

Research Article

Antimicrobial Efficacy and Cell Adhesion Inhibition of In Situ Synthesized ZnO Nanoparticles/Polyvinyl Alcohol Nanofibrous Membranes

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Nanoparticle metal oxides are emerging as a new class of important materials in medical, agricultural, and industrial applications. In this context, free zinc oxide (ZnO) nanoparticles (NPs) have been increasingly shown with broad antimicrobial activities. However, biological properties of immobilized ZnO NPs on matrixes like nanofibrous membranes are still limited. In this study, in situ synthesized ZnO NPs/polyvinyl alcohol (PVA) nanofibrous membranes were fabricated by electrospinning with different zinc acetate concentrations. Characterization results indicated that, with 5 mM zinc acetate, uniform size ZnO NPs (~40 nm) were formed and evenly distributed on the membrane surface. The surfaces became more hydrophobic with higher concentration of zinc acetate. ZnO NPs/PVA nanofibrous membranes showed a broad spectrum of antimicrobial activities and cell adhesion inhibiting effects against four microorganisms including Gram-positive *Staphylococcus aureus*, Gram-negative *Escherichia coli*, fungi *Candida albicans*, and spores of *Aspergillus niger*. Our data revealed that the major antimicrobial mechanism could be attributed to cell membrane damage and cellular internalization of ZnO NPs, while the hydrophobic surface of the membrane primarily contributed to the cell adhesion inhibition. This study suggests that ZnO NPs/PVA nanofibrous membranes could potentially be used as an effective antimicrobial agent to maintain agricultural and food safety.

1. Introduction

Metal oxide nanoparticles have attracted significant attention in recent years due to their distinct physicochemical and biological properties. There are several nanostructured metal oxides that have been reported to be effective antimicrobial agents including graphene oxide (GO), TiO₂, MoO₃, CeO₂, and ZnO [1–5]. Among them, zinc oxide (ZnO) is generally recognized as safe by the U.S. Food and Drug Administration (21CFR182.8991) [6], which has been widely used in daily life such as drug delivery, cosmetics, medical devices, and food industry [7–9]. The development of nanotechnology has made ZnO nanoparticles (ZnO NPs) more and more attractive due to their significantly novel and improved physical, chemical, and biological properties. ZnO NPs have also been used for various applications such as semiconductors,

biosensors, textiles, paintings, and industrial coatings [10–13]. Particularly, ZnO NPs exhibit notable antimicrobial properties and have been developed as a potential antibacterial agent in food industry [14]. Several studies have shown that ZnO NPs have a broad spectrum of antimicrobial activities against both Gram-positive and Gram-negative bacteria, including main foodborne pathogens like *Escherichia coli* O157:H7, *Salmonella typhimurium*, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Campylobacter jejuni* [15–18].

In addition, premade ZnO NPs have been immobilized with different polymers (e.g., polyvinyl alcohol (PVA) and polycaprolactone (PCL)) by electrospinning to fabricate hybrid nanofibrous membranes [19, 20]. Interestingly, these nanocomposite materials showed synergistic/enhanced antimicrobial efficiency. However, the ZnO NPs/polymer hybrid nanofibers were directly electrospun from a ZnO

NPs-containing polymer (e.g., PVA) aqueous solution. Therefore, the premade ZnO NPs are mostly encapsulated in the fiber interior, likely limiting the interactions between ZnO NPs and microbial cells. To overcome this drawback, an in situ preparation of ZnO NPs/PVA nanofibrous membranes has been developed through electrospinning a ZnO precursor (zinc acetate) containing PVA solution [21]. The in situ synthesized ZnO NPs mostly resided on the PVA nanofiber surface, which were further observed with enhanced optical quality [22]. However, the biological properties like antimicrobial and anti-cell adhesion activities of the in situ fabricated ZnO NPs/PVA nanofibrous membranes, to the best of our knowledge, have not been reported to date.

In this work, in situ synthesized ZnO NPs/PVA nanofibrous membranes were produced by electrospinning with different zinc acetate concentrations. Properties of the nanocomposite materials were first characterized, and then their antimicrobial activities and cell adhesion inhibiting effects were investigated based on four different kinds of microorganisms, that is, Gram-positive *Staphylococcus aureus*, Gram-negative *Escherichia coli*, fungi *Candida albicans*, and spores of *Aspergillus niger*.

2. Materials and Methods

2.1. Materials. Zinc acetate dihydrate and polyvinyl alcohol (PVA, MW = 80,000) with a polymerization degree of 1750 ± 50 were purchased from Sinopharm Group Chemical Reagent Co. Ltd. (Shanghai, China). The strains used in this study were Gram-positive bacteria *Staphylococcus aureus* ATCC 6538, Gram-negative bacteria *Escherichia coli* ATCC 8739, fungi *Candida albicans* ATCC 10231, and *Aspergillus niger* L-3.

