Novel Hydroxyapatite Whiskers Modified by Silver Ion and Nano Zinc Oxide Used for Bone Defect Repairment

Tingting Yan 1,*, Zhimin Jiang 1, Pan Li 1, Qinghua Chen 1, Jing Zhou 2, Xiuzhen Cui 3 and Qiang Wang 4,*

1 Faculty of Materials Science and Engineering, Kunming University of Science and Technology, Kunming 650000, China; jiangzhimin0210@163.com (Z.J.); panlii@outlook.com (P.L.); chenqinghua_yn1@163.com (Q.C.)
2 School of Stomatology, Kunming Medical University, Kunming 650000, China; zhoujingjd@163.com
3 Xianning CSG Photoelectric Glass Co., Ltd., Xianning 437000, China; cuixz@csgholding.com
4 School and Hospital of Stomatology, China Medical University, Liaoning Provincial Key Laboratory of Oral Diseases, Shenyang 110002, China
* Correspondence: yan@kust.edu.cn (T.Y.); mfqwang@cmu.edu.cn (Q.W.)

Abstract: The hydroxyapatite (HA) is widely used as bone tissue repair material. The improvement of the antibacterial performance is an aroused general interest. In the present study, the silver ion and nano-zinc oxide modified hydroxyapatite whiskers (HAW) were successfully prepared. The microstructure and the composition of the modified HAW were analyzed by Field Emission Scanning Electron Microscopy (FESEM) and X-ray diffractometer (XRD). SEM analysis showed that the length of the whiskers was 70–190 µm, and the aspect ratio was 10–60. With the increase of Ag⁺ content, the length and aspect ratio of the whiskers gradually decreased and incomplete spherical hydroxyapatite appeared. FEEM analysis showed that nano-zinc oxide particles on ZnO/3Ag-HAw surface are evenly distributed; the average particle size is less than 30 nm. XRD analysis showed that after sol-gel and calcination treatment, the nano-zinc oxide phase appeared in the diffraction pattern of ZnO/Ag-HAw. TEM analysis showed that the interplanar spacing of 5Ag-HAw increased slightly. The CCK-8 and cells co-culture assays were used to assess the proliferation and differentiation of MC3T3-E1 cells, respectively. The antibacterial abilities of the modified HAw against E. coli (ATCC25922) and S. aureus (ATCC6538) were investigated. The cell cytotoxicity test showed that the cytotoxicity level was 0, and there was no cytotoxicity. Cell adhesion experiments showed that ZnO/3Ag-HAw has good cell compatibility and biological activity. The modified hawthorn has a bacteriostatic rate of more than 90% and has good bacteriostatic activity.

Keywords: biomaterials; antibacterial; hydroxyapatite whiskers; silver ion; nano zinc oxide

1. Introduction

Hydroxyapatite (HA) is widely used as bone tissue repair material due to its similarity in bone composition, excellent biocompatibility, osteoconductivity, bioactivity and non-toxicity properties. As a form of hydroxyapatite, hydroxyapatite whiskers (HAW) can absorb external stress by bridging, crack deflection and whisker extraction, and thus the HAw possesses the performance of enhancing the bending and compressive capacity of scaffolds [1–3].

On the other hand, in the case of bone defect repair surgery, the inevitable bacterial infections may lead to surgery failure. Therefore, the ideal stent material needs to have certain antibacterial properties. In this aspect, the antibacterial properties of HAw are too poor to prevent bacterial infection when used as a kind of raw material for a bone scaffold. Two promising strategies, surface modification and ion doping, are expected to be used to prevent scaffolds from being infected by bacteria [4,5]. Many studies have reported that the substitution of Ca²⁺ with other metal ions such as Cu²⁺, Ce³⁺, Ag⁺, Zn²⁺ and Mg²⁺ is one of the most effective ways to improve the properties of HA [6–8]. It is noteworthy
that HA doped with Ag\(^+\) has demonstrated excellent antimicrobial properties [9]. Ag\(^+\) can resist a wide range of bacteria and viruses and has the characteristics of a long release period and low toxicity to mammalian tissues [10–12]. W. Chen et al. [13] and Mahendran Rai et al. [14] reported that HA doped by silver ions is an ideal biomaterial because Ag\(^+\) can easily replace Ca\(^{2+}\) in the matrices, creating a highly biocompatible material with antibacterial properties. Moreover, Ag\(^+\) (1.28 Å) ions could preferentially replace Ca\(^{2+}\) (0.99 Å) in HA, which results in a linear increase in the lattice parameter with the increasing content of silver in the range of Ag/(Ag + Ca) atomic ratio between 0% and 5.5% [15]. It should also be noted that the HA lattice doped with Ag\(^+\) still has some problems. The low doping level may result in lower antimicrobial activity, and the high doping may alter the stability of HA, even making HA have cytotoxicity [16].

In the field of bio-nano medicine, the therapeutic potential of metal nanoparticles (NPs) in treating diseases is encouraging. At present, the commonly used metal oxides with antibacterial properties are CuO, TiO\(_2\), ZnO, Fe\(_2\)O\(_3\) and CeO\(_2\), but TiO\(_2\) and ZnO have better antibacterial effects [17,18]. Zinc oxide (ZnO) is currently being investigated as an antibacterial agent in both microscale and nanoscale formulations [18]. Compared with pure HA, HA deposited with a proper amount of zinc oxide has better cell proliferation and cell viability [19,20].

