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The antimicrobial activity of as-prepared silver-loaded phosphate glasses and zirconium phosphate

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Abstract–The antimicrobial activities of silver-loaded zirconium phosphate (JDG) and silver-loaded phosphate glasses (ZZB) against *Escherichia coli* were studied. Although the silver content in JDG was higher than that in ZZB, ZZB suspensions showed better antimicrobial property than JDG suspensions, especially at low concentrations. The antimicrobial activity was analyzed using minimum inhibitory concentrations, bacterial inhibition ring tests, and detection of silver ions in the suspensions. Furthermore, the amounts of silver ions in suspensions with/without bacterial cells were analyzed. Results revealed that only a portion of released silver ions could be adsorbed by *E. coli* cells, which are critical to cell death. The damaged microstructures of *E. coli* cells observed by transmission electron microscopy may further prove that the adsorbed silver ions play an important role in the antimicrobial process.

Keywords: Silver-loaded Inorganic Materials, Antimicrobial Agent, Antimicrobial Activity, Released Silver Ions, *Escherichia coli*

INTRODUCTION

Silver-containing materials are used in various industries and in daily human activities [1-3] because of their strong antimicrobial effects on different bacteria [4,5]. In studies on silver-loaded antimicrobial materials, many inorganic carriers [6-10], such as zeolite, zirconium phosphate, mesoporous silica, titanium dioxide and hydroxyapatites, have been developed owing to their advantages of chemical stability, biocompatibility, and long lasting action period compared with traditional organic antimicrobial materials. The bactericidal mechanisms of these inorganic silver-containing materials on microorganisms have been partially investigated. One observation shows the strong interaction between the silver ions and the thiol group of vital enzymes, which renders microorganisms inactive [11]. Another mechanism involves the generation of reactive oxygen species by the cells [12], causing the oxidation of silver ions in the aqueous environments that, in turn, disrupts the cells. Such cases have focused on the consequence of released silver ions from the silver-loaded inorganic materials in aqueous solution on antimicrobial efficiency [13-15]. However, antimicrobial efficiency does not follow the expected relationship with the amount of released silver ions, as reported by Ahemd et al. [16]. Hence, the antimicrobial effect of silver ions involved in silver-loaded inorganic materials needs further investigation.

We investigated the antimicrobial activities of two silver-loaded phosphate materials with different silver contents: a commercial product called Novaron AGT330, which is a type of high-efficiency antibacterial agent, and the other is a self-made product. The antibacterial activities of the two agents were compared. The variation of the detected silver ions in different suspensions of the two antimicrobial agents was used to determine the effects of silver ions on the antimicrobial activities. The microstructure of the bacterial cells observed by transmission electron microscopy (TEM) was used to determine the damaging effect of the antimicrobial agents. These findings could reveal the antimicrobial mechanisms of silver-loaded phosphate inorganic materials. Moreover, the superior antimicrobial activity of the self-made product at low concentration suspension may indicate its potential for future practical applications.

MATERIALS AND METHODS

1. Materials

Two different inorganic antimicrobial agents were studied here: silver-loaded zirconium phosphate (JDG) powder (East Asian Synthesis Co. Ltd., Japan) and silver-loaded phosphate glasses (ZZB) powder, which was prepared by using the solid-state fusion method. ZZB powder was prepared by mixing glass powder and $AgNO_3$ (≥99.8%) (Beijing Yili reagent Co. Ltd.) in a mass ratio of 50 : 1. Then, the mixture was heated at 850 °C in oxic atmosphere and quenched in water. After cooling, the mixture was dried, milled, and sifted using micron strainers. The final powders were collected and labeled as ZZB. Antimicrobial agent suspensions were prepared by dissolving the antimicrobial powders into saline. The concentration of the prepared suspensions can be adjusted depending on the test.

The *Escherichia coli* strain (AS1.90) was purchased from China General Microbiological Culture Collection Center. The bacterial cells were cultured in 0.2 wt% nutrient broth at 24 °C with 24 h simul-

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taneous agitation at 100 rpm. Next, the cultured cells were diluted by saline. The concentrations of *E. coli* suspensions were adjusted to 10^5 and 10^8 cells/ml, respectively, for the minimal inhibitory concentration/bacterial inhibition ring test and the analytical determination of the released silver ions.

