prostheses during the healing process because they possess good mechanical strength, low modulus of elasticity, and acceptable biocompatibility. In particular, Ti6Al4V (Ti6) has been extensively studied for orthopedic applications.3,4 However, many biomaterials are bioinert, thus delaying the healing process, and bacterial infection often occurs because they do not possess inherent antibacterial characteristics, leading to implant failure.

Clinically, implant-related bacterial infection is commonly treated by antibiotics, but abuse of antibiotics can lead to bacterial resistance.5 In addition, the therapeutic effects of antibiotics are poor after bacterial biofilms form on the implants. To combat and resist bacterial infection, design of antiadhesive surfaces and release of antimicrobial agents, antibacterial coatings, nanostructured materials, and molecules...
including antibiotics interfering with bacterial biofilms are viable strategies.\textsuperscript{6–13} To avoid drug resistance, inorganic antimicrobial agents have been widely incorporated into coatings to kill bacteria because of the excellent electrical and optical properties.\textsuperscript{14–16} Among them, Ag nanoparticles (AgNPs) and ZnO NPs exhibit a broad-spectrum antimicrobial ability via release of the corresponding ions or production of radical oxygen species (ROS) under light irradiation.\textsuperscript{8,17–20} In addition, a combination of multiple inorganic NPs can produce better effects than a single component because of the synergistic effects.\textsuperscript{21–24} However, some side effects may be introduced by these methods. For example, release of inorganic NPs can cause toxicity,\textsuperscript{14,25} and a large concentration of Ag ions induces cytotoxicity.\textsuperscript{26} Furthermore, many methods cannot improve the osteogenic ability of bioinert implants while maintaining the effective antibacterial activity. The poor bonding between the implants and newly formed bone tissues can also lead to the loosening of implants and final failure,\textsuperscript{27} and so it is urgent to develop new coatings with good osteoinductivity and antimicrobial ability at the same time.

Hydroxyapatite (HA) with almost the same chemical composition as natural bone and tooth has excellent biocompatibility and osteoinductivity.\textsuperscript{28} In addition, nanoscale HA can bind fibronectin and vitronectin, which are the key ligands of integrins playing important roles in cell adhesion.\textsuperscript{29–31} Besides the antibacterial performance, ZnO shows excellent osteogenic properties by affecting bone metabolism. It can promote the expression of osteoblast marker gene and also stimulate mineralization of osteoblasts by depositing calcium on mesenchymal stem cells.\textsuperscript{32,33} In view of this, the combination of HA, AgNPs, and ZnO NPs may produce the dual function of intrinsic bacterial resistance and osteoinductivity. However, it is difficult to immobilize the three components on the surface of metallic implants simultaneously.

Laser cladding can change the specific surface area, surface roughness, and wettability of implants to affect adhesion and differentiation of osteoblasts as well as Ca\textsuperscript{2+} absorption.\textsuperscript{34–37} Furthermore, the roughness and wettability of implants influence the bacterial resistance and inflammation,\textsuperscript{38,39} but the most important issue for ceramic powders is how to fix them tightly on the surface of metallic implants.\textsuperscript{40} In this work, composite coatings composed of AgNPs, ZnO NPs, and HA nanopowders with different ratios are prepared on the Ti6Al4V alloy by laser cladding, and the antibacterial efficacy and osteointegration are evaluated in vitro and in vivo. The process is schematically illustrated in Scheme 1.

2. EXPERIMENTAL PROCEDURE

2.1. Material Preparation. 2.1.1. Ti6Al4V Pretreatment. The Ti6Al4V (Ti6) (Shanghai Baosteel Co. Ltd., China) sample was cut into disks with dimensions of 6 mm × 2.5 mm. The Ti6Al4V plates were ground by SiC paper (240 grits), ultrasonically cleaned with ethanol and deionized (DI) water successively for 30 min, and dried in an oven at 37 °C for further use.

2.1.2. Preparation of Mixed Powders. Five powder samples with different ratios of AgNPs, ZnO NPs, and HA nanopowders were prepared (Table 1). The HA nanopowders (purity >99.5%, BET, Aladdin) had an average particle size of about 100 nm, and ZnO NPs (purity >99.8%, BET, Aladdin) were about 50 nm in size. The AgNPs were synthesized by a chemical reduction method. Briefly, the AgNO\textsubscript{3} crystal particles were dissolved in 30% aqueous ammonia. The HA powders were dissolved in DI water and sonicated for 30 min after adding the 5% dispersing agent of polyvinyl pyrrolidone. The HA solution was stirred for 1 h using a magnetic stirrer, and then the silver ammonia solution was added to the HA solution. The aqueous hydrazide hydrate (N\textsubscript{2}H\textsubscript{4}) solution was added dropwise to reduce and form metallic silver. During the reduction reaction, the white solution gradually changed to gray, and after the solution became gray, the nano Ag/HA powders were filtered, rinsed with alcohol and DI water several times, and dried in an oven at 60 °C. Finally, the Ag/HA mixed with ZnO NPs were ground by a quartz mortar. Composites with different amounts of silver were prepared by adjusting the AgNO\textsubscript{3} solution, and different ZnO contents were determined by weighing. The Ag/ZnO/HA (Ag\textsubscript{x}Zn\textsubscript{y}O\textsubscript{z}HA) composite powders were prepared in a 10 mg/mL suspension in DI water, with proportions shown in Table 1. Ag\textsubscript{x}Zn\textsubscript{y}O\textsubscript{z}HA suspension (100 μL) was added to the Ti6 substrate and dried at 60 °C in a vacuum oven prior to cladding.