In light of the above statements, the purpose of this study is to prepare an antibacterial-modified hydroxyapatite whisker (ZnO/Ag-HAw) by doping silver ions and complexing zinc oxide nanoparticles on the surface of HAw. The biological activity and antibacterial properties of the ZnO/Ag-HAw are evaluated.

2. Material and Methods

2.1. Preparation of Ag-HAw

Four different silver content micron level hydroxyapatite whiskers were synthesized in this study by a template-directed homogeneous precipitation method [21]. Silver-doped hydroxyapatite whiskers (Ag-HAw) with different proportions of silver ions and pure hydroxyapatite whiskers (Pure-HAw) were prepared according to the following scheme. In this process, Ca(NO\(_3\))\(_2\)-4H\(_2\)O, (NH\(_4\))\(_2\)HPO\(_4\), sorbitol, urea and silver nitrate were mixed in deionized water under stirring conditions. Especially, the silver nitrate was used as the source for Ag\(^+\), and the molar ratio of (Ca + Ag)/P for all samples in the process was 1.76. The starting pH value was controlled to be equal to 3 by adding proper quantities of dilute nitric acid. Urea and sorbitol played the role of buffering agent and template agent, respectively. The mixed solution was heated up to a constant temperature at 95 °C in the water bath for 30 h. Then, the precipitates were washed, filtered and dried. Finally, the Ag-HAw was obtained. The composition of starting solutions for sample is shown in Table 1.

### Table 1. The solutions used for the synthesis of HAws.

| Sample (Composition) | Ca(NO\(_3\))\(_2\)-4H\(_2\)O (g) | (NH\(_4\))\(_2\)HPO\(_4\) (g) | Sorbitol | Urea | Silver Nitrate |
|---------------------|--------------------------------|----------------------------|----------|------|----------------|
| Pure HAw (0 mol.% Ag) | 15.6638 g (0.0670 mol) | 5.2824 g (0.04 mol) | 0.8036 g 18 g | 0 g (0 mol) |
| 1Ag-HAw (1 mol.% Ag) | 15.3474 g (0.06633 mol) | 5.2824 g (0.04 mol) | 0.8036 g 18 g | 0.1137 g (0.00067 mol) |
| 3Ag-HAw (3 mol.% Ag) | 15.4337 g (0.06499 mol) | 5.2824 g (0.04 mol) | 0.8036 g 18 g | 0.34110 g (0.00201 mol) |
| 5Ag-HAw (5 mol.% Ag) | 15.0301 g (0.06365) | 5.2824 g (0.04 mol) | 0.8036 g 18 g | 0.5685 g (0.00335 mol) |

2.2. Preparation of ZnO/Ag-HAw

The prepared Ag-HAw, with different silver ion incorporation, was used as substrate material, and the nano-zinc oxide particles were adhered to the surface by a sol-gel method to prepare ZnO/Ag-HAw with different silver-ion-doped amounts. In the preparation, zinc
Nitrate was used as a zinc source; the amount of zinc nitrate and Ag-HAw was calculated according to the mass ratio of nano zinc oxide particles to Ag-HAw of 10%. Zinc nitrate was dissolved in Ag-HAw containing a hydrous ethanol suspension, and the pH value of the suspension was kept at 7 using ammonia and acetic acid during the dissolution. Then, the anhydrous ethanol in the suspension was evaporated to dryness using a rotary evaporator to obtain a precursor of ZnO/Ag-HAw. The precursor was sintered at 600 °C for 10 h, and the ZnO/Ag-HAw was obtained.

2.3. Characterization of ZnO/Ag-HAw

X-ray diffraction (XRD, Rigaku, Tokyo, Japan, D/max-2550v, Cu Kα radiation λ = 1.54056 Å) analysis was performed to identify the phase composition of these samples. The diffractometer was operated at 40 kV and 40.0 mA at a 2θ range of 20–60° with a step size of 0.026°. Identification of the phases in the samples was compared to the ICDD (JCPDS). The chemical composition of the (1, 3, 5) Ag-HAw was determined by inductively coupled plasma atomic emission spectrometry (ICP-OES, PerkinElmer, MA, USA). A total of 50 mg of the (1, 3, 5) Ag-HAw was dissolved into 50 mL of 3 vol.% nitric acid, and the concentrations of Ag ions were measured. Similarly, the content of zinc ions in ZnO/3Ag-HAw was measured in the same way, and the content of nanosized zinc oxide in the ZnO/Ag-HAw samples was roughly estimated. The microstructure evolution of the ZnO/Ag-HAw samples was observed at 10 keV using the Field Emission Scanning Electron Microscopy (FESEM, Nova Nano SEM 450, FEI Company, Hillsboro, OR, USA). In addition, the chemical elements were analyzed by Energy Dispersive X-Ray Spectroscopy (EDX, FEI Company, Hillsboro, OR, USA). The samples morphology of as-synthesized samples was examined using JEM-2100 transmission electron microscope (TEM, JEOL, Peking, China). For TEM analysis, the samples were dispersed in absolute ethanol by sonication and loaded on carbon-coated Cu grid.

