Scientia Iranica F (2021) 28(6), 3806–3815

Sharif University of Technology
Scientia Iranica
Transactions F: Nanotechnology
http://scientiairanica.sharif.edu

Antibacterial properties of zinc oxide nanoparticles on *Pseudomonas aeruginosa* (ATCC 27853)

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Received 2 October 2020; received in revised form 18 February 2021; accepted 8 November 2021

KEYWORDS
Zinc oxide nanoparticles; *Pseudomonas aeruginosa*; Bacterial growth inhibition; Antibacterial property; Scanning electron microscopy; Morphological damage.

Abstract. The application of nanotechnology has brought about advancements in environmental and medical applications. Recently, zinc oxide nanoparticle (ZnO NP) is commonly used to treat a wide range of bacterial and fungal skin infections due to its antimicrobial property. This investigation was intended to study the antimicrobial effect of ZnO NP on *Pseudomonas aeruginosa* by testing the bacterial inhibition and the morphological damages caused by ZnO NP to *P. aeruginosa*. The results of this study exhibited a typical dose-dependent and significant ($p < 0.05$) inhibition of the growth of *P. aeruginosa* treated with 5 to 150 μg/mL of ZnO NP. The polysaccharides and polypeptides from *P. aeruginosa* cell wall were found to be associated with the attachment of ZnO NPs to bacterial cells as illustrated in the Fourier Transform Infrared (FTIR) spectrum. Furthermore, Scanning Electron Microscopy (SEM) images displayed the surface attachment of ZnO NPs on bacteria and the morphological changes, such as disrupted cell wall integrity, cell bending, and cell distortion, as a result of ZnO NPs interaction on the cell wall of *P. aeruginosa*.

1. Introduction

Nanotechnology uses nanoscale materials ranging from 1 to 100 nm. The characteristics of nanoparticles (NPs) are entirely different from the micro-sized bulk materials [1] as the NPs have special features such as high stability against heat and pressure and the ability to change the surface characteristics [2,3]. These exclusive properties have made NPs widely fit for industrial applications [4]. Inorganic NPs have been reported to show an obvious antibacterial activity, even at a low concentration due to the higher surface area to volume ratio [5–7]. Nanoparticles play a role in protecting the environment as NPs can degrade the harmful dyes and antibiotics in wastewater [8,9].

Increased use of antibiotics to treat bacterial infections causes the emergence of antibiotic-resistant bacteria [10]. The antibiotic resistance often results from evolutionary processes [10] that leads to the expression of the enzymes responsible for dissociating the antibiotics and transforming the cellular structures [11]. Advances in nanotechnology have paved

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doi: 10.24200/sci.2021.56815.4974

the way to overcome the problems caused by antibiotic resistance in treating antibiotic-resistant bacterial infections using NPs [12–14].

Among the metallic NPs, zinc oxide nanoparticle (ZnO NP) is mainly utilized as a therapeutic agent due to its lower toxicity and mineral content essential to humans [15–17]. ZnO NP is commonly used in numerous biomedical applications such as anticancer [18], antibacterial, antifungal, anti-inflammatory [19,20], drug delivery [21], and anti-diabetic agents [22]. ZnO NPs are utilized as carriers for a number of chemotherapeutic drugs like cisplatin, doxorubicin, and 5-flurouracil [23]. ZnO NPs also possess a photocatalytic capability to degrade harmful toxic dyes such as rhodamine B [24], methylene blue [25], and malachite green [26] for environmental protection. In addition, ZnO NP has wide applicability in pharmaceutical and food industries because of its antibacterial property [27,28]. The ability of ZnO NPs to hinder the growth of pathogenic microorganisms has made ZnO NPs useful in the food industry as an anti-bacterial agent [29]. Numerous investigations have assumed the antimicrobial effect of ZnO NPs on both gram-positive and gram-negative bacteria, namely *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Salmonella typhimurium*, and *Klebsiella pneumonia* [30–32]. Recently, ZnO NP has been applied in wound dressing creams to promote faster wound healing due to the strong antibacterial activity and the epithelialization stimulating effect of zinc [33,34]. The safety and biocompatibility of ZnO with human skin enables ZnO to be an additive for textiles and surface creams that contact with human skin directly [4]. The US Food and Drug Administration has endorsed ZnO as a safe compound for human applications [3,17,35].