2.1.3. Laser Cladding. Laser cladding was performed using a CW 2 kW Nd:YAG laser (wavelength 1.06 μm, Lumonics, JHM-1GY-300B)
2.2. Material Characterization. The microstructures and composition of the composite coatings were characterized by transmission electron microscopy (TEM; Tecnai G20, FEI, USA), selected-area electron diffraction (SAED), and energy dispersive X-ray spectrometry (EDS). The surface morphology was examined by field emission scanning electron microscopy (JSM7100F), and the chemical states were determined by X-ray photoelectron spectroscopy (XPS, Thermo Fisher Scientific 250Xi) with Al Kα radiation (1486.6 eV).

2.3. Zn2+ and Ag+ Release. The samples were immersed in 30 mL of simulated body fluid (SBF) at 37°C for 1, 4, 7, 14, and 21 days, separately, and the solution was collected and refilled with 3 mL of fresh SBF at preset intervals. The Zn2+ and Ag+ concentrations in the solution were determined by atomic absorption spectrophotometry (AAS W006, Analytik Jena, Germany).

2.4. Contact Angle Measurement. The contact angles were measured at room temperature on a contact angle instrument (OCA40 Filderstadt, Germany), with DI water as the medium. The average calculated from three drops at different positions was calculated.

2.5. Antibacterial Activity. 2.5.1. Spread Plate. To investigate the antibacterial activity of the laser cladding AgsZnO3HA coatings, Gram-negative Escherichia coli (E. coli, ATCC 8099) and Gram-positive Staphylococcus aureus (S. aureus, ATCC 25923) of standard strains were used. The bacteria were cultured in the Luria–Bertani (LB) culture medium and LB broth, and the LB agar plates were sterilized by autoclaving at 121°C for 15 min. Three samples in each group were tested to obtain averages. The samples were placed on a 48-well plate, and 400 μL of the diluted E. coli suspension (1 × 106 CFU/mL) in the sterile phosphate-buffered saline (PBS) broth was kept in a biological safety cabinet and incubated at 37°C for 12 h. Afterward, the E. coli suspension in each hole was diluted 100-fold by sterile PBS, and 20 μL of the diluted E. coli suspension was drawn from each hole to spread on LB agar plates and incubated in the oven at 37°C for 24 h. The same experimental protocol was adopted for S. aureus. The antibacterial efficiency of the samples was calculated by the following formula according to the number of colonies on the plate

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\text{Antibacterial efficiency (\%)} = \frac{\text{control numbers} - \text{experiment numbers}}{\text{control numbers}} \times 100\% \tag{1}
\]

2.5.2. Bacterial Morphology. The bacteria on the samples were fixed with 2.5% glutaraldehyde for 2 h, dehydrated sequentially in a series of ethanol with different concentrations (30, 50, 70, 90, and 100 v/v %) for 15 min each, and freeze-dried. The morphology of the bacteria was observed by scanning electron microscopy (SEM) after gold plating.

2.6. Cell Culture. The mouse calvarial cell line MC3T3-E1 was used in this study. The cells were usually cultured in a dish in a medium composed of α-MEM (HyClone) supplemented with 1% penicillin–streptomycin (HyClone) and 10% fetal bovine serum in an atmosphere of 5% CO2 at 37°C in an incubator. The cells were treated with sterilized PBS and trypsine/ethylenediaminetetraacetic acid (Sigma) to remove dead ones and then diluted to a density of 1 × 103 cells/mL for further use.

2.6.1. Cell Viability and Proliferation. The cell viability was assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. The sterilized samples were put on 96-well culture plates in 150 μL of the medium with a density of 1 × 104 cells/mL in each well and incubated for 1, 3, and 7 days, separately. After removing the medium from each well, 100 μL of MTT solution (0.5 mg/mL) was added and incubated for 4 h at 37°C until the ianthinus precipitate was visible. The MTT solution was removed, and 100 μL of dimethyl sulfoxide was added to each well to dissolve the ianthinus precipitate. Finally, the samples were taken out, and the optical density at 490 nm was determined on a microplate reader (SpectraMax i3, Molecular Devices). The cell viability was calculated by the ratio of the experimental group and control group. The culture medium was refreshed every 3 days, and experiments were done on three parallel groups.