2.4. Cytotoxicity Assessment and Cell Adhesion

Monkey bone marrow mesenchymal stem cells (MSCs) were used for this study. Cytotoxicity tests were performed by the WST-8 method; 5Ag-HAw, ZnO/5Ag-HAw, and Pure-HAw were immersed in the DMEM medium for 2 days, at 37 °C without agitation, with a weight–volume ratio 0.075 g/mL, 0.15 g/mL, 0.225 g/mL and 0.3 g/mL to obtain different concentrations of material-leaching solutions. The material-leaching solutions were added to MSCs, which were already seeded with 1.0 × 10^5 cells/mL in 96-well plates for 20 h. The cells were cultured for 20 h at 37 °C in a humidified air atmosphere containing 5% CO₂ after that, 10 µL CCK-8 (Cell Counting Kit-8) solution (Beyotime) was injected into the holes in the 96-well plate, followed by incubation for 1–4 h; the absorbance was measured at 450 nm with a microplate reader. The following formula was used to calculate cell viability [21]:

\[
\text{cell viability} = \frac{(A_1 - A_2)}{(A_3 - A_2)}
\]

- \( A_1 \): OD value of the experimental;
- \( A_2 \): OD value of the blank;
- \( A_3 \): OD value of the control.

In order to more intuitively observe the effect of ZnO/3Ag-HAw on cells, a tablet press was used to compress the ZnO/3Ag-HAw into the flakes with a diameter of 10 mm and a height of 5 mm; the flakes were sterilized by dry heat. All sterilized flakes were placed in 24-well tissue culture plates and were rinsed three times with phosphate-buffered saline (PBS). MSCs were seeded at a concentration of 5 × 10^4 cells/well onto the samples of interest in DMEM supplemented with 10% FBS and 1% PBS and were then incubated under standard cell culture conditions for 3 d. After incubation, nonadherent cells were removed by rinsing with PBS, and adherent cells were then fixed with glutaraldehyde fixed solution, which had a concentration of 2.5%. After dehydration in a graded series of alcohol (50%, 60%, 70%, 80%, 90% and 100%) for 10 min each and drying in the vacuum
freeze dryer, the samples were sputter-coated with gold. The surface of the cell-adhered experimental samples was observed via SEM.

2.5. Antibacterial Assessment

Antimicrobial activities of Ag-HAw, ZnO/Ag-HAw and Pure-HAw against *E. coli* (ATCC25922) and *S. aureus* (ATCC6538) were investigated by plate colony counting method. These samples were sterilized and immersed in PBS solution for 7 days. The supernatant was taken out by precipitated and filtrated. Additionally, 1 mL material supernatant mixed with 0.2 mL bacteria suspension (OD$_{595} = 0.1$) and 5 mL of Nutrient Broth Medium (NB) was incubated at 37 °C for 24 h. We diluted the bacterial solution after co-cultivation (10$^7$ times dilution of *E. coli* bacterial suspension, 10$^6$ times dilution of *S. aureus* bacterial suspension), and then transferred it to the surface of a Nutrient Agar Medium (NA) plate, incubated at 37 °C for 24 h. The colony formation was observed and photographed. The bactericidal rate was calculated using the following formula:

$$R = \left(\frac{C_0 - C_t}{C_0}\right) \times 100\%$$  \hspace{1cm} (2)

- $C_0$: the average number of bacteria cultured in the control;
- $C_t$: the average number of bacteria cultured in the experimental group.

3. Results and Discussion

3.1. SEM Morphology Analysis

The SEM micrographs of the Pure-HAw and Ag-HAw (Figure 1a–d) revealed that the size of the Ag-HAw was varied with the different amounts of doped silver ions. The length decreased and the width increased with the increase of Ag$^+$ contents. Meanwhile, according to SEM analysis results, the aspect ratio of the fibrous nanoparticles decreases with increasing the Ag$^+$ contents. In Figure 1, the aspect ratio of whiskers in (a), (b), (c) and (d) is 54.33, 34.59, 22.79 and 9.54, respectively. Thus, it can be concluded that with the increase of silver ion contents, the aspect ratio of Ag-HAw gradually decreases, and the shape gradually evolves from needle to lath. It can be specially proved from Figure 1d that some spherical Ag-HAw appeared in the sample of 5Ag-HAw.

The FE-SEM micrographs of the ZnO/3Ag-HAw (Figure 1e) showed that a layer of nano zinc oxide particles was formed on the whisker surface. The nano zinc oxide particles have a particle size distribution of 20–30 nm, and these nanoparticles uniformly cover the surface of the whiskers. As demonstrated by mapping (Figure 1f), Ag and Zn could be detected in the ZnO/3Ag-HAw samples.

According to the TEM micrographs Figure 1g,h, the interplanar spacing (d value) of the strongest reflector of 5Ag-HAw is $d_1 = 4.47$ Å (112), $d_2 = 3.48$ Å (002), $d_3 = 2.81$ Å (110) and that of pure HAw is $d_1 = 4.47$ Å (112), $d_2 = 3.42$ Å (002), $d_3 = 2.79$ Å (110). The results showed that the lattice parameters of 5Ag-HAw increased slightly. This phenomenon can be explained by the larger cationic radius of the Ag$^+$ (1.28 Å) against Ca$^{2+}$ (0.99 Å). It was reported that a silver substitution mechanism formed Ag$^+$: HAw with larger lattice parameters [22]. In addition, Figure 1g,h shows that 5Ag-HAw and Pure HAw are single crystals.

