ORIGINAL ARTICLE

Shape-Dependent Antibacterial Activity against *Staphylococcus aureus* of Zinc Oxide Nanoparticles

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ABSTRACT

Introduction: There is a growing concern in using zinc oxide nanoparticles (ZnO NPs) for medical devices as alternative options in reducing hospital-acquired infections (HAIs). The commensal HAIs; Staphylococcus aureus (S.aureus) infect patients and lead to increased rates of morbidity and mortality. This study aims to investigate the antibacterial action of ZnO NPs in three different shapes; nanorod, nanoflakes and nanospheres impregnated in low-density polyethylene (LDPE) against S.aureus ATCC 25923. **Methods:** The antibacterial efficiency of ZnO NPs was studied through two standard test methods included were based on Clinical Laboratory Standards Institute (CLSI) guidelines MO2-A11 under light conditions of 5.70 w/m2 and American standard test method (ASTM) E-2149. **Results:** Preliminary screening did show a significant growth inhibition against S.aureus with ZnO NPs nanorod and nanoflakes, approximately in 7 to 8 mm zones of inhibition. Further analysis using ASTM E-2149 in dynamic conditions revealed variable activity depending on incubation treatment periods. It demonstrated the ZnO NPs in nanoflakes and nanosphere shape showed better inhibition against S.aureus with maximum reduction (100%). The FESEM results strongly suggest that the structure of ZnO nanoflakes and nanosphere played an importance role in nanomaterial-bacteria interaction which consequently cause cell membrane damage. Additionally, the irradiation under light treatment also enhance the generation of ROS and free radicals which helps the bactericidal activity against S.aureus. **Conclusion:** This study provides new insights for the antibacterial action of ZnO NPs/LDPE thin films in future biomedical appliances to reduce HAIs risks.

Keywords: Zinc oxide nanoparticles, Hospital-acquired infections, Antibacterial nanoparticle, Nanotherapeutics, Nanotechnology

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INTRODUCTION

Nowadays, the conversion of bulk particles into nanoparticles (NPs) is emerging in the biomedical field. The applications of nanosize metal oxides – whose dimensions range from 1 - 100 nanometres (nm) – as antibacterial agents is on the rise as they have been widely studied. Among metal oxide NPs, there is currently great interest in the development of zinc oxide (ZnO) NPs because of their unique properties. Their wide band gap of -3.37 eV, high binding energy of 60 meV at room temperature, high photocatalytic activity, and high optical absorption are beneficial in antibacterial activities (1, 2). The abilities of the NPs to generate reactive oxygen species (ROS) and free radicals in the presence of visible light are currently explored for their feasibility to be used as antibacterial agents to reduce the risk of hospital-acquired infections (HAIs) (3). HAIs are presently a safety concern in worldwide healthcare. There are many factors of the development of HAIs in hospitalized patients, examples of which include impaired immune systems, invasive examinations and treatment, repetitive use of antibiotics (which can promote antibiotic resistance), as well as poor sterilization and disinfection practices among the staff (4). Additionally, poor sterilization and disinfection

of medical devices such as catheters potentially lead to serious risks when in contact with patients (5). These factors create possible routes for the transmission of HAI-causing pathogens into patients, hence increasing the morbidity and mortality rates. A well-known microorganism that is most frequently associated with HAIs is a Gram-positive bacterium, *Staphylococcus aureus* (*S. aureus*) (6, 7).

ZnO NPs are synthesized in different sizes and shapes, as well as impregnated in low-density polyethylene (LDPE) thin film in order to evaluate the antibacterial activities. Biomedical coatings with antibacterial thin films can help resist bacterial adhesion and colonization, apart from reducing the need for surgical removal of implanted biomaterials (such as central venous catheters) in order to minimize HAI risks (8-12). Here, we have synthesized ZnO NPs of three different morphologies: nanorods, nanoflakes, and nanospheres. We characterized our improved ZnO NPs using field emission scanning electron microscopy (FESEM). The antibacterial activities of S. aureus were evaluated using two different standard protocols that were known as CLSI MO2-A11 and ASTM E-2149 (13,14).

MATERIALS AND METHODS

Synthesis and characterization of ZnO NPs
ZnO nanorods were produced by solution precipitation method using zinc nitrate tetrahydrate ($Zn(NO_3)24H_2O$, Merck), hexamethylenetetramine (HMT, $(CH_2)6N_4$, Merck) and polyvinylpyrrolidone (PVP). Two solutions were prepared as follows: solution A – 0.12 M $Zn(NO_3)_2.4H_2O$ was dissolved in 100 ml of de-ionized water; solution B – 0.04 M HMT in 100 ml of de-ionized water; Solution C – 0.09 M PVP in 100 ml of de-ionized water. All solutions were stirred for 30 min at 90°C. Solution A and B were added into the solution C under constant stirring rate at 90°C until white precipitate was formed. Centrifugation was carried out at 3000 rpm for 20 min in order to separate the precipitate from the solution and was dried in the oven for overnight at 100°C.