2.6.2. Osteogenic Differentiation. The osteogenic activity was observed by the alkaline phosphatase (ALP) activity assays according to the manufacturer’s instruction of the microplate test kit (ALP assay kit). The samples were put on 96-well culture plates, and 150 μL of the medium with a density of 1 × 105 cells/mL was added to each well and incubated for 3, 7, and 14 days, separately. After incubation, the medium was sucked out from each well, and 1% Triton X-100 was added. The ALP activity was measured at 520 nm on a microplate reader. Besides, the protein content of the cell in each well was tested using the bicinchoninic acid protein assay kit (Solorbio) at 562 nm on a microplate reader, which can get the ALP activity per unit protein. The culture medium was refreshed every 3 days, and triplicate experiments were performed.

2.6.3. Osteogenic Mineralization. Three groups were compared in this experiment. Each group includes the sterilized samples of Ti6, HA–Ti6, and Ag7ZnO3–Ti6 which were placed on four 96-well plates with 150 μL of the medium with a density of 1 × 105 cells/mL in each well. The medium was refreshed every 3 days. After culturing for 10 days, one group was examined by SEM and EDS to observe the cell morphology and determine the elements. Another group was observed for mineralized nodules after alizarin red staining. Calculation was performed after culturing for 20 days, and the same protocol was implemented on the other two groups. Three parallel experiments were conducted for each group.

2.7. In Vivo Animal Tests. 2.7.1. Surgical Protocol. The in vivo animal experiments were approved by Wuhan Service Biotechnology Co., Ltd., China. Adult male New Zealand white rabbits weighing about 2.5 kg were obtained from the Wuhan Center for Disease Prevention & Control, and 12 rabbits were randomly divided into 2 groups, that is, 6 rabbits in the control group (Ti6) and the others in the experimental group (Ag7ZnO3–Ti6). Before implanting the samples into the rabbit femur, 3% pentobarbital sodium salt was used as an anesthetic to treat the rabbit (1 mL/kg). The hair on the right leg was shaved and disinfected with 1% povidone iodine, and sterilized surgical instruments were used to perform surgery along the direction of the femoral axis. The skin and muscle of the rabbit femur were cut until the femur was exposed. The implants were seeded with 20 μL of S. aureus (108 CFU/mL), and a suitable gap was sawed from the exposed femur. The sample was implanted into the femur and placed into the bone marrow cavity (Figure S7a), and the incision was stitched. The other rabbits underwent the same surgical protocol, and all rabbits were housed in individual sterilized cages.

2.7.2. Characterization. After 2 weeks, three rabbits from each group were sacrificed by injection of excessive anesthetics. The soft and bone tissues in contact with the samples were extracted to evaluate bacterial infection, by hematoxylin and eosin (H&E) staining and Giemsa staining. At 5 weeks postsurgery, the remaining rabbits were sacrificed to assess tissue inflammation and osteogenesis. These evaluation methods included X-ray photography, micro-computed tomography (CT) analysis, H&E staining, Giemsa staining, and van Gieson’s picrofuchsin staining.

2.7.3. X-ray and Micro-CT Assessment. At 5 weeks postsurgery, X-ray pictures were taken from the sacrificed rabbits to observe bone growth. High-resolution micro-CT (Micro-CT; SkyScan 1176) was conducted at 60 kV and 400 μA. A 1 mm aluminum filter was used to determine the newly formed bone around the implants, and two-dimensional (2D) and three-dimensional (3D) osteogenic models were constructed using the micro-CT scanning software.
2.7.4. Histo-Pathological Evaluation. H&E staining and Giemsa staining were used in the histological analysis to evaluate the inflammatory response of soft tissues and bones around the implants. At 2 and 5 weeks after operation, the soft tissues and bones were fixed in 4% paraformaldehyde for 24 h, and the bone tissues were additionally treated with decalcification for a month. The samples were dehydrated for 1 h in each stage with graded alcohol (75, 85, 90, 95, and 100%) separately and treated with xylene. The tissues were embedded into paraffin and sectioned using a microtome (Leica RM2016, Leica Microsystems, Germany). The histological sections were divided into two groups for H&E staining and Giemsa staining, and the inflammatory response and bacterial infection were examined by optical microscopy.

New bone formation around the implants was evaluated by van Gieson’s picrofuchsin staining. At 5 weeks after operation, the implants and surrounding bone tissues were cut off together and fixed in 4% paraformaldehyde for 3 days. The hard tissues were then dehydrated with graded alcohol (70, 90, 95, and 100%) separately and treated with xylene for 3 days. After sealing with bimethyl methacrylate, the embedded hard tissues were sliced using a microtome and polished to about 10 μm. The histological sections were stained with van Gieson’s picrofuchsin. The new bones were dyed red and observed under an optical microscope. The newly formed bone rates and the contact rate between the bone and implants were determined by ImageJ.