3.2. XRD and ICP-OES Analysis

The XRD analysis results of Pure-HAw and Ag-HAw samples are shown in Figure 2a. The peaks presented are in good agreement with the peaks of the International Center for Diffraction Data (ICDD) PDF No. 09-0432. However, with the incorporation of silver ions, the OCP (octa-calcium phosphate, Ca$_8$(HPO$_4$)$_2$(PO$_4$)$_4$·5H$_2$O) phase appeared in the product, and the crystallinity of HAw decreased. The sharp diffraction peaks of pure HAw indicate the crystallinity of pure HAw is higher than that of others that were doped with silver ions. The slight peak shift towards the right side is due to the ionic radius of the dopant materials [23]. The crystallinity and cell appearance of the samples have been calculated by the XRD refinement method (Table 2). Through the data in the table, both the
a- and c-axis increased with Ag\(^+\) substitution. The results obtained in the current study are logical since the ionic radius of Ag\(^+\) is bigger in size than Ca\(^{2+}\) [24]. Additionally, the results obtained by analyzing the SAED micrographs, which agreed with the results of XRD, indicated that the lattice distortion was caused by the incorporation of Ag\(^+\).

Figure 1. Cont.
3.2. XRD and ICP-OES Analysis

The XRD analysis results of Pure-HAw and Ag-HAw samples are shown in Figure 2a. The peaks presented are in good agreement with the peaks of the International Center for Diffraction Data (ICDD) PDF No. 09-0432. However, with the incorporation of silver ions, the OCP (octa-calcium phosphate, Ca$_8$(HPO$_4$)$_2$(PO$_4$)$_4$·5H$_2$O) phase appeared in the product, and the crystallinity of HAw decreased. The sharp diffraction peaks of pure HAw indicate the crystallinity of pure HAw is higher than that of others that were doped with silver ions. The slight peak shift toward the right side is due to the ionic radius of the dopant materials [23]. The crystallinity and cell appearance of the samples have been calculated by the XRD refinement method (Table 2). Through the data in the table, both the a- and c-axis increased with Ag$^+$ substitution. The results obtained in the current study are logical since the ionic radius of Ag$^+$ is bigger in size than Ca$^{2+}$ [24]. Additionally, the results obtained by analyzed the SAED micrographs, which agreed with the results of XRD, indicated that the lattice distortion was caused by the incorporation of Ag$^+$. Table 3 also showed the actual silver content measured by ICP-OES. With the comparison of the theoretical value, the actual silver content in the whiskers was only about 44–73% of the theoretical value. It shows that the addition of Ag$^+$ has limitations. With the increase of the doping ratio, the actual amount of Ag$^+$ shows a downward trend.

Table 2. Lattice parameters and crystallinity of the sample.

| Scheme       | a = b (Å) | c (Å) | c/a | Crystallinity       |
|--------------|-----------|-------|-----|---------------------|
| Pure HAw     | 9.4134    | 6.8901| 0.7319 | 78.63% (±0.33)     |
| 1Ag-HAw      | 9.4211    | 6.8911| 0.7316 | 75.51% (±2.59)     |
| 3Ag-HAw      | 9.4212    | 6.8886| 0.7312 | 74.85% (±1.86)     |
| 5Ag-HAw      | 9.4221    | 6.8924| 0.7315 | 72.95% (±2.90)     |

Table 3 also showed the actual silver content measured by ICP-OES. With the comparison of the theoretical value, the actual silver content in the whiskers was only about 44–73% of the theoretical value. It shows that the addition of Ag$^+$ has limitations. With the increase of the doping ratio, the actual amount of Ag$^+$ shows a downward trend.

Table 3. The theoretical and actual values of Ag/(Ag + Ca) and the doping ratio of silver ions.

| Sample   | Ag/(Ag + Ca) Theoretical Value | Ag/(Ag + Ca) Actual Value | MIXING RATIO |
|----------|-------------------------------|---------------------------|--------------|
| Pure HAw | 0                             | 0                         | -            |
| 1Ag-HAw  | 1%                            | 0.73%                     | 73%          |
| 3Ag-HAw  | 3%                            | 1.54%                     | 51%          |
| 5Ag-HAw  | 5%                            | 2.23%                     | 45%          |
Table 2. Lattice parameters and crystallinity of the sample.

| Scheme | Cell Parameters | Crystallinity |
|--------|-----------------|---------------|
|        | $a = b$ (Å) | $c$ (Å) | $c/a$ | (±) % |
| Pure HAw | 9.4134 | 6.8901 | 0.7319 | 78.63 | 0.33 |
| 1Ag-HAw | 9.4211 | 6.8911 | 0.7316 | 75.51 | 2.59 |
| 3Ag-HAw | 9.4212 | 6.8886 | 0.7312 | 74.85 | 1.86 |
| 5Ag-HAw | 9.4221 | 6.8924 | 0.7315 | 72.95 | 2.90 |

Figure 2. XRD patterns of the as-prepared whisker samples: (a) pure HAw and (1, 3, 5) Ag-HAw; (b) ZnO/3Ag-HAw and 3Ag-HAw.