Hence, this research was designed to investigate the effects of antimicrobial properties of ZnO NPs on gram-negative bacterium *P. aeruginosa* which can cause skin and wound infections [36–38]. The bacterial growth inhibition, surface interaction, and the subsequent morphological damages resulting from the interaction of ZnO NPs on *P. aeruginosa* were investigated to assess the effect of the antibacterial properties of ZnO NPs.

The present study used a wide range of working concentrations of ZnO NPs to explore the inhibitory effect of dose proportionality on *P. aeruginosa*. In addition, the scanning electron microscopy was employed to investigate the surface interaction and surface damages caused by ZnO NPs, and also FTIR spectrum was studied to identify the biomolecules involved in the surface attachment of ZnO NPs on *P. aeruginosa* cell wall. The outcome of the study might be helpful to utilize ZnO NPs as an antibacterial agent for topical applications and wound bandages to cure the skin and wound infections caused by *P. aeruginosa*.

2. Materials and methodology

2.1. Preparation of ZnO NPs suspension

The powder of ZnO NPs was purchased from Sigma-Aldrich, United States of America. A stock solution of 300 μg/mL ZnO NPs was prepared using Luria-Bertani broth (LB broth) and ultra-sonicated to homogenize (Elma, Elmasonic E 100 H, Germany) the NPs in LB broth at 37 kHz for 30 min. The working concentrations of ZnO NPs were prepared by diluting the homogenized stock solution with LB broth.

2.2. Characterization of ZnO NPs

The surface morphology and elemental composition of ZnO NPs were obtained by SEM-EDX (JSM-6701F, JOEL, Japan) which operated at an acceleration voltage of 4 kV. The elemental components were further confirmed with FTIR spectrum (Perkin-Elmer, Spectrum RX1, United States of America).

2.3. Bacterial growth curve

The bacterium *P. aeruginosa* (ATCC 27853) was obtained from the Faculty of Science, Universiti Tunku Abdul Rahman and grown in Luria-Bertani broth (LB broth) at 37°C. The growth curve of the bacterium was considered to identify the mid-exponential phase of *P. aeruginosa* by determining the growth rate of *P. aeruginosa* and obtaining the optical density of the bacterial suspension at OD_{600} at 0, 2, 4, 6, 8, 24, and 48 h following subculture.

2.4. Exposure of P. aeruginosa to ZnO NPs

5 mL bacteria suspension from the mid-exponential phase at 4 h of subculture with an initial concentration of 0.05 at OD_{600} was treated with 5 mL of ZnO NPs suspension to obtain a final concentration of 5, 10, 25, 50, 100, and 150 μg/mL of ZnO NPs and was incubated at 37°C for 24 h.

Negative Control (NC) involved the bacterial culture without ZnO NPs, while Positive Control (PC) included the bacterial culture treated with antibiotic ciprofloxacin (1000 μg/mL) for 24 h at 37°C.

2.5. Investigation of bacterial growth inhibition

2.5.1. Turbidity method

The bacteriostatic effect of ZnO NPs was evaluated by turbidity method. The bacterial suspensions were treated with different concentrations of ZnO NPs and incubated for 24 h at 37°C. The optical density of ZnO NPs treated bacterial suspensions and both positive and negative controls were obtained by a UV-Vis spectrophotometer (Biochrom, Libra S4, UK) at OD_{600}. The background reading from NPs was eliminated by deducting the optical density of working concentrations of ZnO NPs from the test readings. The bacterial growth inhibition after 24 h treatment with ZnO NPs was calculated based on Eq. (1).

Percentage of growth inhibition

$$= \frac{OD_{NC} - OD_T}{OD_{NC}} \times 100\%, \quad (1)$$

where NC is Negative Control and T is Test.