2.2. In Situ Preparation of ZnO NPs/PVA Nanofibrous Membranes by Electrospinning. PVA (8%, w/v) was first completely dissolved in Millipore water at 80°C. Then, different amounts of zinc acetate (0.1, 1, 5, and 10 mM) were added to the 8% PVA solution, followed by gentle stirring for 6 hours. Afterwards, the resulting unique and viscous gel was kept overnight at room temperature. To fabricate ZnO-PVA nanofibrous membranes, the zinc acetate/PVA mixture was transferred into a 20 mL syringe equipped with a metal needle (0.6 mm ID) that was connected to a high-voltage power supply (GDW-A, Beijing Institute of High Voltage Electrical and Mechanical Technology, Beijing, China). The electrospinning flow rate and voltage were set at 0.8 mL/L and 15 kV, respectively, and the distance between the needle tip and the collector was 10 cm. The collected membranes were heated at 125°C for 10 h to form ZnO nanoparticles on the PVA surface [21, 22]. The obtained ZnO NPs/PVA nanofibrous membranes with 0.1, 1, 5, and 10 mM zinc acetate were named ZnO-1, ZnO-2, ZnO-3, and ZnO-4, respectively.

2.3. Characterization of ZnO NPs/PVA Nanofibrous Membranes. The morphology and diameter of the ZnO NPs/PVA nanofibrous membranes were analyzed by scanning electron microscopy (SEM) (XL-30E; Philips, Tokyo, Japan). The morphology and nominal size of in situ synthesized ZnO NPs

in nanofibrous membranes were observed with transmission electron microscopy (TEM) (JEM-2010; JEOL, Tokyo, Japan). The crystal structure of ZnO NPs was determined by X-ray diffraction (XRD) (Focus-D8; Bruker, Germany) in a 2θ range from 10° to 70° at the speed of 2°min^{-1} . The water contact-angle (WCA) measurement was carried out to characterize the hydrophilicity of ZnO NPs/PVA nanofibrous membranes. The images of the droplet (30 μL) on the membrane were visualized through the image analyzer (OCA20; Dataphysics, Germany) and the angles between the water droplet and the surface were measured.

2.4. Inhibition of Bacterial Cell Growth. Bacteria *S. aureus* and *E. coli* were used to investigate the antibacterial activities of ZnO-1, ZnO-2, ZnO-3, and ZnO-4. Overnight cultures of *S. aureus* and *E. coli* were inoculated into 50 mL fresh Luria-Bertani (LB) medium (pH 7.0) with an initial optical density (OD_{600}) of 0.04–0.06. Meanwhile, the same amount (1 cm^2 fiber) of sterilized ZnO-1, ZnO-2, ZnO-3, and ZnO-4 was added to the culture, respectively. Afterwards, the cultivation was carried out at 37°C and 200 rpm for 12 h in a shaker. Cell growth was monitored by OD_{600} measurement every 2 h.

2.5. Antimicrobial Activity. Broad-spectrum antimicrobial activities of ZnO NPs/PVA nanofibrous membranes were further tested against *S. aureus*, *E. coli*, *C. albicans*, and *A. niger* (spores). *S. aureus* and *E. coli* were first incubated at 37°C for 12 h in LB medium, while *C. albicans* was cultivated at 30°C for 24 h in Sabouraud medium (pH 6.0). Afterwards, each culture was centrifuged at 3000 rpm and the pellet was washed twice with sterilized 0.9% NaCl solution. Then, the cells were resuspended with 0.9% NaCl to reach a final concentration of about 10^7 CFU/mL. *A. niger* was initially cultivated on the potato-dextrose-agar plate for 7 days at 30°C. Then, spores of *A. niger* were directly washed with 0.9% NaCl solution and adjusted to around 10^7 CFU/mL.

To each cell suspension, the same amount (1 cm^2 fiber) of sterilized ZnO-3 was added, followed by 12 h cultivation at 200 rpm at appropriate temperatures. The PVA membrane was used instead of ZnO-3 as a negative control. Afterwards, cell suspension was taken out and plated on the agar plates to allow colonies formation and antimicrobial efficiency calculation.

2.6. Cell Adhesion Inhibition. To test cell adhesion inhibiting effects of ZnO NPs/PVA nanofibrous membranes, each cell suspension was prepared as above in Antimicrobial Activity. The same amount of ZnO-3 was added to 5 mL cell suspension, followed by 3 h cultivation at 200 rpm and 37°C (*S. aureus* and *E. coli*) or 30°C (*C. albicans* and *A. niger*). Afterwards, the ZnO-3 membranes were taken out and rinsed twice with sterilized 0.9% NaCl. Then, the washed membranes were soaked in 5 mL fresh 0.9% NaCl solution, followed by 30 s vigorous shaking to detach the adherent cells from the membranes to the solution. The resulting cell suspension was taken out and plated on the agar plates to allow colonies formation and evaluation of cell adhesion inhibition. The PVA membrane was used instead of ZnO-3 as a negative control.

