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Full Length Research Paper

Antibacterial effects of zinc oxide nanoparticles on *Escherichia coli* K88

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This study was conducted to evaluate the antibacterial effects of zinc oxide nanoparticles *in vitro*. *Escherichia coli* K88 was chosen as an indicator of pathogenic bacteria, because it could cause diarrhea in both children and in early-weaned piglets. In this study, the characterization of the nanoparticles was examined. Antibacterial activities against *E. coli* K88 were evaluated by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) and observing the effects on the values of the optical density (OD) at 620 nm and the populations. Results indicate that zinc oxide nanoparticles had strong antibacterial activity against *E. coli* K88. The activity increased as the concentration of the nanoparticles increased. The MIC and MBC were 0.1 and 0.8 µg/ml, respectively. To study the antibacterial mechanisms, atomic force microscopy (AFM) and scanning electron microscopy (SEM) were used to observe morphological changes of *E. coli* K88 treated with 0.8 µg/ml zinc oxide nanoparticles. The results reveal that zinc oxide nanoparticles could damage cell membranes, lead to leakage of cytoplasm and kill the bacterial cells. Our study indicates that zinc oxide nanoparticles could potentially be an antibacterial reagent to treat diseases caused by bacteria.

Key words: Zinc oxide, nanoparticle, *Escherichia coli* K88, antibacterial activity, atomic force microscopy (AFM).

INTRODUCTION

Escherichia coli K88 is an important pathogen, which could cause diarrhea in both children and early-weaned piglets (Guo et al., 2005; Yu et al., 2011). Antibiotics are the most common drugs to inhibit the growth and propagation of *E. coli* K88. However, the use of antibiotics has various side effects, such as the increase in bacterial resistance (Baquero et al., 2011). The exploitations of novel substitutes to antibiotics, especially on inorganic nanoparticles, have recently attracted more attention.

Zinc oxide is an important inorganic material, which has multiple properties, such as semiconducting properties, antibacterial activity and growth promoter. It is widely applied in the field of optoelectronics (Yu et al., 2003; Gao et al., 2005), pharmaceutics (Baldwin et al., 2001), cosmetics (Sheldon et al., 2000; Mitchnick et al., 1999), food science (Daniel et al., 2003) and agriculture (Smith et al., 1997; Carlson et al., 1999). The antibacterial activity of zinc oxide has been widely explored. It has been documented that concentration, size and healing temperature can affect the antibacterial activity. Zinc oxide as an inorganic antibacterial reagent is more stable than the organic reagents (Yamamoto, 2001; Sawai, 2003; Sawai et al., 1996a). Several antimicrobial mechanisms of zinc oxide were supposed; i) hydrogen peroxide, which is generated from the surface of zinc oxide, can penetrate through the cell membrane, produce some type of injury, and inhibit the growth of the cells (Yamamoto, 2001; Sawai, 2003; Sawai et al., 1996b, 1998); ii) the affinity between zinc oxide and bacterial cells is an important factor for antibacterial activity (Stoimenov et al., 2002).

Combined with nanotechnology, zinc oxide nanoparticles can be prepared, which possess some unique characters, such as small particle size and large area surface. Zinc oxide nanoparticles may exhibit

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Abbreviations: MIC, Minimum inhibitory concentration; **MBC,** Minimum bactericidal; **OD,** optical density; **AFM**, atomic force microscopy; **SEM,** scanning electron microscopy.

stronger antibacterial activity than zinc oxide itself (Yamamoto, 2001). Therefore, the interactions of nanoparticles with microorganisms have recently attracted more attention and a wide range of antibacterial effects of zinc oxide nanoparticles have been reported (Jones et al., 2008; Reddy et al., 2007). Moreover, zinc oxide nanoparticles have selective toxicity and are generally regarded as a safe reagent to humans and animals (Reddy et al., 2007; Liu et al., 2009; Fu et al., 2005; Berube, 2008), which could be an ideal potential antibacterial reagent to replace some antibiotics. However, few studies have been conducted to evaluate the antibacterial effects of zinc oxide nanoparticles on *E. coli* K88. Moreover, the antibacterial mechanism of zinc oxide nanoparticles is still unclear. Therefore, our study was undertaken to investigate the antibacterial activity against *E. coli* K88 *in vitro*. The atomic force microscope (AFM) and scanning electron microscopy (SEM) were used to study the mechanism.