2.5.2. Colony count method

The bactericidal effect of ZnO NPs was investigated using colony count method. The bacterial suspensions from NPs treatment, positive control and negative control were plated on Luria-Bertani agar (LB agar) by streak plate technique after 24 h of incubation at 37°C and was further incubated for the next 24 h at 37°C. The number of colonies was counted and the percentage of decrease in bacterial colonies after 24 h of treatment with ZnO NPs was calculated using Eq. (2):

Percentage of growth inhibition

$$= \frac{\text{Colony}_{\text{NC}} - \text{Colony}_{\text{T}}}{\text{Colony}_{\text{NC}}} \times 100\%. \quad (2)$$

2.6. Surface attachment of ZnO NPs on P. aeruginosa

FTIR (Spectrum RX1, Perkin-Elmer, USA) spectroscopy was employed to identify the biomolecules from the cell wall of bacteria that are possibly associated with the attachment of NPs on the bacterial cell surface. 10 mL bacterial suspension treated with ZnO NPs for 24 h was washed three times with 1X Phosphate Buffer Saline (PBS). The pelleted bacterial cells were freeze-dried to remove moisture from the pellet and were subjected to FTIR analysis in the range of 4000 to 400 cm^{-1}.

2.7. Morphological alterations in bacteria resulted from ZnO NPs treatment

The cellular accumulation, surface interaction, and subsequent morphological alterations in *P. aeruginosa* interacted with ZnO NPs were investigated by SEM (JSM-6701F, JOEL, Japan). 10 mL bacterial suspension was washed three times with 1X PBS and fixed with 2.5% glutaraldehyde overnight. The pellet was then dehydrated through a range of increasing concentrations of ethyl alcohol from 50%, 75%, 95% to 100%. The dehydrated pellet was freeze-dried to remove moisture from the pellet and the specimen was mounted on a glass slide using a carbon tape and subjected to SEM analysis.

2.8. Statistical analysis

Experimental data were processed by one-way analysis of variance (ANOVA) to report the significant inhibition of bacterial growth at different concentrations of ZnO NPs using SPSS (ver.24) with the statistical significance at $p < 0.05$. The experiments were performed in triplicates ($n = 3$) and the data are presented as mean ± standard deviation.

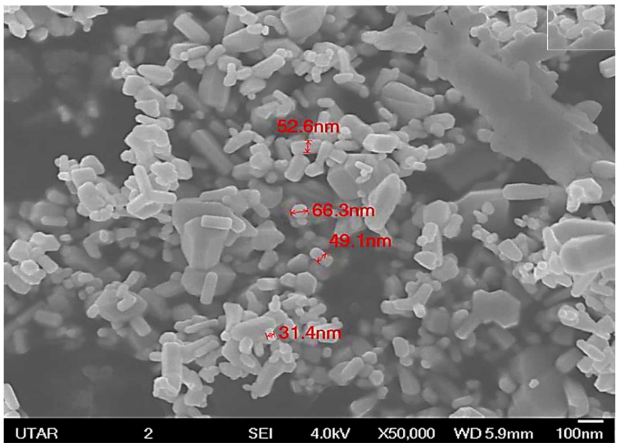
3. Results and discussion

3.1. ZnO NPs characterization

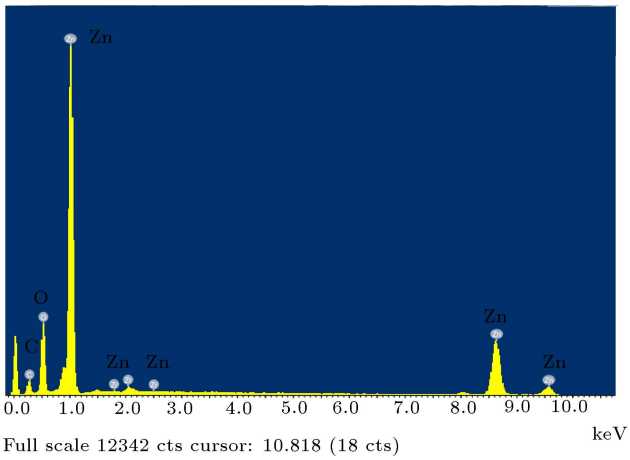
The ZnO nanopowder was identified as rod-shaped with a mixture of spherical particles in the size range of 31.4 to 66.3 nm. The average magnitude of the NPs was found to be 49.9 nm (Figure 1(a)). The EDX spectrum points to the existence of zinc and oxygen elements in ZnO nanopowder examined (Figure 1(b)). Figure 2 shows the elemental composition of ZnO nanopowder by FTIR spectrum. From the FTIR analysis, the region characterized by the peak at 540 cm^{-1} corresponded to ZnO [35,36] and other peaks at 3430 and 1629 cm^{-1} represented O-H stretching of water and angular deformation of water H-OH, respectively [39,40]. A study by Varadavenkatesan et al. (2019) demonstrated that the peaks at 557 cm^{-1}, 511 cm^{-1}, and 433 cm^{-1} confirmed the existence of characteristic zinc and oxygen bonding [24].