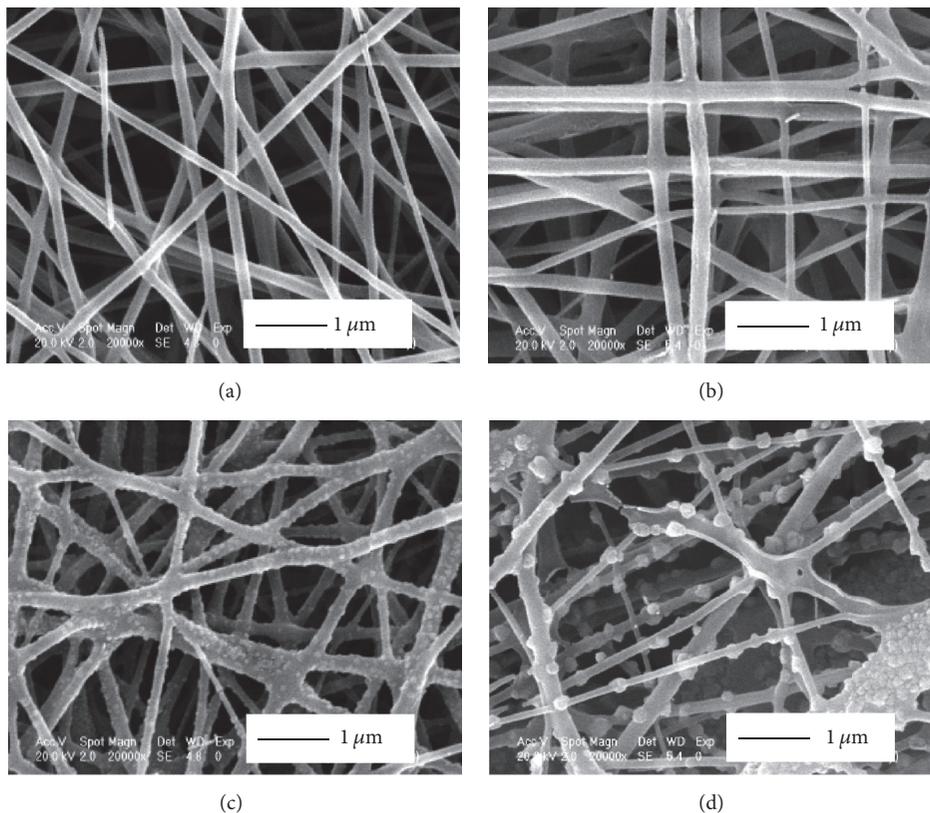


FIGURE 1: SEM images showing ZnO-PVA nanofibrous membranes with 0.1 mM (a), 1 mM (b), 5 mM (c), and 10 mM (d) zinc acetate.

3. Results and Discussion

3.1. Characterization of ZnO NPs/PVA Nanofibrous Membranes. ZnO NPs/PVA nanofibrous membranes were fabricated by electrospinning with different zinc acetate concentrations and their surface morphologies were analyzed by SEM (Figure 1). At low concentrations of zinc acetate (0.1 and 1 mM), no obvious ZnO NPs could be observed on the membrane surface. With 5 mM zinc acetate, uniform size ZnO NPs were formed and evenly distributed on the surface (Figure 1(c)). The highest concentration of zinc acetate (10 mM) led to larger sphere-like ZnO NPs without uniform distribution on the membrane (Figure 1(d)). In addition, the highest zinc acetate caused the formation of nonuniform nanofibers with different diameters. This is likely due to the fact that more salt (zinc acetate) reduced the viscosity of the PVA solution, which is not sufficient enough to generate uniform fibrous structures during the electrospinning process [23].

A representative TEM image of the sample with 5 mM zinc acetate (ZnO-3) is shown in Figure 2(a). The results indicated that the in situ synthesized ZnO NPs were crystalline on the fibrous PVA surface with an average diameter of around 40 nm. The crystal structure of ZnO-3 was further analyzed by XRD in a 2θ range from 10° to 70° (Figure 2(b)). The observed broad peak at 20° was attributed to the plane of crystalline PVA, and peaks at 2θ values of 31.9° , 34.5° , 36.4° , 47.6° , 56.7° , and 62.9° corresponded to the crystal planes

of (100), (002), (101), (102), (110), and (103), respectively. These diffraction peak positions can be assigned to a pure hexagonal ZnO wurtzite structure [24]. Our results suggested that nanocrystalline ZnO NPs were well synthesized in the composite nanofibrous membranes, which is in agreement with previous reports [21, 22].

The WCA of the surface was measured to analyze the change in the hydrophilicity of the membrane surface. As shown in Figure 3, a significant increase in the WCA was observed from 36° to 107° , demonstrating that the membrane surfaces become more hydrophobic with higher zinc acetate. This is attributed to the increasing roughness of the ZnO NPs/PVA membrane surface, where the water droplet forms a more stable water-air-solid contact line than on the smooth surface [25].