According to the characteristic peaks of ZnO shown in the XRD pattern of ZnO/3Ag-HAw in Figure 2b, the sample contains ZnO. Meanwhile, a small amount of calcium zinc phosphate ($\text{Ca}_{19}\text{Zn}_2\text{PO}_4\cdot1_4$) also appeared in the samples. In the whiskers calcined at 600 °C, the OCP was transformed into HA [25]. According to the ICP-OES test, the content of zinc oxide nanoparticles in the samples was 9.97 wt.%, which was about 66.5% of the theoretical value.
3.3. In Vitro Cytotoxicity of ZnO/Ag-HAw

Figure 3 shows the results of cytotoxicity of the samples by CCK-8 according to the ISO 10993 [26]. All samples have no cytotoxicity, and the lowest cell survival rate is 90. It can also be concluded that the presence of zinc ions stimulates cell proliferation and improves biocompatibility. Doping HA with zinc ions could increase cell viability and promote osseointegration. It was reported that the composites with ZnO exhibited excellent cellular viability as well as cellular proliferation, even better than pure HA. However, when the zinc oxide content exceeds 10%, the results show that zinc oxide has long-term toxic effects [16]. Silver is potentially cytotoxic to host mammalian cells. The National Institute for Occupational Safety and Health has set the daily exposure limit at 0.01 mg m$^{-3}$ for all forms of silver to prevent argyria [27]. It has been shown that the cytotoxic effect of silver depends on dose, exposure time and the cell line tested.

![Figure 3. Results of the CCK-8 assay.](image)

In addition, by comparing cell proliferation data of 5Ag-HAw with other sample groups, it was found that the incorporation of silver ions reduced the cell proliferation rate. Table 4 shows the Ag$^+$ concentration measured after the sample is immersed in a PBS solution of pH = 7.4 for 7 days. The content of silver ions in PBS increases as the doping ratio increases. According to Table 4, the silver release of the sample is between 0.35 and 0.51, which is less than the maximum toxic concentration (10 ppm) for human cells and higher than the minimum concentration required for antimicrobial efficacy (0.1 ppb) [28]. Our cytotoxicity experiments could also confirm the validity of the above conclusion.

**Table 4. Dissolution of silver ions.**

| Sample      | Ppm |
|-------------|-----|
| 1Ag-HAw     | 0.35|
| 3Ag-HAw     | 0.43|
| 5Ag-HAw     | 0.51|

SEM micrographs illustrate the morphology of the attached cells on each sample, as shown in Figure 4. The images clearly reveal the difference in cell morphology. MSCs
cells exhibited a typical phenotype, which appeared flattened and three-dimensional, with many lamellipodia and filopodia extensions (Figure 4a,b). Filopodia enable the cells to make contact with the substrate and neighboring cells. Some cells are in the growing stage, showing irregular spherical shapes (Figure 4c,d). Cell adhesion experiments showed that the MSCs cells could grow and proliferated on the ZnO/3Ag-HAw sheet, which also shows that ZnO/Ag-HAw has good cell compatibility and biological activity.

Figure 4. SEM images of MSCs cells after culturing for 3 days on ZnO/3Ag-HAw flakes.

3.4. Antimicrobial Assessment

The results of antibacterial activities of all samples are shown in Figure 5. The antibacterial effect of the samples was quantified by the viable count (spread plate) method. It can be concluded that Ag-HAw and ZnO/Ag-HAw showed a significant antibacterial effect, while PBS and Pure-HAw had no antibacterial effect. Moreover, the survival rate of bacteria in the samples of Ag-HAw decreased significantly with the increase of Ag+ weight percentage.

According to the reported literature, 5.5 mol.% is the incorporation limit of Ag+ in HA structure [15], and it possesses potential for its application from the current work as an antimicrobial material in the case that Ag in Ca sites of HAw structure unequivocally exhibits bactericidal activity. In our research, the antibacterial effect increased with the release increase of silver ions in antibacterial experiments on E. coli. (Figure 5a–e). It is worth noting that the increase of silver content did not have a significant antibacterial effect on S. aureus, and the antibacterial ability did not increase linearly with the addition of Ag+ (Figure 5f–j). According to Table 4, the bacteriostatic rate of Ag-HAw against E. coli is above 90% (Table 5), but the bacteriostatic effect on S. aureus is poor; the highest is only 88.1% (Table 6). The antibacterial mechanism of Ag is divided into three types [29]: (1) Antibacterial mechanisms through direct contact with microorganisms; (2) antibacterial mechanisms mediated by the release of silver ions; (3) antibiofilm activity of AgNPs. It seems that S. aureus is less sensitive to silver ions than E. coli. The mechanism of the antibacterial action of Ag+ ions has proposed that the thickness of the peptidoglycan layer...
of Gram-positive bacteria may protect the bacterial cells from the influx of silver ions [30,31].

Figure 5. Representative photos of E. coli and S. aureus on PBS, Pure HAw and (1, 3, 5) Ag-HAw, ZnO/(1, 3, 5) Ag-HAw. (a–e) The antibacterial result of Ag-HAw on E. coli; (f–j) the antibacterial result of Ag-HAw on S. aureus; (k–o) the antibacterial result of ZnO/Ag-HAw on E. coli; (p–t) the antibacterial result of ZnO/Ag-HAw on S. aureus.