2.8. Statistical Analysis. The results were calculated as means ± standard deviation (SD) based on at least three experiments, according to Kruskal–Wallis in the one-way analysis of variance.

3. RESULTS AND DISCUSSION

3.1. Surface Characterization. The mixed powders of AgxZnOyHA were characterized by TEM. As shown in Figure S1a, the commercial HA nanopowders are composed of many needlelike nanorods. The SAED pattern shows that these nanorods have different orientations (Figure S1b). The mixed powders with reduced AgNPs exhibit similar structures (Figure S1c) and SAED patterns (Figure S1d) because of the small amount of AgNPs from the AgNO3 solution. The size of the AgNPs ranges from 30 to 60 nm. As shown in Figure S1e, O, Ag, Ca, and P are detected by EDS, demonstrating the coexistence of HA and Ag in the mixed powders. The copper signal originates from the copper mesh.

The surface morphology of the AgxZnOyHA composite coating on Ti6 in Figure 1a shows a regular texture with parallel ridges because rapid melting and freezing occur as the laser spot moves back and forth quickly on the surface. There are bulging areas (zone 1) and flat areas (zone 2). The high-magnification image of the bulging areas (Figure 1b) reveals raised molten peaks, whereas that of flat areas shows a loccular nanostructure (Figure 1c). The cross-sectional SEM image of AgxZnOyHA–Ti6 shows good bonding between the coating and substrate, and the largest cladding depth is about 460 μm, as indicated by red arrows (Figure 1d). In the Ag5ZnO5HA–Ti6 sample, the
EDS scan discloses homogeneous distributions of Ca, P, Ti, Zn, and Ag in the coating (Figure 1e), confirmed by the XPS survey scan (Figure 2a). As shown in Figure 2b, the XPS narrow scan discloses Ag 3d$_{5/2}$ and Ag 3d$_{3/2}$ peaks at 368.0 and 374.0 eV, respectively, and the difference between Ag 3d$_{5/2}$ and Ag 3d$_{3/2}$ is 6.0, indicative of the metallic state in the coating.41

As shown in Figure S2, the contact angle on the untreated Ti6 is 73.7°, whereas that on the laser-treated Ti6 (L-Ti6) decreases to 24°. After cladding of HA, the contact angles on Ag$_x$Zn$_y$O$_z$HA decrease further, with the HA-cladded sample showing the smallest contact angle of 10.5°. On flat surfaces, the wettability depends on the surface free energy that can be expressed by Young’s equation

$$\gamma_{sv} = \gamma_{sl} + \gamma_{lv} \cos \theta_Y$$

(2)

where $\theta_Y$ is the contact angle, $\gamma$ refers to the surface tension (or surface free energy), and s, l, and v represent the solid, liquid, and gas, respectively. For the droplet on the untreated Ti6, the relationship between the contact angle and surface tension is expressed by eq 2, but for uneven surfaces, the surface roughness plays an important role in the wettability. The relationship between the roughness and contact angle is expressed by Wenzel equation

$$\cos \theta_w = r \cos \theta_Y$$

(3)

where $r$ represents the surface roughness factor, $\theta_w$ is the contact angle in the Wenzel model, and $\theta_Y$ is the contact angle in the Young’s model. The roughness affects the wettability when the contact angle of the flat surface is less than 90°.42,43 Compared to the untreated Ti6, the contact angle on L-Ti6 decreases because of the rough surface after the laser treatment, during which there is heat transfer from the HA and Ag$_x$Zn$_y$O$_z$HA composite coatings to the Ti6Al4V substrate. The laser beam size is 0.6 mm, and the cooling rates at the center and periphery are slightly different, forming nanoscale microspheres during solidification. The molten pool structure forms at a laser frequency of 20 Hz, and the low laser energy produces bulging areas in the coating between the pulses. The parallel ridges and nanoscale molten pools form a hierarchical structure (shown in Figure 1), further increasing the hydrophilicity of the HA and Ag$_x$Zn$_y$O$_z$HA samples. The OH group in HA increases the hydrophilicity, and so the HA-cladded Ti displays the smallest contact angle. It is believed that a hydrophilic surface favors adhesion of osteoblasts, fostering subsequent growth and proliferation.5

3.2. Release Behavior of Zn$^{2+}$ and Ag$^+$. Zn$^{2+}$ and Ag$^+$ are linked to the antibacterial activity of the coatings.8 Zn is an essential trace element playing an important role in the regulation of osteoblast functions and bone formation.32 The release behavior of Zn$^{2+}$ in the SBF solution is shown in Figure 3a. In the earlier stage within 4 days of immersion in SBF, all Ag$_x$Zn$_y$O$_z$HA–Ti6 samples exhibit a burst release of Zn$^{2+}$ because of the reaction between the exposed ZnO and the electrolyte at a pH of 7.42

$$\text{ZnO} + \text{H}_2\text{O} \leftrightarrow \text{Zn}^{2+} + 2\text{OH}^-$$

(4)

As the ZnO content in the composite coatings increases, the amount of released Zn$^{2+}$ increases, as shown in eq 4. The cumulative concentration of Zn$^{2+}$ released from Ag$_0$Zn$_{10}$-HA–Ti6 is 46.2 μg/L after immersion for 4 days, and the

Figure 2. (a) XPS survey spectrum of Ag$_5$Zn$_5$O$_5$HA–Ti6 and (b) XPS spectrum of Ag of the Ag$_5$Zn$_5$O$_5$HA–Ti6 sample.