2. Characterizations

The phase identification was performed by X-ray diffractometer (XRD; Bruker D-8) with 2θ ranging from 10° to 80°. The morphology and particle size of ZZB and JDG powders were investigated using a scanning electron microscope (SEM; Quanta 250FEG) and laser granulometer (Mastersizer2000MU). The silver content in the powders and suspensions was quantified by using atomic absorption spectroscopy (AAS; Aurora AI1200), and the microstructure of bacterial cells was acquired with TEM (Hitachi H-7650).

3. Specimen Preparation for TEM

Two kinds of antimicrobial powders were separately dissolved in saline to prepare 1 mg/ml concentration of the suspensions. Each suspension was mixed with the prepared *E. coli* solution at a volume ratio of 1 : 1 for 3 h. The mixture was further diluted with saline and centrifuged at 5,000 rpm for 5 min. The resulting precipitation was collected, prefixed in a fixing solution containing 2.5 wt% glutaraldehyde and 2 wt% paraformaldehyde, and kept in the refrigerator at 4 °C for 2 h. Then, the mixture was immersed in a phosphate buffer solution for 10 min and further centrifuged at 5,000 rpm for 5 min. The precipitation was then dehydrated at room temperature with 95% ethanol for 10 min and embedded in propylene oxide resin. For cross-section observation, the specimens were cut into 100 nm thick slices with an ultramicrotome and then stained with 1% uranyl acetate and 0.2% lead citrate solution prior to TEM measurement.

4. Minimal Inhibitory Concentration (MIC) Test

The MIC value of antimicrobial agents refers to the lowest concentration at which the antimicrobial agent can completely inhibit visible bacterial growth, as judged by the naked eye [16]. Each antimicrobial agent was dissolved in saline to make suspensions with 0.2, 0.4, 1.0, 2.0, 5.0, and 50.0 mg/ml concentrations. For the MIC test, 1 ml of each suspension was dropped onto different sterile petri plates, and 1 ml of the prepared *E. coli* suspension and 20 ml of nutrient agar medium were added to each plate. The mixtures were incubated for 24 h at 37 °C, and the numbers of colonies were counted. The test was repeated thrice for each sample, and the average value was recorded.

5. Bacterial Inhibition Ring Test

The bacterial inhibition ring test was carried out according to standard operation procedures in the test standard QB/T 2591-2003 (Antimicrobial plastic-Test for antimicrobial activity and efficacy). In an agar plate with a diameter of 90 mm, 100 µl of the prepared *E. coli* suspensions were pipetted and spread throughout the surface. Then, 50 mg of each of the antimicrobial powders was placed on filter papers with a diameter of 6 mm and onto the agar plate, which was incubated at 37 °C for 24 h. The diameters of inhibition zones were measured in millimeter (mm).

6. Analytical Determination of Released Silver

AAS was used to quantify the amount of silver released from different media (culture media or culture media containing bacteria). For the analytical determination of released silver in culture media containing bacteria, 4 ml of bacterial cell suspensions (10^8 cells/ml) was added under sterile conditions into plastic tubes containing 24 ml of each kind of antimicrobial agent solution at a certain concentration. Dispersions were incubated at 37 °C for 0.1, 1, 3, 5, and 24 h in an orbital incubator at 150 rpm, and then centrifuged at 5,000 rpm for 5 min. The collected supernatants were analyzed by using AAS.

RESULTS

1. Phase Identification

The XRD patterns of JDG and ZZB particles are shown in Fig. 1. Compared with the standard diffraction pattern, the JDG sample is identified as a mixture of zirconium phosphate $Zr_{2.25}(PO_4)_3$ and silver phosphate Ag_3PO_4, [Fig. 1(a)]. The amorphous characteristic peak is observed in the ZZB sample.

2. Morphology Characterization

The morphologies of two antimicrobial agents were observed by using SEM. As seen in Fig. 2(a), the JDG sample is a mixture of large cubic particles (around 1 µm) and small particles (0.1 µm) consistent with the size distribution result in Fig. 2(a) inset. Fig. 2(b) shows that ZZB particles are irregular and with a broad size distribution. The size distribution plot in Fig. 2(b) inset illustrates that the average particle size of ZZB is above 10 µm.