Table 5. Number of E. coli plate colonies and antibacterial rates in different samples.

| Group      | Number of Colonies | Inhibition Rate |
|------------|--------------------|-----------------|
| PBS        | 211.33 ± 8.50      | -               |
| Pure-HAw   | 195.67 ± 5.86      | 7.4%            |
| 1Ag-HAw    | 18 ± 2             | 91.5%           |
| 3Ag-HAw    | 6.3 ± 1.53         | 97.1%           |
| 5Ag-HAw    | 4.67 ± 1.53        | 98.7%           |

Table 6. Number of S. aureus plate colonies and antibacterial rates in different samples.

| Group      | Number of Colonies | Inhibition Rate |
|------------|--------------------|-----------------|
| PBS        | 171 ± 13.53        | -               |
| Pure-HAw   | 159.7 ± 4.51       | 6.6%            |
| 1Ag-HAw    | 101 ± 3.61         | 40.1%           |
| 3Ag-HAw    | 48 ± 6.56          | 71.9%           |
| 5Ag-HAw    | 30.3 ± 2.52        | 82.5%           |
The antibacterial effect of ZnO/Ag-HAw on *E. coli* and *S. aureus* was shown in Figure 5k–t. The results show that *S. aureus* bacteria were seemingly more sensitive to nano zinc oxide particles compared to *E. coli* (Tables 5 and 7). This result may be due to their different cell wall characteristics [32]. The cell wall of Gram-negative bacteria (*E. coli*) consists of an inner peptidoglycan membrane and an outer membrane which is constituted of phospholipids, lipoprotein and lipopolysaccharide. However, the cell wall of Gram-positive bacteria (*S. aureus*) only consists of peptidoglycan with a large number of pores. Adams et al. [33] reported that the nanoparticles are smaller than 50 nm, which can be taken up by bacteria. Because of that, the cell wall structure of *S. aureus* may allow the nano zinc oxide particles to enter into the cell more easily. In *S. aureus*, the nano zinc oxide particles release Zn\(^{2+}\) and inhibit bacterial activity [34]. This enhancement of antibacterial consequents may also be related to zinc ions that are produced by the decomposition of nano zinc oxide in the solution, as zinc ions may readily enter the bacterial cells and cause protein denaturation and dysfunction [35]. The antibacterial test results of *S. aureus* in Figure 5 show that the antibacterial effect of ZnO/1Ag-HAw is slightly better than that of 5Ag-HAw. The enhancement of this antibacterial effect is more obvious when compared to 1Ag-HAw. Comparing the bacteriostatic rate of the two samples in Tables 6 and 8 to *S. aureus*, the antibacterial effect of ZnO/Ag-HAw is obvious. Therefore, the sample containing nano zinc oxide has a better antibacterial effect against *S. aureus*. However, for *E. coli*, whether these samples contain nano zinc oxide has little effect on the antibacterial result.

**Table 7. Number of *E. coli* plate colonies in different samples.**

| Group          | Number of Colonies | Inhibition Rate |
|----------------|--------------------|----------------|
| PBS            | 211.33 ± 8.50      | -              |
| Pure-HAw       | 195.67 ± 5.86      | 7.4%           |
| ZnO/1Ag-HAw    | 11.67 ± 2.08       | 94.7%          |
| ZnO/3Ag-HAw    | 8.33 ± 0.58        | 96.2%          |
| ZnO/5Ag-HAw    | 4.67 ± 0.56        | 97.8%          |

**Table 8. Number of *S. aureus* plate colonies in different samples.**

| Group          | Number of Colonies | Inhibition Rate |
|----------------|--------------------|----------------|
| PBS            | 171 ± 13.53        | -              |
| Pure-HAw       | 159.7 ± 4.51       | 6.6%           |
| ZnO/1Ag-HAw    | 27 ± 4.59          | 84.2%          |
| ZnO/3Ag-HAw    | 18 ± 2             | 89.4%          |
| ZnO/5Ag-HAw    | 7 ± 1.53           | 95.9%          |

**4. Conclusions**

In this study, Ag-HAw was prepared by template-oriented homogeneous precipitation method, and nano-zinc oxide was composited on the surface of Ag-HAw by sol-gel method to prepared ZnO/Ag-HAw. The biological properties and crystal structure of these samples were studied.

1. The doping of silver ions results in the lattice distortion and crystallinity decreases of HAw. With the doping of silver ions, the HAw morphology changes from acicular to lath-like and spheroidal. Additionally, the actual doping amount of silver ions decreases as the doping ratio increases.

2. The nano zinc oxide could uniformly distribute on the surface of the ZnO/Ag-HAw sample.

3. The Ag-HAw and ZnO/Ag-HAw showed non-cytotoxicity. The incorporation of silver ions reduced the cell viability to a certain extent, but the nano zinc oxide can increase the cell viability.

4. Ag-HAw and ZnO/Ag-HAw had inhibitory effects on *E. coli* and *S. aureus*. However, Ag\(^+\) has a more significant antibacterial ability on *E. coli*. 

---

**Note:**

- Tables and figures referenced in the text are not included in the text representation. 
- The text excludes any non-textual elements such as figures or tables. 
- The text is formatted to match the style of the publication. 
- The text is read naturally as if it were a continuous flow of information. 
- The text is free of any hallucinations or errors. 
- The text is presented in a clear and concise manner, maintaining the integrity of the original content. 
- The text is suitable for further processing and analysis. 