3.2. Bacterial growth curve

The growth curve of *P. aeruginosa* is shown in Figure 3.



(a)



(b)

Figure 1. The SEM image (a) and EDX spectrum (b) of ZnO nanopowder.

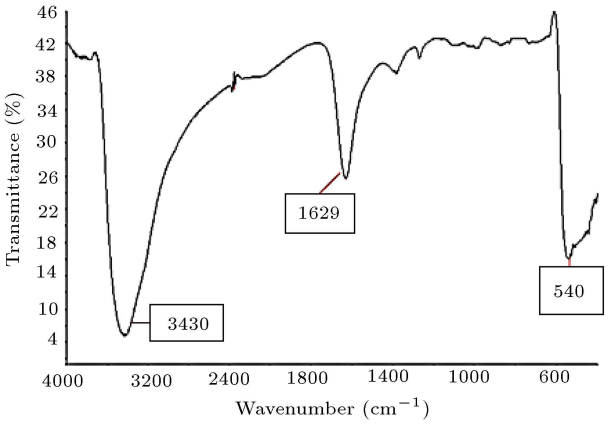


Figure 2. The FTIR spectrum of ZnO nanopowder.

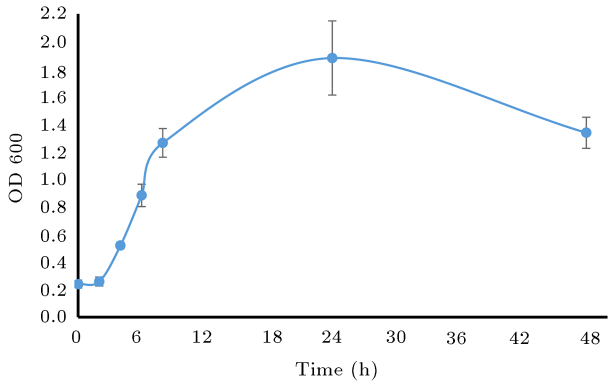


Figure 3. The growth curve of *P. aeruginosa* in Luria-Bertani broth (LB broth) at 37°C.

From 0 to 2 h of sub-culturing, the bacteria were in the lag phase wherein bacterial cells would not increase in number, but there might not be any increase in the size of the individual bacterial cells [41]. From 2 to 8 h, the bacterial population entered a drastic log phase wherein the cells multiply actively and this increase continues in many folds as long as there is the availability of sufficient nutrients and a conducive environment [41]. From 24 to 48 h, the bacterial cells progressed into the death phase wherein they began to die and hold no capacity to multiply further [41]. In this study, the mid-log phase was found to be at the time interval of 4 h in which the cells are actively dividing and metabolically active and this is the most suitable time for them to interact with the antibacterial agents [42–44].

3.3. Bacterial growth inhibition studies

3.3.1. Turbidity method

The present study revealed a significant bacterial growth inhibition ($p < 0.05$) at 24 h for all the tested concentrations (5 to 150 μg/mL) of ZnO NPs as compared to negative control, with the resulting values of 8.34 ± 0.14, 13.72 ± 0.58, 25.42 ± 2.19, 41.96 ± 3.85, 65.73 ± 3.88, and 84.62 ± 3.06% for 5, 10, 25, 50, 100, and 150 μg/mL ZnO NPs, respectively (Figure 4). However, Positive Control (PC) exhibited 98.93±0.20% of growth inhibition. The results clearly demonstrated a dose-dependent bacterial growth inhibition as the growth inhibition increased with the increasing concentrations of ZnO NPs. Similar to the present study results, Ann et al. (2014) reported that the highest concentration of 160 μg/mL ZnO NPs exhibited 72% growth inhibition, whereas the lowest concentration of 40 μg/mL caused 29% growth inhibition in *P. aeruginosa* [45].