Figure 3. (a) Release of Zn from different ZnO NPs and (b) release of Ag from different Ag NPs.
release rate diminishes gradually afterward. After immersion for 14 days, the cumulative concentration of Zn\(^{2+}\) reaches a stable value for all samples, that is, 20.11, 28.74, 36.49, and 54.5 \(\mu\text{g/L}\) for Ag\(_7\)ZnO\(_3\)HA\(_-\)Ti\(_6\), Ag\(_5\)ZnO\(_5\)HA\(_-\)Ti\(_6\), Ag\(_3\)ZnO\(_7\)HA\(_-\)Ti\(_6\), and Ag\(_0\)ZnO\(_{10}\)HA\(_-\)Ti\(_6\), respectively. The corresponding increments are 1.71, 1.70, 3.13, and 1.91 \(\mu\text{g/L}\) after immersion for 21 days, indicating chemical stability. It is noted that the largest cumulative Zn\(^{2+}\) concentration (56.41 \(\mu\text{g/L}\) from Ag\(_0\)ZnO\(_{10}\)HA\(_-\)Ti\(_6\)) is far below the safety level of 3 mg/L recommended by the World Health Organization.\(^{44}\) The release profiles of Ag\(^+\) from the Ag\(_x\)ZnO\(_y\)HA\(_-\)Ti\(_6\) samples are presented in Figure 3b. Compared with Zn\(^{2+}\), there was no obvious burst release of Ag\(^+\), and all samples exhibit a slow and sustained release of Ag\(^+\) at lower concentrations because release of Ag\(^+\) from nano-silver in the composite coatings arises from the ionization of the outer Ag atoms of the nano-silver in PBS. As the Ag content increases, the cumulative amount of Ag\(^+\) released increases. The cumulative concentration of Ag\(^+\) released from Ag\(_{10}\)ZnO\(_{0}\)HA\(_-\)Ti\(_6\) increases to 33.59 \(\mu\text{g/mL}\) after immersion for 14 days, and afterward, the released amount increases gradually to 37.68 \(\mu\text{g/mL}\) after 21 days. With regard to Ag-containing antibacterial composites, previous studies have shown a larger Ag release.\(^{45,46}\) The doping process by physical mixing of HA or brushite cements in the AgNO\(_3\) solution leads to rapid release of Ag\(^+\) at a higher concentration of over 200 \(\mu\text{g/L}\) after immersion in PBS for 7 days,\(^{45}\) whereas that released from the HA/Ag composite coating prepared by plasma spraying is larger than 300 \(\mu\text{g/L}\) after immersion for 14 days,\(^{46}\) indicating that Ag is immobilized in the composite coatings. A larger Ag concentration kills bacteria more effectively but comes with toxicity.\(^{43}\) In addition, rapid release of Ag\(^+\) does not bode well for the long-term antibacterial activity. Here, laser cladding can fix the composite coating tightly on the Ti\(_6\) substrate because of the high transient temperature, consequently blocking the rapid release of Zn\(^{2+}\) and Ag\(^+\) from the AgZnO\(_y\)HA composite coatings and reducing the associated toxicity. A slow and gradual release of Ag\(^+\) is preferred for long-term prevention of bacterial infection. Although the melting point of nano-silver is size-dependent, it is similar to that of bulk Ag if the particle size is over 10 nm.\(^{48}\) Hence, owing to the lower melting point (961.78 °C) and higher thermal conductivity (473 W\(\cdot\)m\(^{-1}\)\(\cdot\)K\(^{-1}\)),\(^{49,50}\) tight fixation of Ag is accomplished.