MATERIALS AND METHODS

Characterization of zinc oxide nanoparticles

Zinc oxide nanoparticles were provided by Institute of Feed Science, Zhejiang University, Zhejiang, China. The particle sizes of zinc oxide nanoparticles were determined by AFM (AFM, SPM-9500J3, Shimadzu CO., Japan) and Zetasizer Nano-ZS90 (Malvern Instruments) by the method of Du et al. (2008). For AFM, the zinc oxide nanoparticles suspended into water were placed onto cleaved mica and observed in contact mode with Si_3N_4 probes. For Zetasizer Nano-ZS90, the analysis was performed at a scanning angle of 90° at 25°C using samples diluted with water. The water used throughout this study was the reagent-grade water produced by Milli-Q SP Ultra-Pure-Water Purification System of Nihon Millipore Ltd. (Tokyo, Japan).

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined by a method recommended in NCCLS (2000) with some modifications. Briefly, the sterile tubes were incubated aerobically at 37°C for 24 h, which contained 5 ml Muller- Hinton (MH) broth (Difco, USA) with approximate 5×10^9 CFU bacterial cells and 0 (the control group), 0.025, 0.05, 0.1, 0.2, 0.4, and 0.8 µg/ml zinc oxide nanoparticles. The concentration of tube without visible growth of the bacterial cells was the MIC. To evaluate the MBC, 100 µl of sample from each tube without visible growth was transferred into MH agar plate (Difco, USA), and then incubated aerobically for another 24 h. The concentration of the tube without growth was the MBC (in this test, the population in agar plate less than 10 was regarded no growth). All the measures were triplicate.

Antibacterial effects on values of optical density (OD) at 620 nm and the populations

The sterile tubes contained 5 ml Luria–Bertani (LB) culture fluids, approximate 5×10^9 CFU bacterial cells and 0 (the control group), 0.025, 0.05, 0.1, 0.2, 0.4, 0.8 µg/ml zinc oxide nanoparticles, and were incubated aerobically at 37°C for 24 h. Then, samples were collected to measure the values of OD at 620 nm and bacterial populations. Values of OD at 620 nm were determined using an ultra violet visible (UV-Vis) spectrophotometer (Hitachi Ltd, Tokyo, Japan). A sample of 1 ml from each tube was serially diluted using phosphate buffer solution (PBS) for enumeration of bacterial populations. Bacterial populations were enumerated on LB agar plate. All the measures were in triplicates.

Morphological changes of *E. coli* K88

AFM (SPM-9500J3, Shimadzu CO., Japan) and SEM (XL30-ESEM Philips company, Japan) were used to examine morphological changes of *E. coli* K88 treated with 0.8 µg/ml zinc oxide nanoparticles. For AFM, the morphological changes were determined as described by Du et al. (2008). Briefly, the sterile tubes, which contained 5 ml, 0.8 µg/ml zinc oxide nanoparticles LB culture fluid and approximate 5×10^9 CFU bacterial cells, were cultured aerobically in a shaken thermostat (200 rmp/min) at 37°C. Samples were collected from the tubes at 0 (the control), 0.5, 1.0, 2.0, 3.0, 4.0 h, dried on slices, and observed by AFM in contact mode with Si_3N_4 probes.

For SEM, samples were collected from the tubes at 0 (the control) and 0.5 h. The samples were immersed in the solution of 2.5% glutaraldehyde (Merck EM grade, Auer Bittman Soulie AG, Basel, Switzerland) for 24 h and were washed by PBS. Then, they were fixed by 1% OsO_4 solution for 2 h and dehydrated in a grade series of ethanol (50, 70, 80, 90, 95, 100, and 100%) for 15 min at each step. Subsequently, the samples were treated with solution of ethanol and iso-amyl acetate(1:1) for 30 min, then treated with pure iso-amyl acetate for 2 h and dried by Hitachi HCP-2 critical point dryer (Hitachi Ltd, Tokyo, Japan). The samples were then coated with gold using Eiko IB-5 ion coater (Eiko Engineering Co. Ltd., Tokyo, Japan), after which, they were observed with SEM.

Statistical analysis

Data were analyzed statistically by one-way analyses of variance (ANOVA), using the SPSS statistical software package for Windows (version 16.0, SPSS, Chicago, USA). Probability values below 0.05 were considered to be statistically significant.