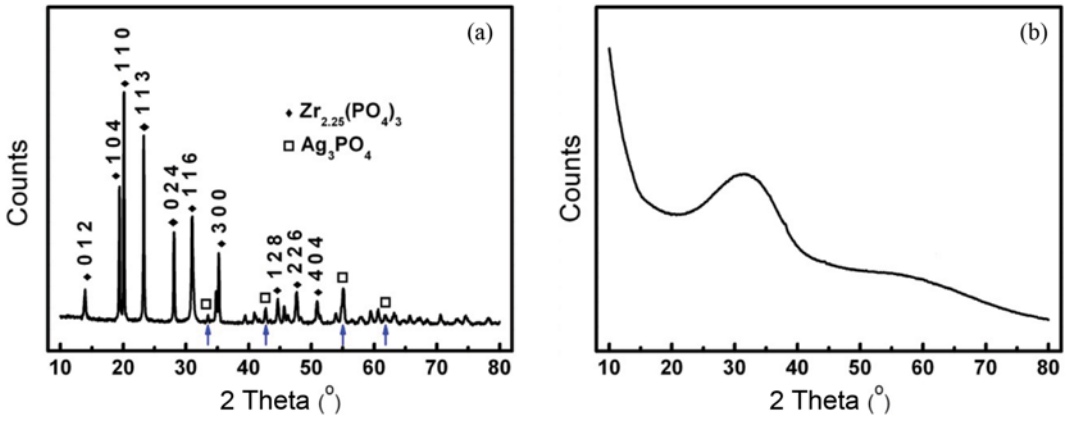

Fig. 1. XRD patterns of (a) JDG powders and (b) ZZB powders.

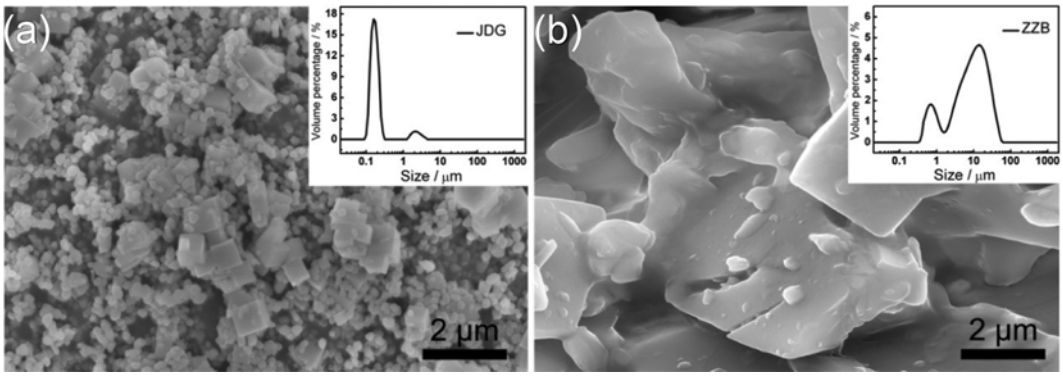

Fig. 2. SEM images of (a) JDG powders and (b) ZZB powders and powders' particle size distribution (inset).

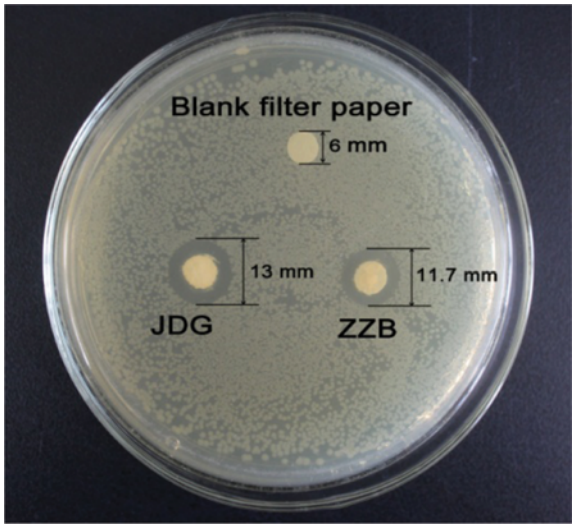

Fig. 3. Picture of the bacterial inhibition rings.