### 3.3. In Vitro Antibacterial Activity of Ag\(_x\)ZnO\(_y\)HA\(_-\)Ti\(_6\)

The antibacterial activity of the Ag\(_x\)ZnO\(_y\)HA composite coatings is evaluated by the plate counting method. As shown in Figure S3a, with the exception of L-Ti6, the other samples exhibit different levels of antibacterial efficacy against E. coli relative to pure Ti6, as determined by the specific bacterial count on the LB agar plates (indicated with the red number) ascribed to the antibacterial action of released Ag\(^+\) and Zn\(^{2+}\) or the synergistic effects of both ions. L-Ti6 shows more E. coli than the untreated sample because of the hierarchical structure produced by the laser treatment (Figure S4), which enhances the hydrophilicity (shown in Figure S2) and favors the adhesion of bacteria. HA–Ti6 exhibits a slight reduction in

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**Figure 4.** Antibacterial properties against E. coli and S. aureus: (a) antibacterial efficiency of the samples compared to Ti6 for E. coli; (b) antibacterial efficiency of the samples for S. aureus; (c) SEM images of E. coli and S. aureus on Ti6, Ag0ZnO10HA–Ti6, Ag7ZnO3HA–Ti6, and Ag10ZnO0HA–Ti6 (scale bar = 1 \(\mu\text{m}\)). The error bars indicate means ± SD [\(*P < 0.05, **P < 0.01, and ***P < 0.001 (t-test)\)].
the bacterial strains than the untreated sample because of the following two factors. On the one hand, the surface hydrophilicity for a contact angle of 10.5° favors adhesion of bacteria but on the other hand, HA NPs have some antibacterial activity. The E. coli numbers decrease significantly with contents of AgNPs in the Ag5ZnO5HA coatings. According to formula 1 and the bacterial strain numbers in Figure S3a, the antibacterial efficacy of samples against E. coli is calculated (Figure 4a). The antibacterial efficiency was –9.4% for L-Ti6 but 5.34% for HA–Ti6. Addition of ZnO (10 wt %) improved the antibacterial efficacy by up to 53.3% against E. coli because of the released Zn2+. As the Ag content is increased to 3 wt %, the antibacterial efficacy against E. coli increases sharply to 79.6%, and the antibacterial rates against E. coli are 90.4, 96.5, and 99.2% for Ag5ZnO5HA–Ti6, Ag7ZnO3HA–Ti6, and Ag10ZnO0HA–Ti6, respectively. Figure S3b shows the plate counting results of S. aureus showing a similar trend. That is, L-Ti6 has more S. aureus than the untreated Ti6, but HA–Ti6 has less bacteria than the control. A hydrophilic and nanostructured surface has been shown to favor adhesion of bacteria. The antibacterial rates of L-Ti6, HA–Ti6, Ag0ZnO10HA–Ti6, Ag3ZnO7HA–Ti6, Ag5ZnO5HA–Ti6, Ag7ZnO3HA–Ti6, and Ag10ZnO0HA–Ti6 against S. aureus are –21.9, 6.5, 35.9, 54.3, 77.1, 85.8, and 93.0%, respectively (Figure 4b). Addition of ZnO and Ag NPs endow the coatings with antibacterial ability. Addition of ZnO (10 wt %) inhibits the growth of S. aureus by about 35.9% without the assistance of AgNPs, whereas the addition of Ag (10 wt %) only enhances antibacterial efficacy to 93%, indicating the predominant role of AgNPs in bacteria killing. Figure 4c shows the morphology of the bacteria on different samples. Both E. coli and S. aureus show an irregular smooth shape with intact cell walls, but on Ag0ZnO10HA–Ti6, Ag7ZnO3HA–Ti6, and Ag10ZnO0HA–Ti6, the cell walls show different degrees of damage such as sunken, cracked, and deformed features (indicated by red arrows). The damage becomes more serious as the AgNP content is larger. It has been reported that AgNPs and Ag+ released from AgNPs lead to intracellular oxidation and membrane potential variation, resulting in the destruction of bacteria proteins, and AgNPs act as a vehicle to deliver Ag+ to generate ROS to destroy the bacteria DNA.

3.4. In Vitro Evaluation of Cell Viability and Mineralization. The cell viability evaluated by MTT on days 1, 3, and 7 is shown in Figure 5a. After culturing for 1 day, except Ag10ZnO0HA–Ti6, all samples exhibit better cell viability than the pristine Ti6 because of the increased amount of Ag+ released from the Ag10ZnO0HA–Ti6 sample than other samples (Figure 2). The better cell viability of L-Ti6 is ascribed to the hierarchical nanostructure. Among the Ag5ZnO5HA–Ti6 samples, HA–Ti6 shows the largest cell survival rate because HA has a composition similar to the natural bone, thus facilitating osteoblast proliferation. However, after culturing for 3 days, except for HA–Ti6 and L-Ti6, the modified samples show reduced cell viability because of the higher cumulative concentrations of released Zn2+ and Ag+, as shown in Figure 2a.b. Ag10ZnO0HA–Ti6 shows the lowest cell viability because of the largest concentration of Ag+. Compared to Ag NPs, ZnO NPs show lower cytotoxicity. It has been reported that AgNPs induce oxidative stress, impacting cell proliferation. On the seventh day, the cell viability of all samples exhibited a trend similar to that on the third day. Furthermore, except Ag10ZnO0HA–Ti6, the four composite coatings display an increased cell viability on the seventh day compared to that on the third day, indicating...
decreasing cytotoxicity with culture time. The continuous decrease in the cell viability observed from Ag10ZnO0HA−Ti6 provides evidence that more Ag⁺ release produces cytotoxicity.