RESULTS AND DISCUSSION

Particle size

The primary sizes of zinc oxide nanoparticles are one of the important factors for the antibacterial activity (Zhang et al., 2007a). In our study, zinc oxide nanoparticles were characterized by Zetasizer Nano-ZS90 (Malvern Instruments) and AFM. The sizes of zinc oxide nanoparticles were 75±20 nm as determined by Zetasizer Nano-ZS90, which indicated that the zinc oxide nanoparticles in this study were small sized and could exhibit antibacterial activity of nanoparticles. Images observed by AFM are shown in Figure 1. The images show that the zinc oxide nanoparticles were small sized, which were in line with the results of Zetasizer Nano-ZS90. Moreover, Figure 1b reveals that the morphologies of the zinc oxide nanoparticles were not quasi-spherical morphology, as reported by Wu et al. (2010). The nonspherical morphology coming from the

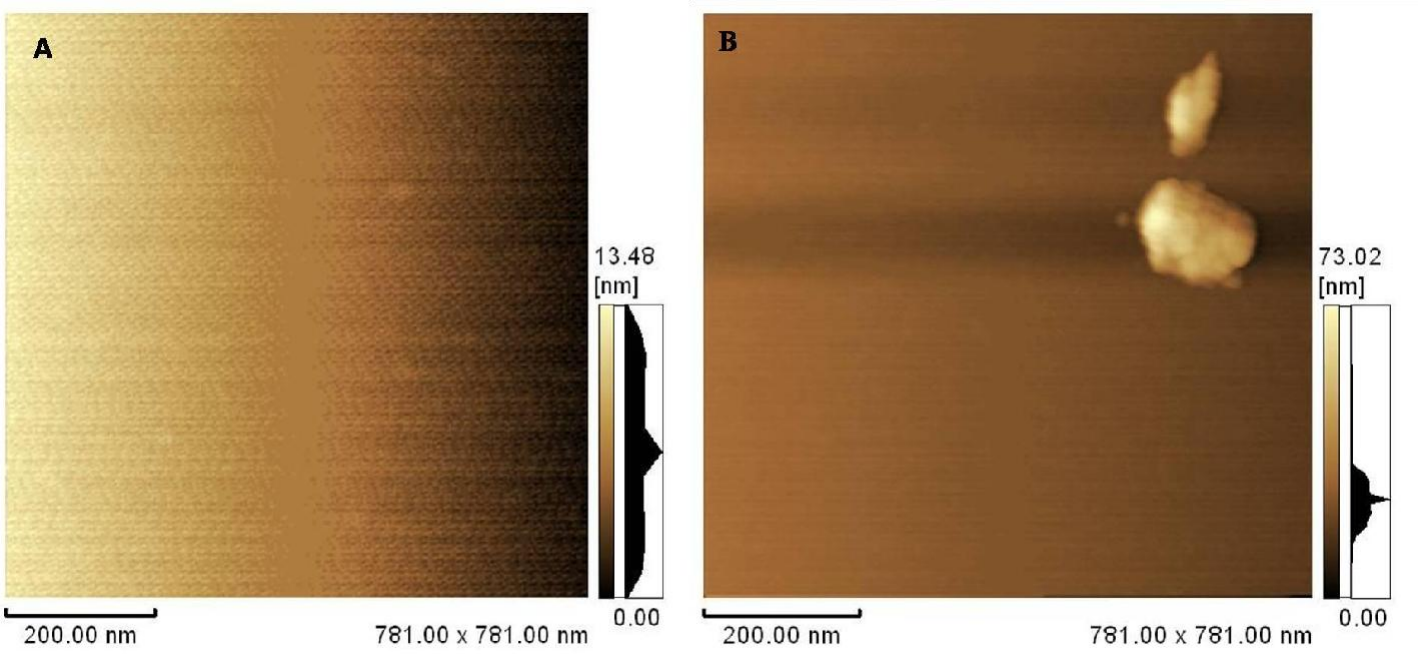

Figure 1. AFM images of mica surface (a) and zinc oxide nanopartcles (b).

preparation method may influence the antibacterial activity, which could be studied in the future.