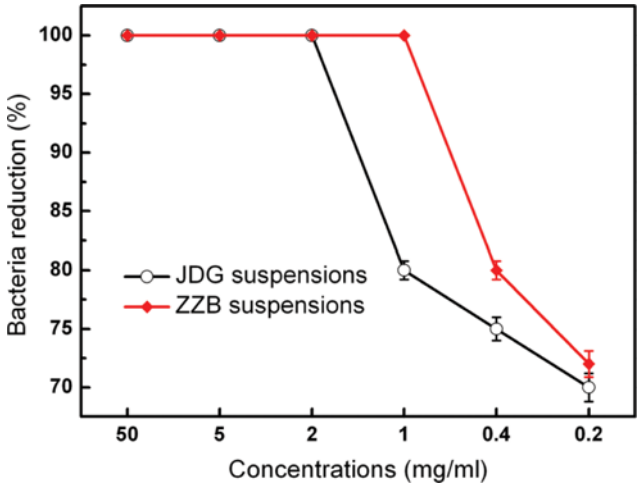

Fig. 4. The relationship of bacteria reduction rate with the concentration of *E. coli* strain suspensions.

3. Detection of Silver Content

AAS results show that 5.19 wt% and 1.6 wt% of silver are detected in the JDG and ZZB powders, respectively.

4. Results of the Bacterial Inhibition Ring Test

The bacterial inhibition ring test was performed to evaluate the bacterial activities of bacterial agent powders according to the standard QB/T 2591-2003. The measurements of the bacterial rings are shown in Fig. 3. A blank filter paper in the agar plate was used for comparison. According to the test standard, the antimicrobial agent that produces an inhibition ring greater than 8.5 mm in diameter has an antibacterial effect. Each test was repeated at least thrice, and the average diameters were calculated. The diameters of inhibition rings for JDG and ZZB are 13.0 and 11.7 mm, respectively.

5. Result of the MIC Test

The MIC test was performed and the results shown in Fig. 4. The minimum concentration required for each sample to achieve 100% bacterial reduction is the MIC value. The MIC values for JDG and ZZB were 2 and 1 mg/ml, respectively.

DISCUSSION

The inhibition ring of JDG is larger than that of ZZB, indicating better antimicrobial activity. Silver ions suppress the growth of bacterial cells [17], and thus, the amount of silver ions released from antimicrobial agents is significant to the antimicrobial effect. The more pronounced antimicrobial effect of the JDG powder may have been caused by its higher silver content (5.19%) compared with that of the ZZB powder (1.6%).

MIC is another critical parameter for evaluating the antimicrobial effect. For JDG suspensions with concentrations above 2 mg/ml, almost no colony was observed after the reaction. As the concentration of the suspensions decreased to 1 mg/ml and below, the number of observed colonies increased. Thus, the MIC values for JDG and ZZB are 2 and 1 mg/ml, respectively. MIC values can also be related to the bacterial reduction rate, which is calculated based on Eq. (1). The relationship of bacteria reduction with the suspension concentrations is shown in Fig. 4. The error bars represent the evaluation error during the counting process of colonies.

For JDG and ZZB suspensions with concentrations lower than the MIC value, the percentage of bacteria reduction is calculated from the equation [18,19]

$$\text{Bacterial ratio (\%)} = (N_0 - N_1)/N_0 \times 100\%, \quad (1)$$

where N_0 and N_1 are the number of colonies in the strain suspen-

sion at the initial and culture time, respectively.

As seen in Fig. 4, the bacteria reduction rate of ZZB suspensions is greater than that of JDG suspensions at the same concentration, indicating that ZZB is a more effective antimicrobial agent against *E. coli* than JDG. This result is confirmed by the lower MIC value of ZZB than JDG. However, this finding is inconsistent with the conclusion obtained from the bacterial inhibition ring test. In fact, the "conflict illusion" is what led us to do the mechanism investigation. We found that this phenomenon is normal because the inhibition ring test is performed using powders, whereas the MIC test is based on solutions. Antimicrobial powders used in the bacterial inhibition ring test can be taken as suspensions with extremely high concentrations. As detected by AAS, the silver content of the JDG powder (5.19%) is significantly higher than that of the ZZB powder (1.6%), explaining the larger inhibition ring of the JDG sample. Suspensions of antimicrobial agents were used for the MIC test; however, the amount of silver ions in suspensions is much different from that in powdered materials. Thus, AAS traced the released silver ions in different suspensions, as shown in Fig. 5. The error bar in Fig. 5(a) represents the evaluation error during the solution preparation.