ZnO nanoflakes were produced by using 0.41 M of zinc chloride ($Zn(Cl_2)_24H_2O$) dissolved in 100 ml of ethanol and kept under constant stirring using magnetic for 1 hour. 0.8 M of Potassium hydroxide (KOH) was also prepared in 100ml with stirring of 1 hour. After complete dissolution of zinc chloride, KOH aqueous solutions were added under high speed constant stirring drop by drop for 45 min. Further, the supernatant solution was separated carefully. The remaining solution was centrifuged for 20 min and the precipitate was removed. Thus, precipitated ZnO nanoparticles were cleaned with de-ionized water and ethanol then dried in oven for 8 hours at 60°C.

In the typical experiments, ZnO nanopsheres were produced by using 0.12 M of zinc acetate dihydrate ($Zn(CH_3COO)_2.2H_2O$ (99.5%)) dissolved in 200 ml deionized water (DI) and heated at 65°C under constant stirring. Simultaneously, 0.53 M of potassium hydroxide (KOH) solution was prepared in 100 ml deionized water. When KOH solution was added dropwise into the above solution, white gel was formed. The gel was allowed to precipitate at 100°C for 2 h in normal oven for characterization.

Preparation and characterization of ZnO NPs/LDPE composite
The low density polyethylene (LDPE) matrix was prepared from pallets mixture of the industrial LDPE (TITANLENE) without additives. The thin film was prepared by wet casting method. In order to prepare NPs/LDPE composite, 10 mg of NPs was added to 10 ml dichlorobenzene and ultrasonicated. Simultaneously, a LDPE/dichlorobenzene was prepared by dissolving 1 g LDPE in 20 ml dichlorobenzene, using a magnetic stirrer at 120°C. Nanoparticles/dicholorobenzene was added to LDPE/dichlorobenzene solution and was vigorous stirred for 1 minute. Then, this mixture was transferred to petri dish and dried at 80°C for 24 hours in an oven to evaporate the solvent.

Antibacterial Disc Susceptibility Tests (Semi quantitative test)
The disc diffusion assay of ZnO NPs was conducted according to the guidelines of CLSI MO2-A11 (13). It was also known as the agar diffusion Kirby-Bauer assay, which was commonly used as an antibacterial pre-screening test. S. aureus ATCC 25923 was cultured aerobically at 37°C on Luria–Bertani agar plates (Merck, Germany) for 24 hours. The positive control was represented by the standard antibiotic (ampicillin) while negative control discs (sample diluent) 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich). The samples were cut into thin film of diameter 6 mm and carefully placed onto the agar surface. All test samples were minimally exposed to visible light (Electricity fluorescent fixture T5 8W of intensity 5.70 w/m2) 2 hours prior to incubation. All tests were done in triplicates. The plates were incubated at 37°C for 18-24 hours.

Standard Test Method under Dynamic Contact Condition: ASTM E-2149 (Quantitative test)
The antibacterial activities of LDPE nanocomposites (which contained different shapes of ZnO nanoparticles) and blank control substrate materials (2.7 x 2.7 cm2) were also evaluated using a shake flask method along with phosphate buffer solution to investigate the reduction in bacterial reduction after certain incubation periods. The conical flasks were shaken (115 rpm) for 1, 3, and 6 hours at 37°C using a mechanical shaker under visible light (Electricity fluorescent fixture T5 8W with intensity of 5.70 w/m2). After 1 h of stirring, 100-µl aliquots of appropriate dilutions were aseptically pipetted to determine the bacterial concentrations via

standard plate count techniques. The colonies were counted after 24 hours of incubation and compared with those on the control plates to measure the changes in the cell growth inhibition. The percentage reduction of bacteria (R %) was calculated using the following equation:

$$R\% \; (CFU/mL) = [(B-A)/B] \times 100$$

where R is antibacterial rate (%), B are the average number of cell colony of sample (CFU/sample) at t0 (1 hour) contact time and A are the average number of colony of treated sample (CFU/sample) at specified contact time (14).

Statistical analysis

All data were analyzed using GraphPad PRISM VERSION 7. Data was presented as mean ± standard deviation. The ANOVA was applied to calculate the statistical significant of the experiment data and the difference between mean values was compared by Tukey's test ($p < 0.05$).

RESULTS

SEM images of ZnO NPs

Fig. 1 shows representatives SEM images of the prepared ZnO NPs samples with three different morphologies. The samples are well-defined in three dimensional nanoparticles, From the FESEM images, it is clear that the ZnO NPs in nanorood shapes (Fig. 1 a) showed rod-shapes NPs, ZnO in nanoflakes shapes (Fig. 1 b) showed a tiny plate-like form with a uneven ridges at the outer surface and ZnO NPs in nanosphere shapes (Fig. 1 c) with high specific surface area.