Antibacterial activity

In this study, results of MIC and MBC show that the MIC and MBC were 0.1 and 0.8 µg/ml, respectively. The ratio of MBC/MIC was 8. These results indicate that zinc oxide nanopaticles had antibacterial effects on *E. coli* K88, which is partly in accordance with the reports by Soderberg et al. (1990), Sawai (2003), Hernandez-Sierra et al. (2008), and Liu et al. (2009). They reported that zinc oxide or its nanoparticles had antibacterial activity against *Staphylococcus aureus*, *Streptococcus*, *E. coli* 745 and *E. coli* O157:H7. However, the antibacterial concentrations were inconsistent. Soderberg et al. (1990) revealed that 179 and 1790 µg/ml zinc oxide could exhibit a clear antibacterial effect on *S. aureus.* Results of Liu et al. (2009) indicated that 3 mmol/l zinc oxide nanoparticles could inhibit the growth of *E. coli* O157:H7 and 12 mmol/l or higher concentrations completely inhibited the growth. The main factors for the variable antibacterial concentrations may be that the different microbes and zinc oxide or its nanoparticles were used. Interestingly, Roselli et al. (2003) reported that 1 mmol/l zinc oxide (greatly exceeding the level of 0.8 µg/ml zinc oxide nanoparticles) did not affect the growth of *E. coli* K88, which partly explained the reports that zinc oxide nanoparticles exhibited stronger antibacterial activity than zinc oxide (Yamamoto, 2001). It has been reported that the values of OD at 620 nm can be an important indicator for bacterial growth (Missotten et al., 2009; Zhang et al., 2007b). Therefore, effects of zinc oxide nanoparticles on values of OD at 620 nm were measured and are shown in Figure 2a, which indicated that 0.025 and 0.05 µg/ml zinc oxide nanoparticles did not affect the bacterial growth. Compared with the control group, 0.1, 0.2, 0.4, and 0.8 µg/ml zinc oxide nanoparticles could significantly decrease the values of OD at 620 nm, especially the 0.8 µg/ml. These results are consistent with the results of MIC and MBC. Figure 2a also reveals that the antibacterial activities increased as the concentration of zinc oxide nanoparticles increased, which are in line with results of Brayner et al. (2006) and Liu et al. (2009).

Effects of zinc oxide nanoparticles on the populations of *E. coli* K88 are shown in Figure 2b, which indicates that compared with the control group, 0.05, 0.1, 0.2, 0.4 and 0.8 µg/ml zinc oxide nanoparticles significantly decreased the population of *E. coli* K88. These results are partly inconsistent with the results of the MIC and effects on values of OD at 620 nm, in which 0.05 µg/ml zinc oxide nanoparticles did not affect the bacterial growth. This difference may indicate that concentrations of zinc oxide nanoparticles in MH broth or LB media could affect the results of the MIC and values of OD at 620 nm.

Morphological changes of *E. coli* K88

The mechanisms of antibacterial activities of zinc oxide or its nanoparticles against some microbes have been explored. However, no study has been reported to investigate the mechanisms of antibacterial effects of zinc