3.2. Effect of ZnO NPs/PVA Nanofibrous Membranes on Inhibition of Bacterial Cell Growth. To test the inhibiting effect of ZnO NPs/PVA nanofibrous membranes on bacterial cell growth, we chose Gram-positive *S. aureus* and Gram-negative *E. coli* as target bacteria. Both cells were cultivated with ZnO NPs/PVA membranes for 12 h and cell growth curves were shown in Figure 4. In the *S. aureus* group (Figure 4(a)), lower ZnO NPs (ZnO-1 and ZnO-2) on the membranes had no obvious inhibition on cell growth, while cell growth was significantly inhibited by ZnO-3 after only 2 h. With ZnO-4 in the culture, cells almost stopped growing for 8 h probably due to the high toxicity of ZnO-4 on cells.

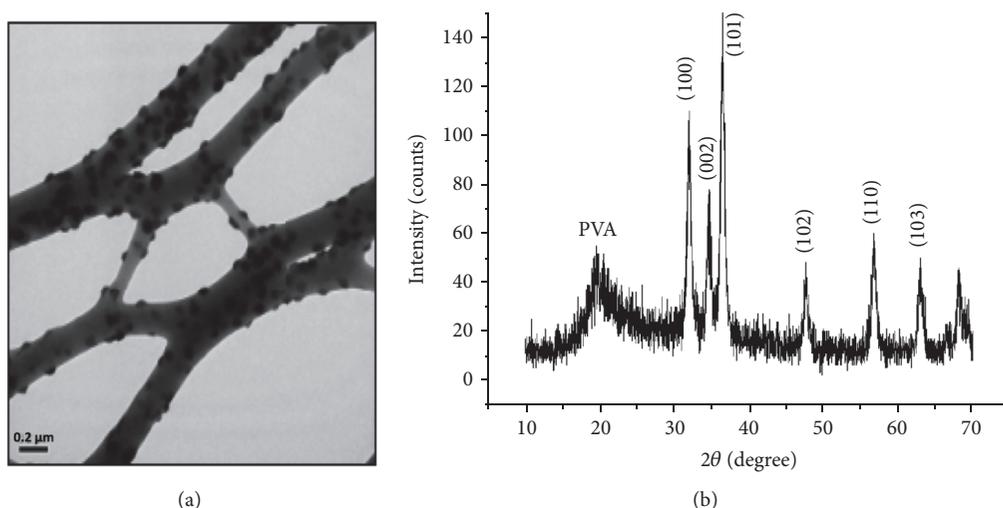


FIGURE 2: TEM image (a) and XRD spectra (b) of ZnO-3.

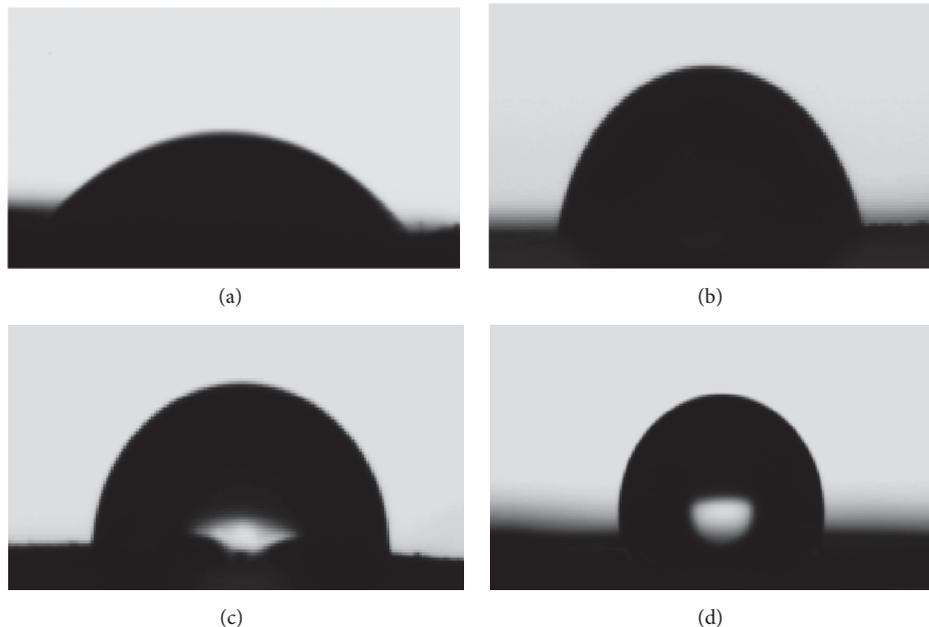


FIGURE 3: WCA measurements of PVA and ZnO-PVA nanofibrous membranes. (a) PVA (WCA = 36°), (b) ZnO-2 (WCA = 78°), (c) ZnO-3 (WCA = 86°), and (d) ZnO-4 (WCA = 107°).