Fig. 5(a) shows the various amounts of released silver ions in different concentrations of suspensions. As can be seen, the initial silver content of the JDG powder (5.19%) is higher than that of ZZB powder (1.6%), leading to more silver ions detected in JDG suspensions at 100 mg/ml. However, the detected silver ions released in JDG suspensions are significantly reduced with decreasing concentrations. When the concentration of suspensions reaches lower than 1.7 mg/ml, JDG suspension releases less silver than ZZB suspension. These results show that the release of silver ions from ZZB is easier than that from JDG suspensions, especially at relatively low concentrations, which may explain the lower MIC value of ZZB suspensions. Another factor should be considered in analyzing the different antimicrobial activities of the two studied materials. When the JDG and ZZB suspensions were treated with *E. coli* cells, the released silver ions detected in the suspensions were significantly reduced. The amounts of detected silver ions in the suspensions with and without *E. coli* cells change with time. The results in Figs. 5(b) and (c) indicate that only one in 10000 silver ions could be released from both ZZB and JDG suspensions at the first 10 min. After incubation for 24 h, the amount of released silver ions increases four times but is sharply reduced after the addition of *E. coli*. This result may be attributed to the adsorption of silver ions by *E. coli* cells [20].

The blue arrows in Figs. 5(b) and (c) represent the reduction scale of detected silver ions in JDG and ZZB suspensions treated

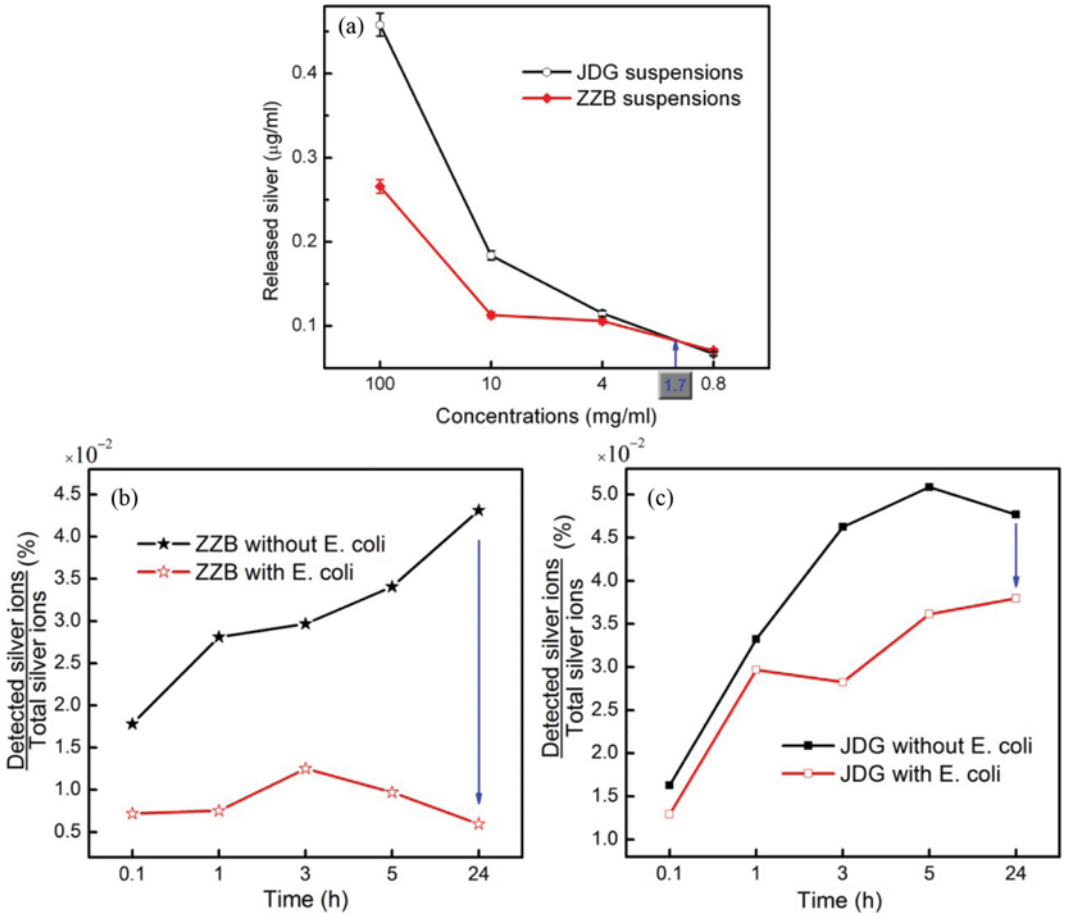

Fig. 5. (a) Varied amounts of released silver ions with concentrations of JDG and ZZB suspensions; (b) and (c) are the released silver ions in JDG and ZZB suspensions with and without *E. coli*.

with *E. coli*. The reduction in ZZB suspensions was greater than that in JDG, indicating that greater portions of the released silver could be adsorbed by the *E. coli* cells in ZZB suspensions. This phenomenon may also explain why the antimicrobial ability of ZZB is better than that of JDG at a concentration of 1 mg/ml, despite the minor difference in the amount of detected silver ions in the two suspensions without bacterial cells.