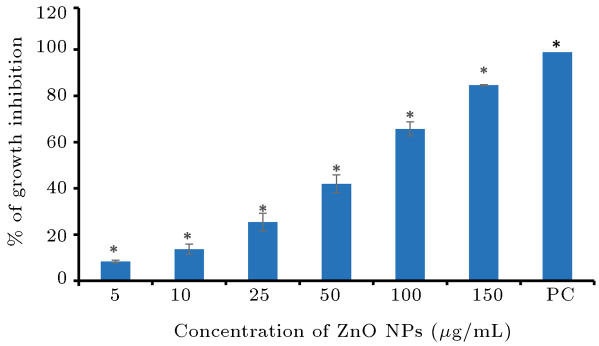


Figure 4. The percentage of bacterial growth inhibition by turbidity method. * represents the significant difference ($p < 0.05$) between negative control and the tested concentration of ZnO NPs.

3.3.2. Bacterial colony count method

Similar to the results of the turbidity method, the results of the bacterial colony counting demonstrated a significant ($p < 0.05$) dose-dependent reduction in the number of bacterial colonies for all the tested concentrations (5 to 150 μg/mL) of ZnO NPs at 24 h as compared to negative control, with the reported growth inhibition of 6.16 ± 0.56, 26.48 ± 1.40, 36.57 ± 2.89, 55.05 ± 1.52, 81.53 ± 1.68, and 93.84 ± 0.56% for 5, 10, 25, 50, 100, and 150 μg/mL ZnO NPs, respectively (Table 1, Figure 5), while no bacterial colony was found at PC. Likewise, Dhas et al. (2014) demonstrated that 10, 25, 50, and 100 μg/mL of ZnO NPs caused 5, 18,

Table 1. The number of bacterial colonies formed on LB agar in 24 h.

ZnO NPs (μg/mL)	Number of colonies
	Mean ± standard deviation
0 (NC)	16 ± 2
5	15 ± 2
10	12 ± 1
25	10 ± 1
50	7 ± 1
100	3 ± 0
150	1 ± 0
Ciprofloxacin (PC)	0 ± 0

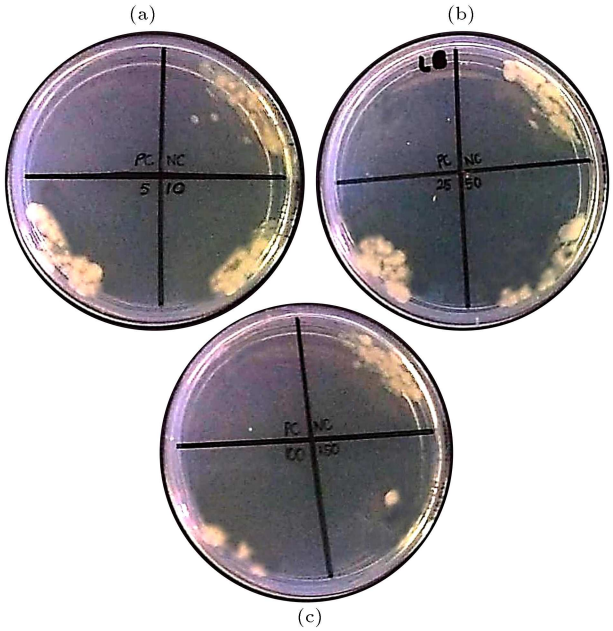


Figure 5. The number of colonies of *P. aeruginosa* formed on LB agar at 37°C after 24 h in Negative Control (NC), Positive Control (PC), and bacterial suspensions treated with (a) 5 and 10 μg/mL of ZnO NPs, (b) 25 and 50 μg/mL of ZnO NPs, and (c) 100 and 150 μg/mL of ZnO NPs.

42, and 62% of growth reduction in *P. aeruginosa* [46]. In addition, another study by Sharma et al. (2010) pointed to the reduction of the colony-forming units of *P. aeruginosa* from 160 to 120 cfu/mL upon interaction with 8 μg/mL of ZnO NPs at 24 h [5]. Further, Azam et al. (2012) reported that ZnO NPs had a greater antibacterial capability when compared to copper oxide nanoparticles (CuO NPs) and iron (III) oxide nanoparticles (Fe_2O_3 NPs) as ZnO NPs exhibited the inhibition zone of 19 mm against *E. coli*, while CuO NPs and Fe_2O_3 NPs caused the inhibition zones of 15 and 3 mm, respectively [39].