A similar inhibiting effect on cell growth was also found in the *E. coli* group (Figure 4(b)), although ZnO-2 showed a little more inhibition on *E. coli* than on *S. aureus*. In order to investigate the broad-spectrum antimicrobial activities of ZnO NPs/PVA nanofibrous membranes, ZnO-3 is therefore used in our following experiments since it displays notable inhibiting effect on cell growth and possesses potentially low risks of toxicity for future applications.

3.3. Broad-Spectrum Antimicrobial Activities of ZnO NPs/PVA Nanofibrous Membranes. The antimicrobial efficacy of ZnO-3 was further tested against four representative microorganisms, that is, bacteria *S. aureus* and *E. coli* as well as fungi

C. albicans and *A. niger*. Figure 5 shows that ZnO-3 had the highest antibacterial effect on *S. aureus* (nearly 100% inhibition rate), followed by *C. albicans* (~90%) and *E. coli* (~70%). The antimicrobial efficiency on *A. niger* was less than 50%, which is likely due to the fact that the spores have distinctive resistance to harsh conditions. Obviously, our results indicated that ZnO NPs/PVA nanofibrous membranes (herein ZnO-3 as an example) have a wide range of antimicrobial activities, and the effect is more significant on the Gram-positive microorganisms (*S. aureus* and *C. albicans*) than on the Gram-negative *E. coli* and the fungus *A. niger*. Our data also support previous reports that ZnO NPs showed a broad-spectrum antimicrobial activity and stronger inhibiting effect

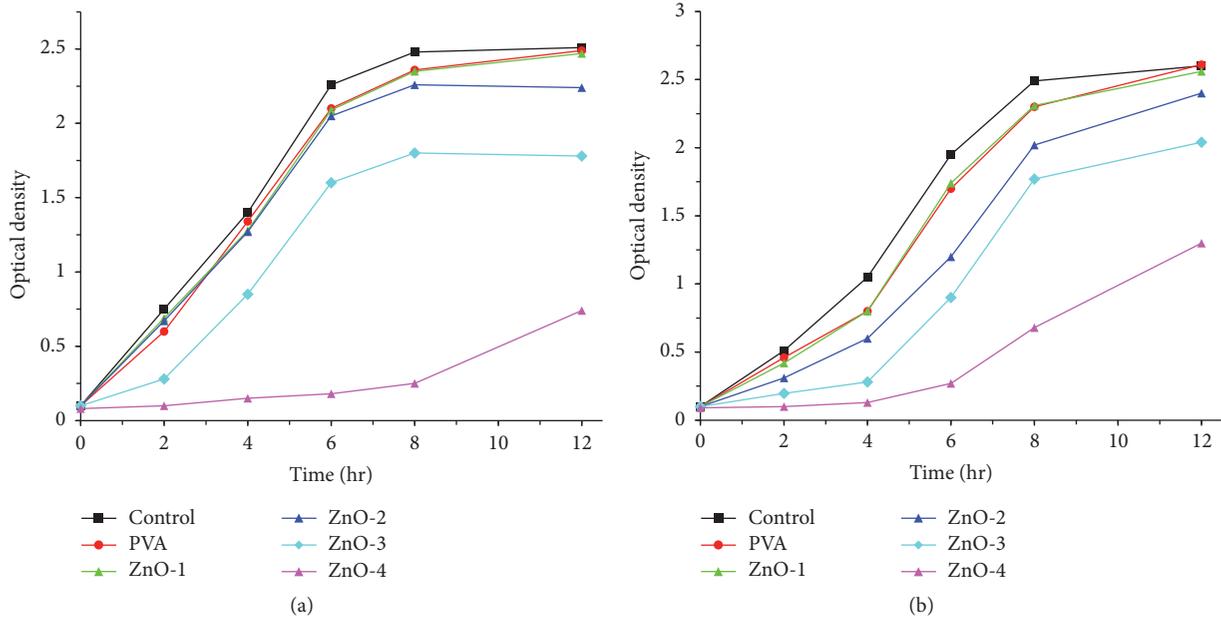


FIGURE 4: Effect of ZnO-PVA nanofibrous membranes on inhibition of bacterial cell growth. (a) *S. aureus* and (b) *E. coli*. Cell growth was monitored by measuring optical density at 600 nm.

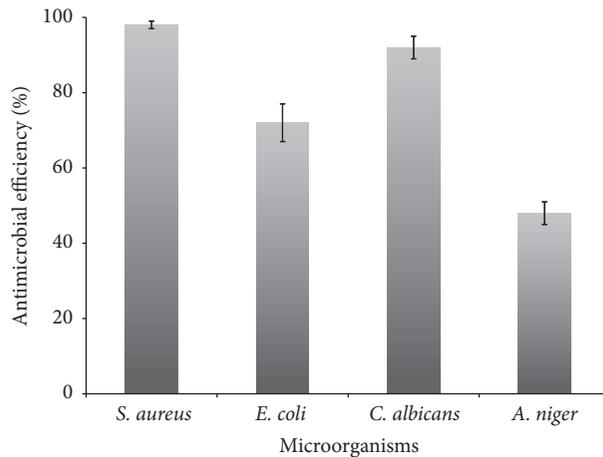


FIGURE 5: Antimicrobial activity of ZnO-3 against *S. aureus*, *E. coli*, *C. albicans*, and spores of *A. niger*.

on Gram-positive microorganisms than on Gram-negative ones [16, 26].