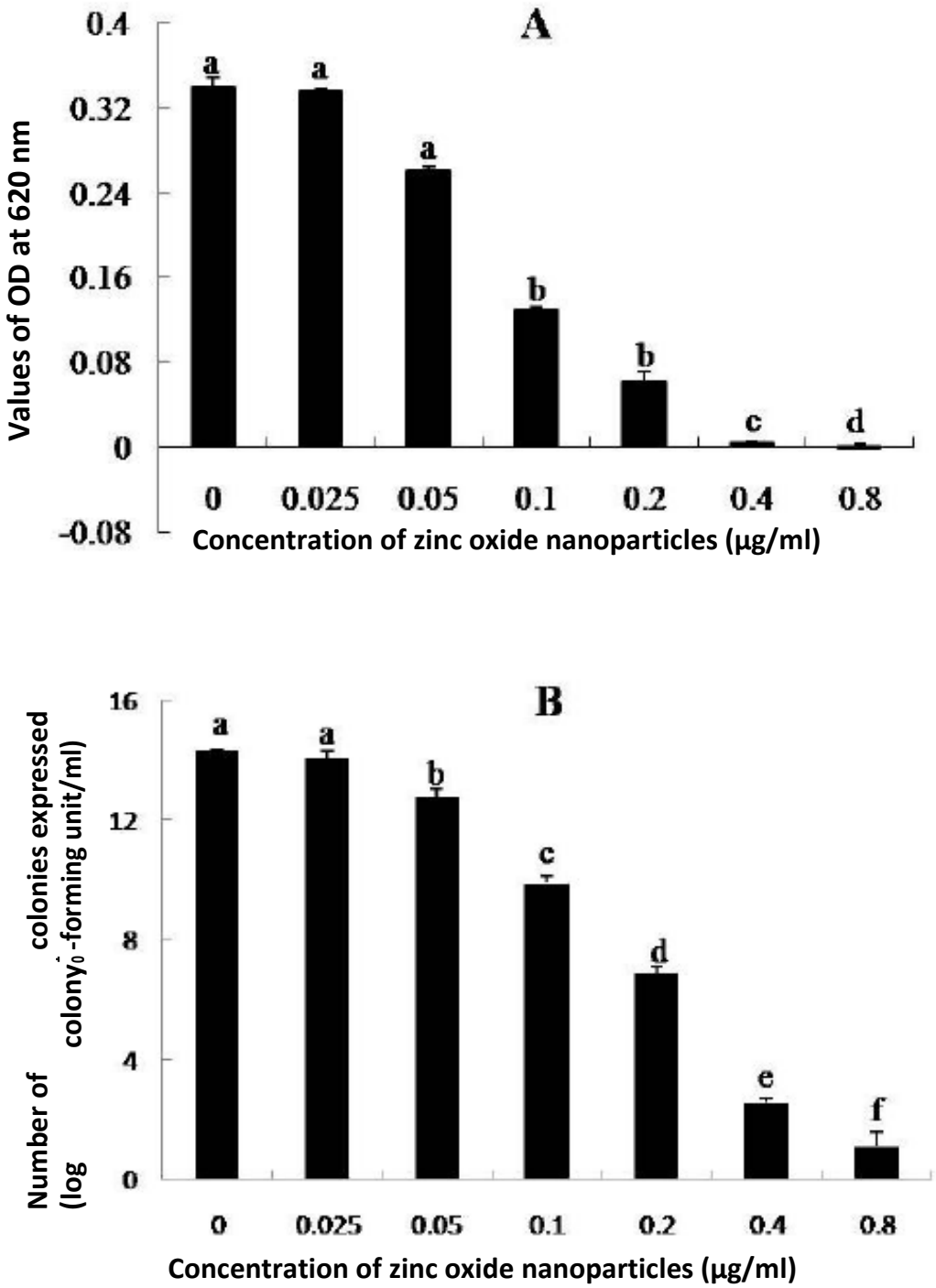

Figure 2. Effects of zinc oxide nanoparticles on values of OD at 620 nm (a) and populations (b) of *E. coli* K88. Data given represents means ± standard error of mean (n=3).

oxide nanoparticles on *E. coli* K88. Therefore, morphological changes were observed by AFM and SEM to investigate the preliminary mechanisms.

The AFM is a scanning probe technique. The probe allows imaging in any environment instead of only vacuum for conventional probes (Binning et al., 1986). AFM has been widely used in researches for yeast cells (Touhami et al., 2003), biopolymers (Morris et al., 2001), proteins (Yan et al., 2003) and bacteria (Kasas et al., 1994; Doctycz et al., 2003; Du et al., 2008; Kailas et al., 2009). It is an ideal tool to determine the morphological changes of cells (Camesano et al., 2000). In our present