3.4. Surface attachment of ZnO NPs on *P. aeruginosa*

Figure 6 shows the FTIR spectrum of negative control and test, while Table 2 shows the biomolecules involved in the binding of ZnO NPs on the bacterial cell surface. The FTIR spectrum generated from the negative control demonstrated O-H and N-H stretching at 3413 cm^{-1}, C-H stretching at 2926 cm^{-1}, C-N

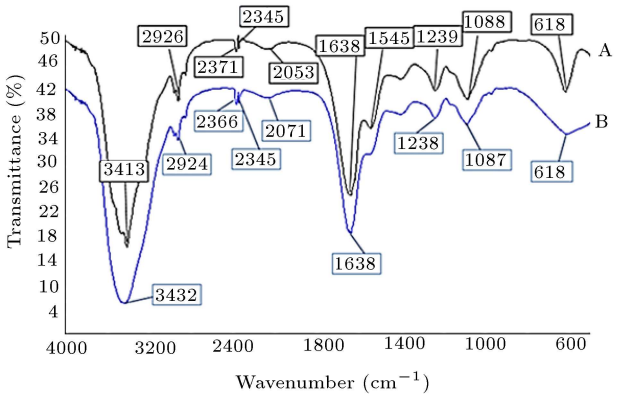


Figure 6. FTIR spectrum of negative control (A-black line) and test cells treated with 150 μg/mL ZnO NPs (B-blue line).

stretching of amine group at 2371 cm^{-1}, COO^- in amine group at 1638 cm^{-1}, ring stretching of mannose and galactose at 1545 cm^{-1}, PO_2^- asymmetric stretching at 1239 cm^{-1}, CN stretching of aliphatic amine at 1088 cm^{-1}, and carbonate moieties at 618 cm^{-1}. The FTIR spectrum of bacterial cells treated with ZnO NPs points to the stretching of O-H and N-H (3413 to 3432 cm^{-1}) and C-N stretching of amine group (2371 to 2366 cm^{-1}). The conceivable association between alcohol and amide A groups (3432 cm^{-1}) and amine groups (2366 cm^{-1}) was observed. The range between 3413 and 3432 cm^{-1} was composed by the O-H and N-H group stretching of protein, polysaccharides, and intermolecular bond [47–49]. The region between 2371 and 2366 cm^{-1} represents the stretching of C-N of amine group of the polypeptides and protein backbone [50,51]. The present study results explicitly reported the role of proteins and polysaccharides in the surface attachment of ZnO NPs on *P. aeruginosa* cell surface.

3.5. Morphological alterations in bacteria resulting from ZnO NPs treatment

SEM micrographs of bacterial cells in negative control displayed rod-shaped bacterial cells with smooth external cell wall layer, as shown in Figure 7(a). The bacterial cells interacted with ZnO NPs displayed the aggregation of bacterial cells (Figure 7(b) and (c)), cell membrane disruption, cell bending and roughening of cell surface (Figure 7(d)), and cell distortion (Figure 7(e) and 7(f)). Similar findings were reported in freshwater sediment microorganism isolates [52],

Table 2. The biomolecules from bacterial cell wall involved in binding of ZnO NPs on bacterial cell surface by FTIR analysis.

Absorption (cm^{-1})	Molecular motion	Functional group	Biomolecules
3413 → 3432	O-H and N-H stretching	Alcohol, amide A	Proteins, polysaccharides
2371 → 2366	C-N stretching	Amine	Polypeptide, protein backbone