Therefore, for silver-loaded antimicrobial materials, it is the amount of silver ions absorbed by the bacterial cells that indicates the antimicrobial ability, rather than the silver content in the powdered antimicrobial materials. The evident differences in the particle size and crystallinity of the two antimicrobial materials studied may have also influenced the dispersion of silver ions in suspensions and their attachment to the antimicrobial materials [21]. Consequently, the release of silver ions from the materials and the absorption of silver ions by *E. coli* cells may thus be influenced.

The morphology of *E. coli* bacterial cells before and after the interaction with antimicrobial agents was observed by using TEM. Similar results were obtained when *E. coli* cells were incubated in JDG or ZZB suspensions for 3 h. A series of cross-section view images in Fig. 6 demonstrate the microstructures of *E. coli* cells. *E. coli* cells were in good condition before interacting with silver ions, as shown in Figs. 6(a) and (b). The multilayered surface consisting of the outer membrane, the peptidoglycan layer in periplasmic space, and the cytoplasmic membrane are clearly displayed in each cell. After exposure in the antimicrobial suspensions for 3 h, the amount of observed *E. coli* cells is reduced, indicating that a portion of the bacterial cells may have been affected. The remaining *E. coli* cells are damaged, as confirmed by the inhomogenous cytoplasm, the fuzzy cell walls, and leakage of some intracellular substances. Vacuole degeneration is also observed in some cells encircled by blue lines in Fig. 6(c). Some black spots labeled by the red arrow in Fig. 6(d) are also found in the treated *E. coli* cells, indicating that some coagulated particles might have been formed during the permeation of adsorbed silver ions.

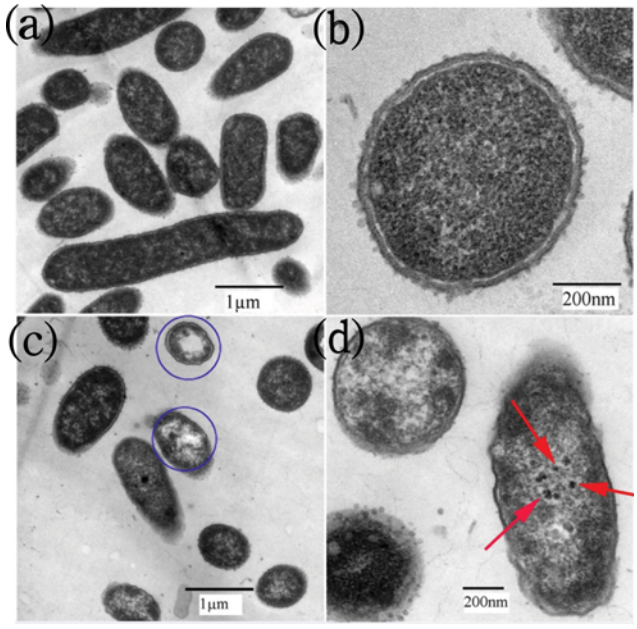

Fig. 6. TEM images of thin sections of (a)-(b) untreated *E. coli* and (c)-(d) *E. coli* treated with ZZB or JDG suspensions for 3 h.

CONCLUSIONS

The antimicrobial activity of ZZB and JDG against *E. coli* has been studied. The two agents had different particle sizes, structures, and silver contents, which influenced the amount of silver ions released from antibacterial materials depending on the concentration. More importantly, the amount of adsorbed silver by *E. coli* cells served as the key factor in the efficiency of the antimicrobial suspensions, instead of the content or the released silver ions in the suspensions. A relatively larger portion of released silver ions could be absorbed by ZZB, leading to better antimicrobial effect than that produced by JDG in low suspension concentrations. Furthermore, the microstructure of bacterial cells exposed in the antimicrobial suspensions was observed by using TEM. The results illustrated that the silver ions could damage the *E. coli* cells by breaking the cell membrane, thus causing the leakage of intracellular substances and the degeneration of vacuoles.

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