The ALP activity is an indicator of osteoblast differentiation and osteogenesis. As shown in Figure 5b, on the third day, the ALP activity is 39.5 U/g protein for the untreated sample and 42.1 U/g protein for L-Ti6. All AgxZnOyHA−Ti6 samples show a higher ALP expression than the untreated Ti6. After culturing for 7 days, the cells on AgxZnOyHA−Ti6 samples show enhanced ALP expressions indicating excellent osteogenesis properties. All AgxZnOyHA composite coatings have higher ALP activities than the pure HA coating because of the release of small amounts of Ag and Zn. On the 14th day, the ALP activity is similar to that after the 7th day. The slightly higher ALP activity of L-Ti6 than the untreated sample is attributed to the larger roughness and hydrophilicity.59,60 Moreover, Ca and P from HA provide the osteogenetic mineralization factors.30,61 The released Zn can be easily absorbed to promote bone proliferation and osteogenic mineralization by stimulating osteoblast metabolism. Zn also promotes deposition of calcium in HA,33 and it has been reported that the local microgalvanic couples formed between the AgNPs and the Ti substrate favor bone formation.51,62

The morphology of the MC3T3-E1 cells is examined by SEM and fluorescence staining. As shown in Figure 5c, after culturing for 1 day, the MC3T3-E1 cells attach well and grow on all samples. The MC3T3-E1 cells on the untreated Ti6 show symmetrical adhesion with smooth edges (indicated by the red arrow), but there are no obvious filamentous pseudopods, whereas those on L-Ti6 show short pseudopods (indicated by the red arrow). Compared to the untreated Ti6, larger spindles and long pseudopods are observed from the HA and AgxZnOyHA coatings (indicated by the red arrow), especially the AgxZnOyHA coatings. The fluorescence images (Figure 5d) show a normal morphology. The number of cells on the AgxZnOyHA−Ti6 samples decreases gradually with Ag concentration in agreement with SEM and MTT, after culturing for 1 day.

Considering the effective in vitro antibacterial activity and excellent cytocompatibility of Ag7ZnO3HA, this group is selected for the mineralization and in vivo tests. Mineralization of the MC3T3-E1 cells can be evaluated by examining the formation of mineralization nodules by SEM and EDS. As shown in Figure 6, after culturing for 10 days, the osteoblasts exhibit a smooth morphology on the untreated Ti6, and EDS shows an intense signal of Ti from the substrate, indicating that the MC3T3-E1 cells do not undergo mineralization. Some dotlike protrusions appear on HA−Ti6 ls (indicated by blue arrows), and the EDS results obtained from a nodule show a more intense Ca peak than Ti, suggesting partial mineralization of the MC3T3-E1 cells. The mineralized nodules of the MC3T3-E1 cells on Ag7ZnO3HA−Ti6 are larger than those on HA−Ti. In addition, the Ca signal is stronger than that from HA−Ti6, indicating better mineralization on Ag7ZnO3HA. The Ca concentration in the mineralized nodule of MC3T3-E1 is larger than that in the nonmineralized areas (Figure S5a). After culturing for 20 days, the MC3T3-E1 cells spread well and adhere tightly to the untreated Ti6. The corresponding
EDS data show almost no Ca signal but an intense Ti peak indicative of no mineralization. Many mineralized nodules appear on HA−Ti6, and EDS shows a strong Ca peak but a weak Ti peak compared to the sample cultured for 10 days, indicating further mineralization. The MC3T3-E1 cells on Ag7ZnO3HA−Ti6 exhibit a higher mineralization level than those on HA−Ti6, as shown by the larger mineralization nodules, intense Ca signal, and weak Ti signal. The EDS spectrum obtained from the nonmineralized zone shows a stronger Ti peak than that from the mineralized nodules (Figure S5b). The alizarin red stained images of different samples are shown in Figure S6a, and no mineralized nodules appear on the untreated Ti6 after culturing for 10 days, whereas both HA−Ti6 and Ag7ZnO3HA−Ti6 display obvious red mineralized nodules. After culturing for 20 days, the untreated sample still does not show obvious stained nodules, but HA−Ti6 and Ag7ZnO3HA−Ti6 exhibit more obvious alizarin red stained nodules, especially the latter one. The quantitative measurement of mineralization by alizarin red staining is shown in Figure S6b. The quantitative measurement of mineralization by alizarin red staining is shown in Figure S6b. After culturing for 10 and 20 days, the mineralization level on HA−Ti6 is the best (Figure 6). The excellent in vitro osteogenic differentiation ability of Ag7ZnO3HA can be attributed to two factors. First of all, HA with the composition of the natural bone benefits adhesion and proliferation of osteoblasts, and Ca and P in HA are conducive to cell differentiation and mineralization. Second, Ag and Zn released from Ag7ZnO3HA stimulate the metabolism and osteogenesis of osteoblasts as well as subsequent bone mineralization.39,40