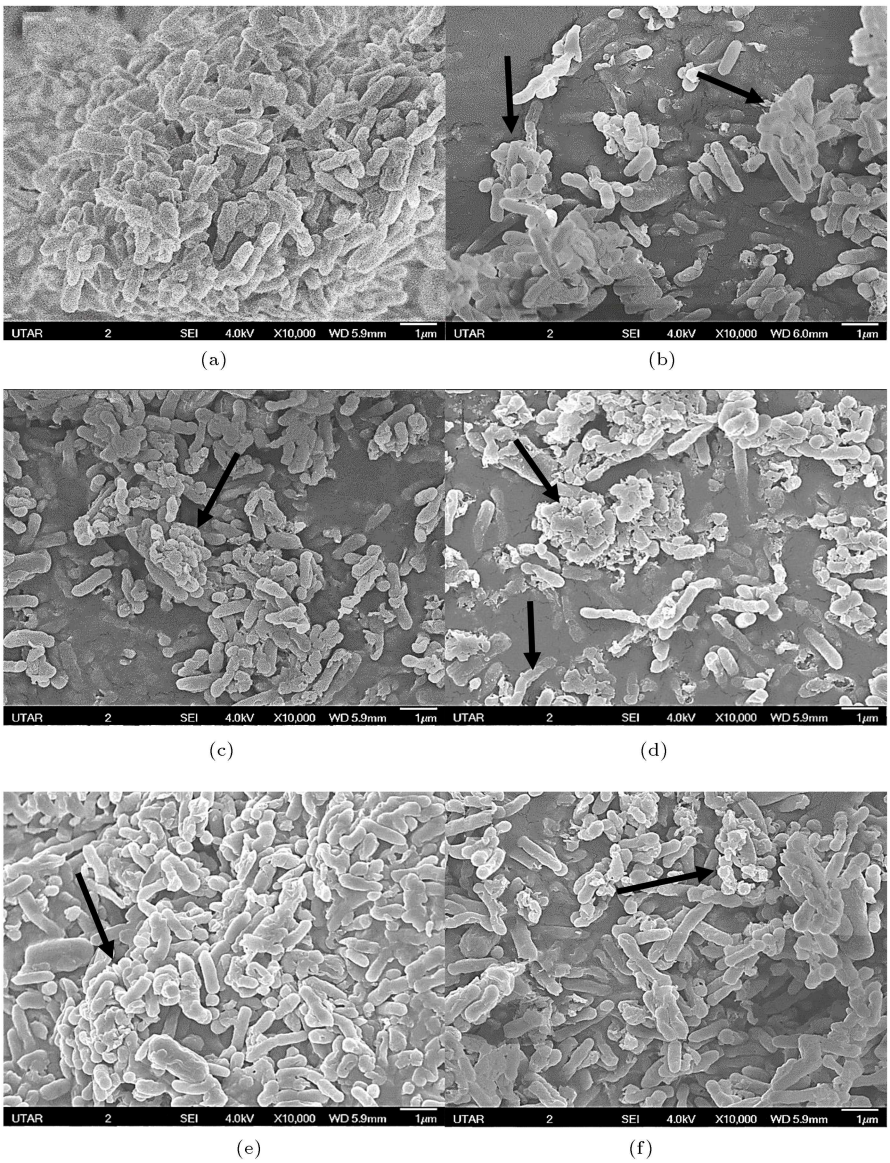


Figure 7. (a) SEM images of negative control, (b and c) bacteria treated with 150 μg/mL of ZnO NPs for 24 h showing aggregation of bacterial cells, (d) cell membrane disruption, cell bending, roughening of cell surface, and (e and f) cell distortion observed under $10,000\times$ magnification and scale bars are in 1 μm.

Bacillus licheniformis [53], *E. coli* [54], *Campylobacter jejuni* [3], *Spirulina platensis* [55], and *Chlorella vulgaris* [56] upon treatment with metallic NPs. The rationale for cell aggregation might result from a need to promote self-protection and to reduce the total surface area that is available for binding of ZnO NPs [50]. The binding of NPs on the cell wall leads to the aggregation of NPs on the cell surface and subsequently causes alterations to the cell morphology and cell membrane rupture, eventually leading to cell death [57].

4. Conclusion

The present study demonstrated a dose-dependent growth inhibition and the morphological damages induced by ZnO NPs on *P. aeruginosa*. Further, the results also illustrated the role of the association between functional groups from polysaccharides and polypeptides of *P. aeruginosa* cell wall in the attachment of ZnO NPs on the cell surface. Hence, the findings of the study propose that the potential of ZnO NPs be utilized in topical applications and wound dressings to curb the skin and wound infections caused by *P. aeruginosa* without completely relying on the use of conventional antibiotics.

Acknowledgment

This research work was funded by Ministry of Education,Malaysia (Grant No. FRGS-1-2014-SG03-INTI-

02-1) and Universiti Tunku Abdul Rahman (Grant No. IPSR/RMC/UTARRF/2016-C1/L3a).

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