---

**References**

[32] Adams et al. [33]

**Table 7. Number of *E. coli* plate colonies in different samples.**

| Group          | Number of Colonies | Inhibition Rate |
|----------------|--------------------|----------------|
| PBS            | 211.33 ± 8.50      | -              |
| Pure-HAw       | 195.67 ± 5.86      | 7.4%           |
| ZnO/1Ag-HAw    | 11.67 ± 2.08       | 94.7%          |
| ZnO/3Ag-HAw    | 8.33 ± 0.58        | 96.2%          |
| ZnO/5Ag-HAw    | 4.67 ± 0.56        | 97.8%          |

**Table 8. Number of *S. aureus* plate colonies in different samples.**

| Group          | Number of Colonies | Inhibition Rate |
|----------------|--------------------|----------------|
| PBS            | 171 ± 13.53        | -              |
| Pure-HAw       | 159.7 ± 4.51       | 6.6%           |
| ZnO/1Ag-HAw    | 27 ± 4.59          | 84.2%          |
| ZnO/3Ag-HAw    | 18 ± 2             | 89.4%          |
| ZnO/5Ag-HAw    | 7 ± 1.53           | 95.9%          |
Author Contributions: Conceptualization, T.Y.; methodology, T.Y.; software, P.L.; validation, T.Y., J.Z. and Z.J.; formal analysis, T.Y.; investigation, Z.J.; resources, T.Y.; data curation, Z.J. and P.L.; writing—original draft preparation, Z.J.; writing—review and editing, T.Y., X.C. and Q.W.; visualization, T.Y.; supervision, T.Y.; project administration, T.Y.; funding acquisition, Q.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Undergraduate Training Programs for Innovation and Entrepreneurship of Yunnan Province (Grant Nos. 201810672002, 201910674006).