Normally, three main mechanisms of action have been proposed to interpret the antimicrobial activity of ZnO NPs. First, free Zn²⁺ ions released from ZnO NPs may interact with nucleic acids and deactivate enzymes of the respiratory system, leading to cell damage and finally death [27, 28]. Second, cellular internalization of ZnO NPs could result in physical damage and membrane disorganization [29]. Third, reactive oxygen species (ROS) generated by ZnO NPs in the aqueous solution could also contribute to the antimicrobial activity [17, 19, 30, 31].

In this work, to demonstrate the possible antimicrobial mechanism of ZnO NPs/PVA nanofibrous membranes, the Gram-positive *S. aureus* and the Gram-negative *E. coli* were

treated with ZnO-3 for 12 h, followed by TEM analysis (Figure 6). It is clear that some intracellular contents leaked out from *S. aureus* cells (Figure 6(a)), which is probably due to the cell membrane damage caused by the cellular internalization of ZnO NPs (Figure 6(b)). By contrast, in the treated *E. coli* cells neither ZnO NPs inside of the cells nor the leakage of intracellular contents could be observed (Figures 6(c) and 6(d)). Clearly, the outer membrane of *E. coli* was decomposed by ZnO NPs. However, the *E. coli* spheroplast still could survive. This result also confirms our findings that ZnO NPs have stronger antimicrobial activity on Gram-positive bacteria than on Gram-negative ones. The observed difference could be due to many reasons while the main difference between Gram-positive and Gram-negative bacteria is the nature of their cell wall structure. Additionally,

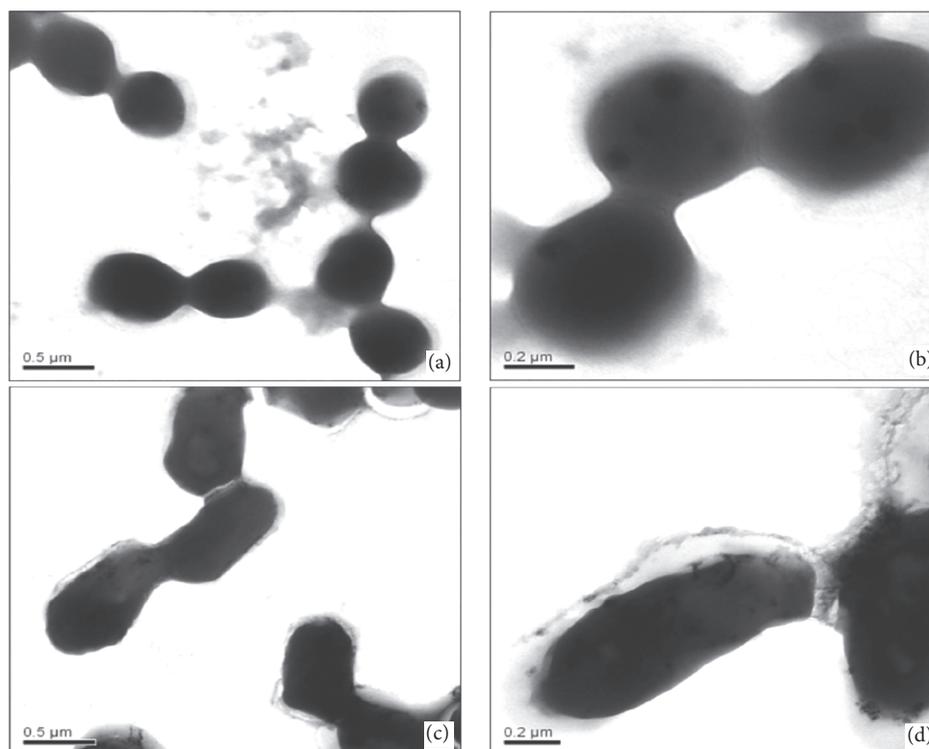


FIGURE 6: TEM images of *S. aureus* (a, b) and *E. coli* (c, d) after 12 h treatment with ZnO-3. (a) and (c) were in view of low magnification, while (b) and (d) were in view of high magnification.

the Gram-negative bacteria possess an additional outer membrane comprised of lipopolysaccharide that protects the peptidoglycan layer from chemical attacks [1]. While we ascribe the cell membrane damage to the ZnO NPs, it is possible, though further investigation is needed, that the damage comes from the effect of dissolved Zn species as an example. The mechanism underlying the antimicrobial activity of ZnO NPs is still not well understood and much remains unknown about the function, specificity, and toxicity of ZnO NPs which need to be investigated in the future study.