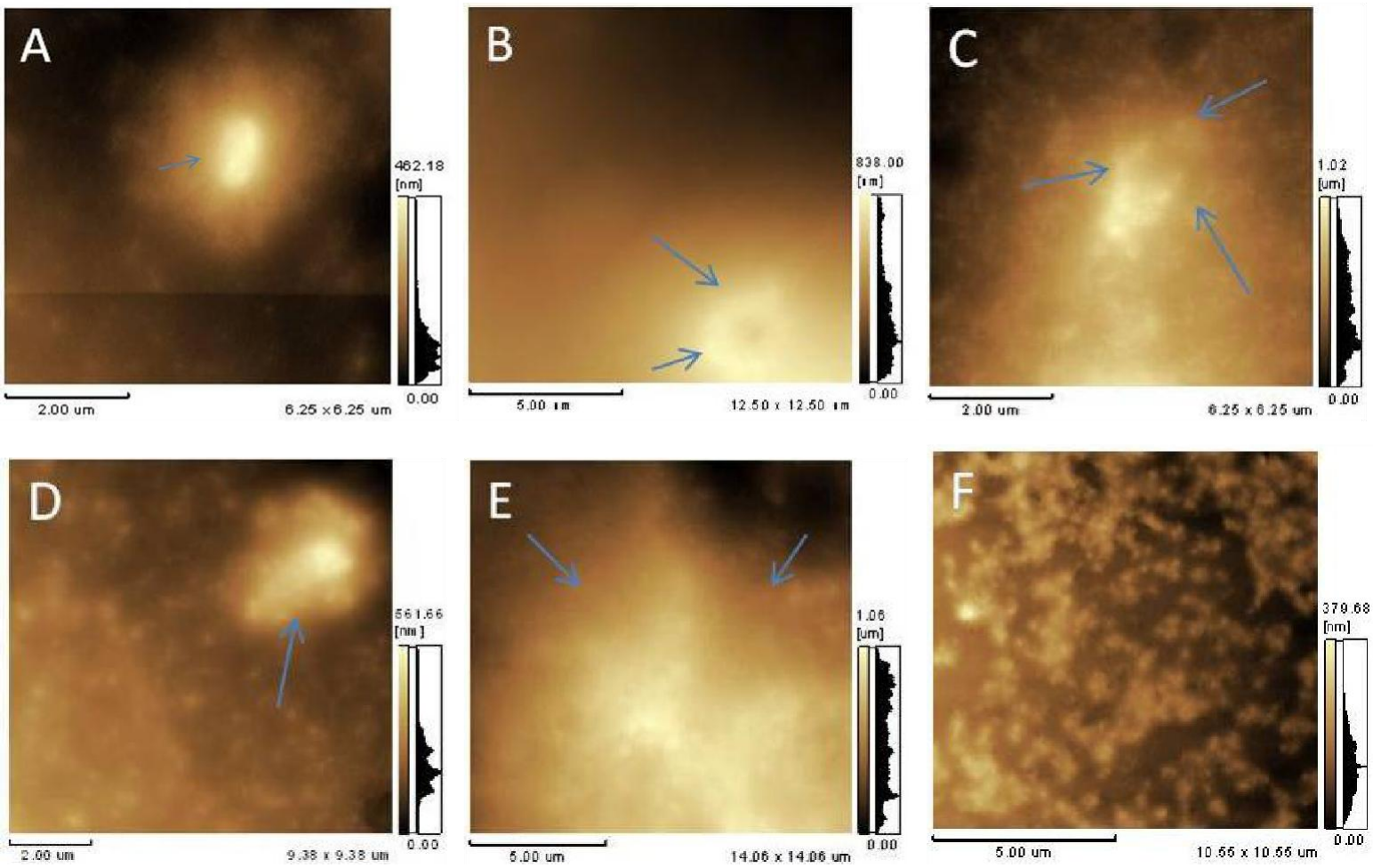

Figure 3. AFM images of *E. coli* K88 treated by zinc oxide nanoparticles for 0.0 (a), 0.5 (b), 1.0 (c), 2.0 (d), 3.0 (e), and 4.0 h (f).

study, AFM in contact mode was used to examine the morphological changes of *E. coli* K88. The images are shown in Figure 3. Figure 3a shows that *E. coli* K88 was a rod shape of about 1 µm wide and 2 µm long, and the membrane was intact. This result is similar to previous report (Du et al., 2008). After 0.5 h of treatment with 0.8 µg/ml zinc oxide nanoparticles, the membrane of *E. coli* K88 was affected, and the boundary became blurry as shown in Figure 3b. When treated for 1.0 h, the membrane of *E. coli* K88 was further affected, some cell contents leaked out of the cell and a lot of debris was found around the cell (Figure 3c). Results of the cells treated for 2.0 h are similar to the results for 1.0 h, as shown in