3.5. In Vivo Antibacterial Activity. The in vivo antibacterial efficiency is assessed by the inflammatory response of soft tissues and bone tissues around the implanted model in vivo. H&E staining is a common immunoassay to evaluate inflammatory cells in tissues, and Giemsa staining is employed to detect the cells’ eosinophil and bacteria. As shown in Figure 7, after the samples have been implanted for 2 weeks, the soft tissues and bone tissues in the untreated Ti6 group show substantial inflammatory cell infiltration by H&E staining. The inflammatory cells are mainly lobulated neutrophils (indicated by red arrows), and as an inflammatory response in the immune system, neutrophils quickly attack the bacteria.63,64 Many bacteria are observed from the soft tissues and bone tissues by Giemsa staining (indicated by black arrows). By contrast, on the Ag7ZnO3HA−Ti6 implant, only a small number of lobulated neutrophils are distributed in the soft tissues and bone tissues, as shown by the H&E staining images. Giemsa staining shows few bacteria in the soft tissues and bone tissues as well. After 5 weeks, H&E staining shows a lot of inflammatory cell infiltration, including neutrophils in the soft and bone tissues of the untreated Ti6 group, and many bacteria are shown in the Giemsa staining image. In comparison, the soft tissues and bone tissues around the Ag7ZnO3HA−Ti6 implant show almost no inflammatory cells and bacteria in the H&E and Giemsa staining image. These results disclose that the Ag7ZnO3HA coating has excellent in vivo antibacterial efficacy, in agreement with the in vitro antibacterial results. Ag and ZnO play synergistic roles favoring long-term antibacterial activity.

3.6. Bone−Implant Formation in Vivo. To evaluate the in vivo bone formation ability of Ag7ZnO3HA−Ti6 during bacterial infection, the rabbits injected with 20 μL of S. aureus at 10⁵ CFU/mL are sacrificed after 5 weeks and inspected by X-ray radiography. As shown in Figure S7a,b, the implant is tightly fixed to the surrounding tissues in the rabbit femur, and newly formed bone tissues can be detected around the implant by micro-CT. The 2D and 3D models reconstructed from the CT images show the formation of new bone tissues around the implant. In the 2D model (Figure 8a), it is difficult to find new
bone tissues around the untreated Ti6 implant, but the two sides of Ag7ZnO3HA−Ti6 are completely covered by newly formed bone tissues (indicated by red arrows). In the 3D model, the implants and newly formed bone tissues are marked red and yellow, respectively, and more bone tissues are observed from Ag7ZnO3HA−Ti6 than from the untreated Ti6 implant. The osteogenesis ability can be evaluated by analyzing the ratio of object volume (Obj.V) and tissue volume (TV). The Obj.V/TV value is 46.01% for the Ag7ZnO3HA−Ti6 implant but only 20.10% for the untreated Ti6 implant, as shown in Figure 8c.

New bone formation around the implants and bone−implant contact is evaluated by van Gieson’s picrofuchsin staining. As shown in Figure 8b(1), more new bone tissue (red color) forms around Ag7ZnO3HA−Ti6 than around the untreated Ti6 implant. The high-magnification images of the interface between the implant and surrounding tissues disclose larger bone−implant areas on Ag7ZnO3HA−Ti6 than on the untreated Ti6. The new bone area coverage of the former is 87.15% and that of the latter is 44.36%. The contact area between bone tissues and Ag7ZnO3HA−Ti6 is 83.8%, and so new bone tissues almost completely cover the implant. By contrast, most of the surface on the untreated Ti6 implant is covered by soft tissues (indicated by blue arrows). In the early recovery stage after surgery, the composite coating possesses the hierarchical nanostructure, which benefits adhesion of osteoblasts and osteointegration, and Ca and P facilitate formation of new bone tissues by promoting osteoblast mineralization. Besides killing bacteria, Zn and Ag promote bone cell metabolism and absorption of Ca and P to accelerate bone formation.

Figure 8. (a) 2D and 3D micro-CT images showing new bone formation around the Ti6 and Ag7ZnO3HA−Ti6 samples implanted into the rabbit femur for 5 weeks; (b) quantitative new bone model based on 3D micro-CT images; (c) new bone tissues evaluated by van Gieson’s picrofuchsin staining; (d) histomorphometric measurement of new bone areas. The error bars indicate means ± SD [*P < 0.05, **P < 0.01, and ***P < 0.001 (t-test)].
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Supporting information

Nano Ag/ZnO Incorporated Hydroxyapatite Composite Coatings: Highly Effective Infection Prevention and Excellent Osteointegration

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Figure S7. (a) Image of the sample implanted in the rabbit femur and (b) X-ray photo after 5 weeks.