3.4. Evaluation of Cell Adhesion to ZnO NPs/PVA Nanofibrous Membranes. Microbial cell first adhesion to surfaces and subsequent biofilm development is a survival strategy employed by virtually all bacteria [32]. Microbial biofilms allow bacteria to better resist environmental extremes like high antibiotic concentrations. Therefore, it is an important step to inhibit cell adhesion to surfaces before biofilm formation. To evaluate the effect of ZnO NPs/PVA nanofibrous membranes on cell adhesion, four microorganisms were tested, that is, *S. aureus*, *E. coli*, *C. albicans*, and *A. niger*. As shown in Figure 7, cell adhesion inhibiting effects of ZnO-3 on bacteria (*S. aureus* and *E. coli*) were much better than on fungi (*C. albicans* and *A. niger*). This is likely due to the fact that *S. aureus* [33] and *E. coli* [34] are hydrophilic bacteria and their adhesion is better to hydrophilic matrix rather than to the hydrophobic ZnO-3 surface (Figure 3(c)).

SEM analysis revealed that ZnO NPs/PVA nanofibrous membranes were covered by film-like substance in the groups

of *S. aureus* (Figure 8(a)) and *E. coli* (Figure 8(b)). In addition, only low amounts of cells and no cell clusters (i.e., cell-cell adhesion) were observed on the membrane surface. Therefore, we suspect that they are intracellular contents originated from lysed cells but not the biofilm. In the *C. albicans* group, cells were still clustered together with no continuous film-like substance (Figure 8(c)), suggesting that these cells are not significantly damaged and are still alive. This result is consistent with our previous finding that the cell adhesion inhibiting effect on *C. albicans* is low (Figure 7(c)). It has been reported that capillary forces play a dominant role in the *A. niger* spore adhesion process [35]. The hydrophobic surface of spores could especially promote adhesion [36], which suggests that *A. niger* spore could well adhere to the hydrophobic ZnO-3 membrane surface and therefore lower cell adhesion inhibiting effect by ZnO-3 (Figures 7(d) and 8(d)).

In conclusion, ZnO NPs/PVA composite nanofibrous membranes were in situ synthesized and their physical/chemical properties were characterized. This material showed significant antimicrobial activities and cell adhesion inhibiting effects on a broad spectrum of microorganisms including Gram-positive *S. aureus*, Gram-negative *E. coli*, fungi *C. albicans*, and spores of *A. niger*. Our data revealed that the main antimicrobial mechanism could be attributed to cell membrane damage and cellular internalization of ZnO NPs, while the hydrophobic surface of ZnO NPs/PVA nanofibrous membranes primarily contributed to the cell adhesion inhibition. These results suggest that ZnO NPs/PVA nanofibrous