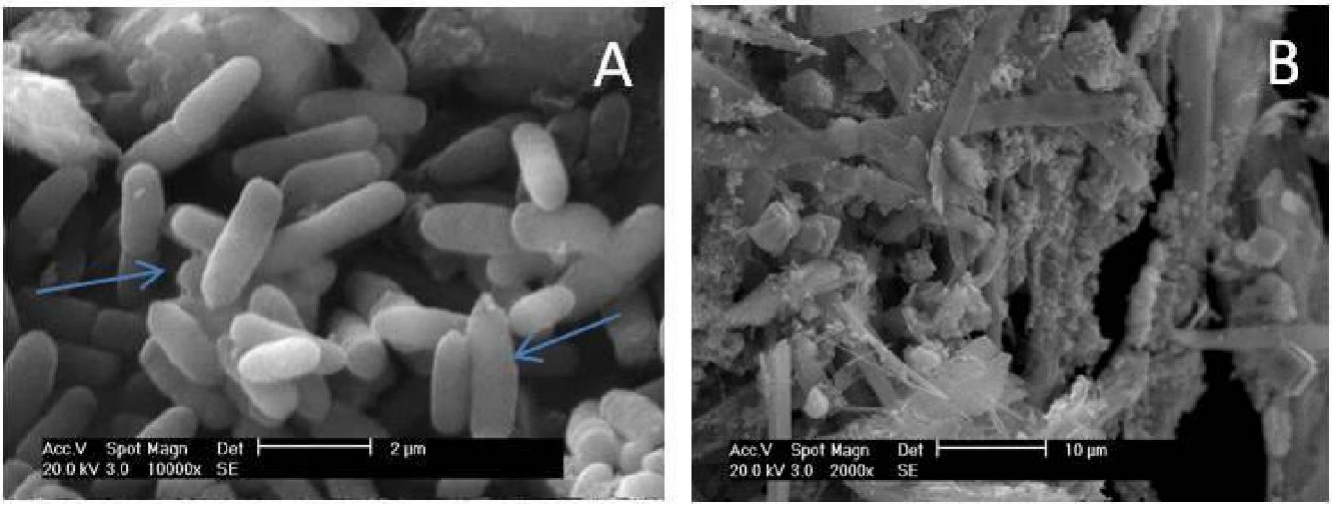

Figure 4. SEM images of *E. coli* K88 without (a) and with (b) treatment of zinc oxide nanoparticles.

Figure 3d. After 3.0 h of treatment, the boundary of the cell was further damaged and almost disappeared, and only high concentrations of debris were left (Figure 3e). When treated for 4.0 h, debris filled in the vision field instead of bacterial cells (Figure 3f).

In agreement with the AFM image (Figure 3a), SEM image in Figure 4a shows that most of the *E. coli* K88 in our present study were 1.0×2.0 µm rod shape and had no flagellum. However, after 0.5 h of treatment with 0.8 µg/ml zinc oxide nanoparticles, no intact cell was found as shown in Figure 4b, which was different from the AFM image (Figure 3b). One of the reasons for no intact cell may be that the complicated treatments for SEM images further damaged the structures of bacterial cells, and led them to debris.

Results of morphological changes show that zinc oxide nanoparticles could damage the membrane of *E. coli* K88, lead to the leakage of cytosolic components and kill the bacterial cells, which is partly consistent with the previous reports of Sawai et al. (1996b, 1998), Yamamoto (2001), Stoimenov et al. (2002) and Sawai (2003). They reported that zinc oxide could damage the membrane of bacterial cell by hydrogen peroxide or the affinity between zinc oxide and bacteria surface. From these studies, we preliminarily supposed that the mechanisms of antibacterial activities of zinc oxide nanoparticles against *E. coli* K88 were closely in line with those against other microbes. The unique characters of nanoparticles largely increased the surface of zinc oxide, or enhanced the affinity, so, zinc oxide nanoparticles could exhibit stronger antibacterial activity than zinc oxide (Yamamoto et al., 1998; Yamamoto, 2001). However, the concentration of nanoparticles should also exceed the MIC to produce enough hydrogen peroxide or the affinity to damage the bacterial membrane. Interestingly, results of SEM images observed by Liu et al. (2009) indicated that 12 mmol/l zinc oxide nanoparticles (the concentration which completely inhibited the bacterial growth) did not result in the morphological changes, which was inconsistent with our present results. Reasons for the difference of the images are still unknown, which require further research. In addition, the mechanisms underlying the damage of bacterial membrane are yet to be studied.

Conclusion

Results in our present study indicate that zinc oxide nanoparticles had strong antibacterial activity against *E. coli* K88 and the activity increased as the concentration of zinc oxide nanopartices increased. The mechanisms may be that zinc oxide nanoparticles could damage the membrane, lead to the leakage of cytosolic components and kill the bacterial cells. In summary, our study reveals that zinc oxide nanoparticles could potentially be an antibacterial reagent to treat diseases caused by bacteria. In future, these nanoparticles might replace conventional antibiotics in humans and animals. However, antibacterial effects, safety, and detailed mechanisms of zinc oxide nanoparticles should be further studied *in vitro* and *in vivo*.

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