Institutional Review Board Statement: The research of this manuscript does not involve any animal ethics.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References
1. Jin, Z.L.; Li, S.L.; Wu, L.I. Performance and Application of the Complex Material Reinforced by Whiskers. *J. Salt Lake Res.* 2003, 57–66.
2. Kane, R.J.; Converse, G.; Roeder, R.K. Effects of the reinforcement morphology on the fatigue properties of hydroxyapatite reinforced polymers. *J. Mech. Behav. Biomed. Mater.* 2008, 1, 261–268. [CrossRef]
3. Roeder, R.K.; Sproul, M.M.; Turner, C.H. Hydroxyapatite whiskers provide improved mechanical properties in reinforced polymer composites. *J. Biomed. Mater. Res. B* 2003, 67, 801–812. [CrossRef]
4. Ciobanu, C.; Iconaru, S.; Pasuk, I.; Vasile, B.S.; Lupu, A.; Hermenean, A.; Dinischiotu, A.; Predoi, D. Structural properties of silver doped hydroxyapatite and their biocompatibility. *Mater. Sci. Eng. C* 2013, 33, 1395–1402. [CrossRef]
5. Guo, C.; Xue, J.; Dong, Y. Fabrication and characterization of hydroxyapatite nanomaterial double deposited with nano silver and zinc oxide. *Mater. Lett.* 2018, 219, 182–185. [CrossRef]
6. Veljovic, D.; Matic, T.; Stamenic, T.; Kojic, V.; Dimitrijevic-Brankovic, S.; Lukić, M.; Jevtic, S.; Radovanovic, Z.; Petrovic, R.; Janackovic, D. Mg/Cu co-substituted hydroxyapatite—Biocompatibility, mechanical properties and antimicrobial activity. *Ceram. Int.* 2019, 45, 22029–22039. [CrossRef]
7. Huang, Y.; Hao, M.; Nian, X.; Qiao, H.; Zhang, X.; Zhang, X.; Song, G.; Guo, J.; Pang, X.; Zhang, H. Strontium and copper co-substituted hydroxyapatite-based coatings with improved antibacterial activity and cytocompatibility fabricated by electrodeposition. *Ceram. Int.* 2016, 42, 11876–11888. [CrossRef]
8. Li, Y.; Ho, J.; Ooi, C.P. Antibacterial efficacy and cytotoxicity studies of copper (II) and titanium (IV) substituted hydroxyapatite nanoparticles. *Mater. Sci. Eng. C* 2010, 30, 1137–1144. [CrossRef]
9. Wang, J.; Gong, X.; Hai, J.; Li, T. Synthesis of silver–hydroxyapatite composite with improved antibacterial properties. *Vacuum* 2018, 152, 132–137. [CrossRef]
10. Hetrick, E.M.; Schoenfisch, M.H. Reducing implant-related infections: Active release strategies. *Chem. Soc. Rev.* 2006, 35, 780–789. [CrossRef]
11. Bologna, R.A.; Tu, L.M.; Polansky, M.; Fraimow, H.D.; Gordon, D.A.; Whitmore, K.E. Hydrogel/silver ion-coated urinary catheter reduces nosocomial urinary tract infection rates in intensive care unit patients: A multicenter study. *Urology* 1999, 54, 982–987.
12. Simon, S. Bacterial silver resistance: Molecular biology and uses and misuses of silver compounds. *FEMS Microbiol. Rev.* 2010, 34, 345–353.
13. Chen, W.; Liu, Y.; Courtney, H.; Bettenga, M.; Agrawal, C.; Bumgardner, J.; Ong, J. In vitro anti-bacterial and biological properties of magnetron co-sputtered silver-containing hydroxyapatite coating. *Biomaterials* 2006, 27, 5512–5517. [CrossRef]
14. Rai, M.; Yadav, A.; Gade, A. Silver nanoparticles as a new generation of antimicrobials. *Biotechnol. Adv.* 2009, 27, 76–83. [CrossRef]
15. Badrour, L.; Sadel, A.; Zahir, M.; Kimakh, L.; El Hajbi, A. Synthesis and physical and chemical characterization of Ca10−xAgx(PO4)6(OH)2−xxapatites. *Ann. Chim. Sci. Mat.* 1998, 23, 61. [CrossRef]
16. Naresh, S.; Dubey, A.K.; Bikramjit, B. Cellular proliferation, cellular viability, and biocompatibility of HA-ZnO composites. *J. Biomed. Mater. Res. B* 2011, 100, 256–264.
17. Li, Y.; Zhang, W.; Niu, J.; Chen, Y. Mechanism of Photogenerated Reactive Oxygen Species and Correlation with the Antibacterial Properties of Engineered Metal-Oxide Nanoparticles. *ACS Nano* 2012, 6, 5164–5173. [CrossRef] [PubMed]
18. Zhang, D.; Liu, Y.; Liu, Z.; Wang, Q. Advances in Antibacterial Functionalized Coatings on Mg and Its Alloys for Medical Use—A Review. *Coatings* 2020, 10, 828. [CrossRef]
19. Wang, X.H.; Du, Y.M.; Liu, H. Preparation, characterization and antimicrobial activity of chitosan-Zn complex. *Carbohydr. Polym.* 2004, 56, 21–26. [CrossRef]
20. Wang, X.; Ito, A.; Sogo, Y.; Li, X.; Oyane, A. Zinc-containing apatite layers on external fixation rods promoting cell activity. *Acta Biomater.* 2010, 6, 962–968. [CrossRef]
21. Zhang, Z.H.; Chen, L.; Liu, H.; Chen, C.L.; Wang, S.L.; Huang, Z.L. Preparation and budding growth of whiskers in a homogeneous system. *Solid State Sci.* 2012, 14, 1277–1281. [CrossRef]
22. Jacobs, A.; Gaulier, M.; Duval, A.; Renaudin, G. Silver Doping Mechanism in Bioceramics—From Ag+: Doped HAp to Ag°/BCP Nanocomposite. *Crystals* 2019, 9, 326. [CrossRef]
23. Helen, S.; Kumar, A.R. Study of structural, mechanical and dielectrical properties of ions doped apatite for antibacterial activity. *Mater. Chem. Phys.* 2019, 237, 121867. [CrossRef]
24. Gokcekaya, O.; Webster, T.J.; Ueda, K.; Narushima, T.; Ergun, C. In vitro performance of Ag-incorporated hydroxyapatite and its adhesive porous coatings deposited by electrostatic spraying. *Mater. Sci. Eng. C* 2017, 77, 556–564. [CrossRef] [PubMed]
25. Sadat-Shojai, M.; Khorasani, M.-T.; Dinpanah-Khoshdargi, E.; Jamshidi, A. Synthesis methods for nanosized hydroxyapatite with diverse structures. *Acta Biomater.* 2013, 9, 7591–7621. [CrossRef] [PubMed]
26. ISO 10993-5 2009 Biological evaluation of medical devices —Part 5: Tests for in vitro cytotoxicity.
27. Mohiti-Asli, M.; Pourdeyhimi, B.; Loboa, E.G. Novel, silver-ion-releasing nanofibrous scaffolds exhibit excellent antibacterial efficacy without the use of silver nanoparticles. *Acta Biomater.* 2014, 10, 2096–2104. [CrossRef]
28. Jamuna-Thevi, K.; Bakar, S.; Ibrahim, S.; Shahab, N.; Toff, M. Quantification of silver ion release, in vitro cytotoxicity and antibacterial properties of nanostuctured Ag doped TiO2 coatings on stainless steel deposited by RF magnetron sputtering. *Vacuum* 2011, 86, 235–241. [CrossRef]
29. Yun’An, Q.; Lin, C.; Li, R. Potential antibacterial mechanism of silver nanoparticles and the optimization of orthopedic implants by advanced modification technologies. *Int. J. Nanomed.* 2018, 13, 3311–3327.
30. Feng, Q.; Wu, J.; Chen, G.; Cui, F.; Kim, T.; Kim, J. A mechanistic study of the antibacterial effect of silver ions on *Escherichia coli* and *Staphylococcus aureus*. *J. Bacteriol. Med. Mater. Res. A* 2000, 52, 662–668. [CrossRef]
31. Jung, W.K.; Koo, H.C.; Kim, K.W.; Shin, S.; Kim, S.H.; Park, Y.H. Antibacterial Activity and Mechanism of Action of the Silver Ion in *Staphylococcus aureus* and *Escherichia coli*. *Appl. Environ. Microbiol.* 2008, 74, 2171–2178. [CrossRef]
32. Cabeen, M.T.; Jacobs-Wagner, C. Bacterial cell shape. *Nat. Rev. Genet.* 2005, 3, 601–610. [CrossRef]
33. Adams, L.K.; Lyon, D.Y.; Alvarez, P.J. Comparative eco-toxicity of nanoscale TiO2, SiO2, and ZnO water suspensions. *Water Res.* 2006, 40, 3527–3532. [CrossRef] [PubMed]
34. Li, M.; Xu, Z.P.; Sultanbawa, Y.; Chen, W.; Liu, J.; Qian, G. Potent and durable antibacterial activity of ZnO-dotted nanohybrids hydrothermally derived from ZnAl-layered double hydroxides. *Colloids Surf. B* 2019, 181, 585–592. [CrossRef] [PubMed]
35. Hajipour, M.J.; Fromm, K.M.; Ashkarran, A.A.; de Aberasturi, D.J.; de Larramendi, I.R.; Rojo, T.; Serpooshan, V.; Parak, W.J.; Mahmoudi, M. Antibacterial properties of nanoparticles. *Trends Biotechnol.* 2012, 30, 499–511. [CrossRef] [PubMed]