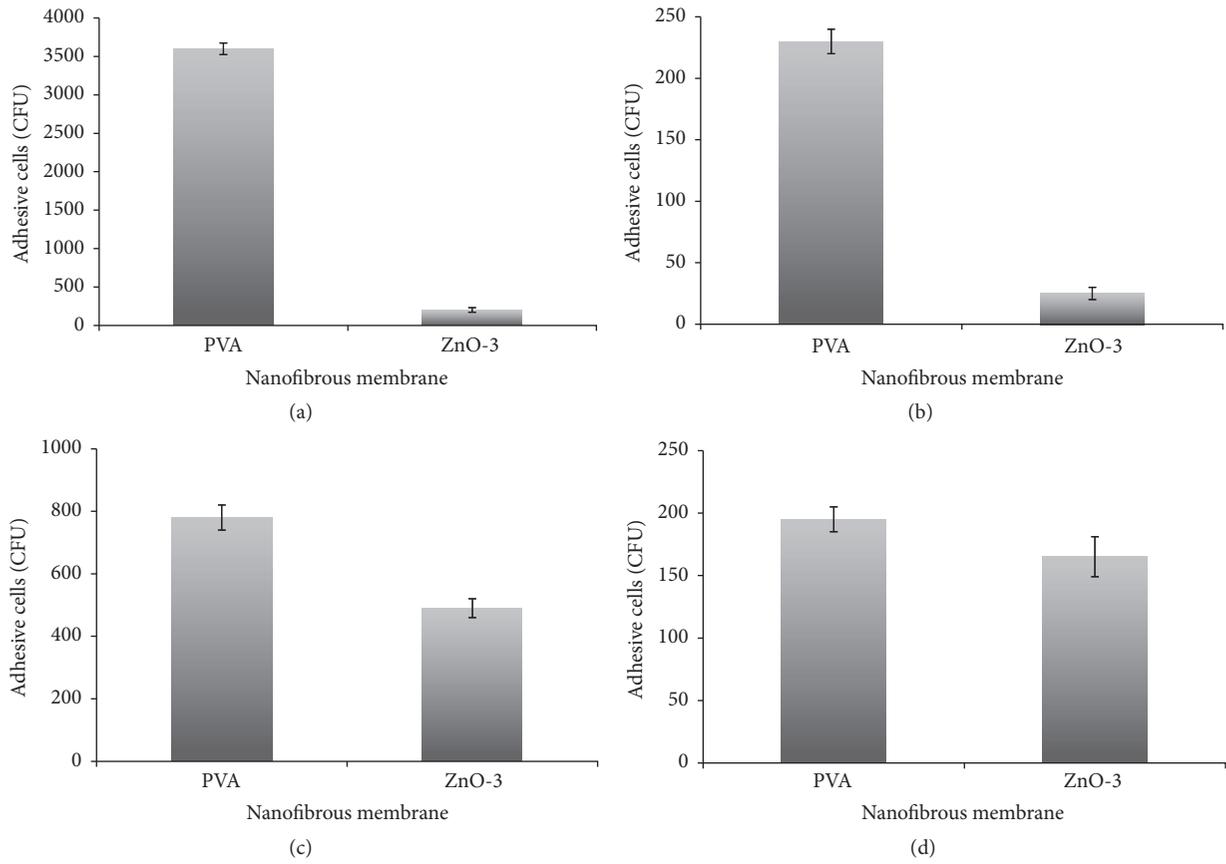


FIGURE 7: Cell adhesion tests of *S. aureus* (a), *E. coli* (b), *C. albicans* (c), and *A. niger* (d).

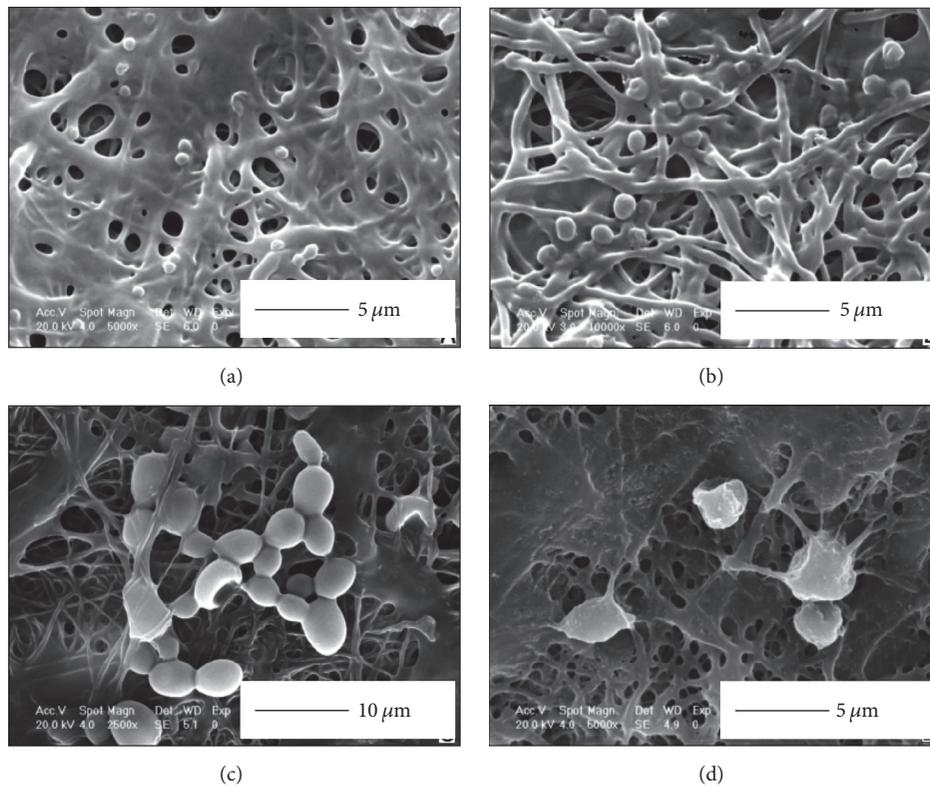


FIGURE 8: SEM images on morphology of cell adhesion to ZnO-3 for 12 hours. (a) *S. aureus*, (b) *E. coli*, (c) *C. albicans*, and (d) *A. niger*.

membranes could potentially be used as an effective antimicrobial agent to maintain agricultural and food safety.

Competing Interests

The authors declare that they have no competing interests.

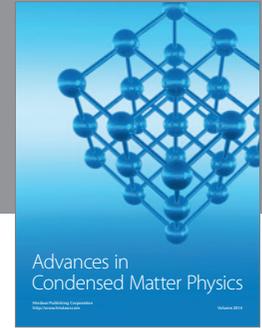
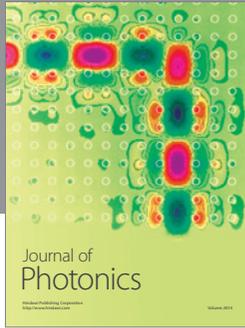
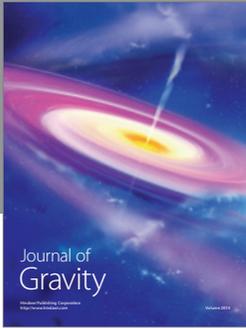
Acknowledgments

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