Antibacterial activity

The results of antibacterial activity of ZnO NPs are presented in Fig. 2 and Table I. Figure 2 shows the ZnO NPs in nanoflakes shapes have larger inhibition zones with mean zone of inhibition of (7.83 ± 0.29) mm following with ZnO in nanorod shapes (7.33 ± 1.16) mm compared to untreated ZnO NPs-LDPE thin films (6.0 ± 0.0) mm.

The antibacterial activity was confirmed by another standard protocol in aqueous solution treated with visible light for 6 hours. A noteworthy bactericidal activity was observed after 1 hour treatment for S.aureus (100% mortality) with ZnO NPs in nanoflakes and nanospheres shapes under dynamic condition (Table 1).

DISCUSSION

ZnO NPs have been explored for their role as antibacterial agents against a variety of microorganisms
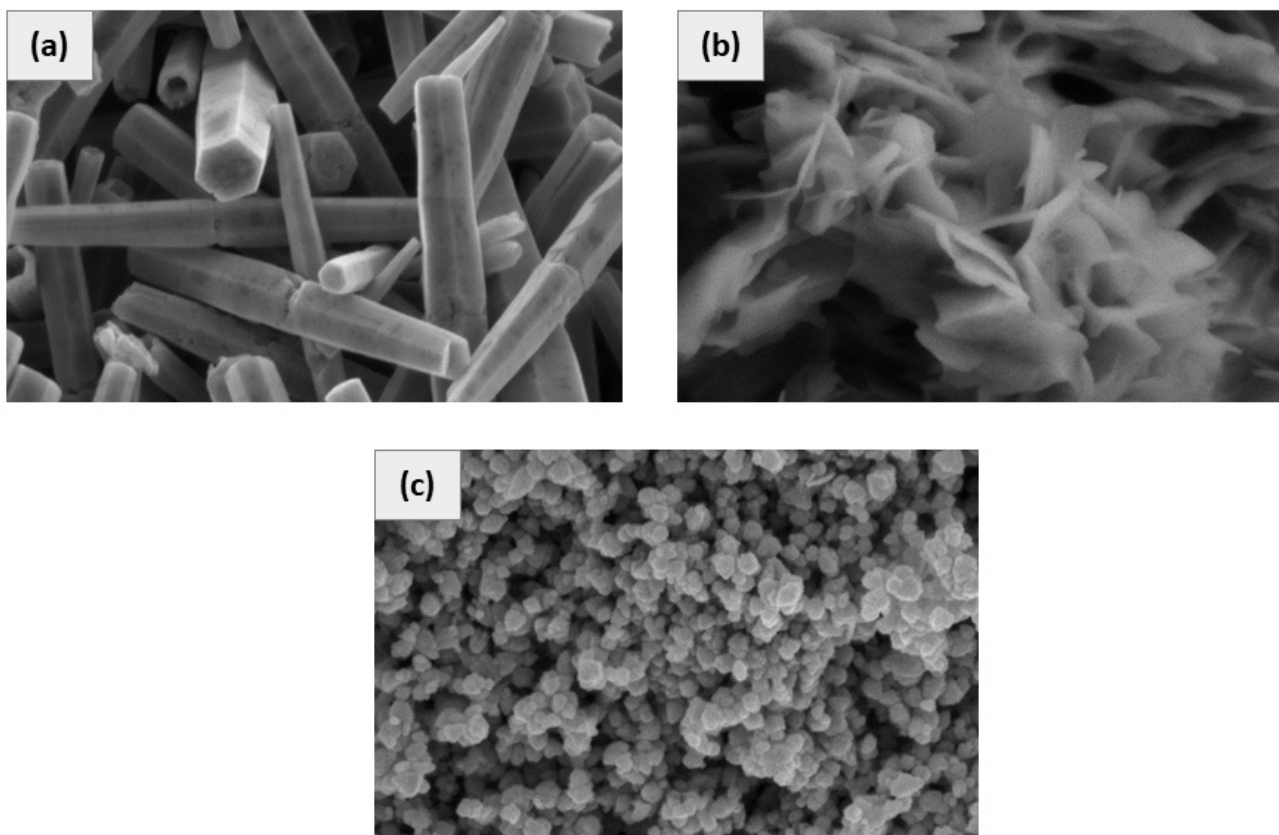

Figure 1: SEM images of ZnO NPs with different morphology. (a) ZnO in rod-shaped shapes, (b) ZnO with tiny plate-like form nanoflakes shapes and (c) ZnO in nanospheres shapes (30.00 KX, 200 nm)
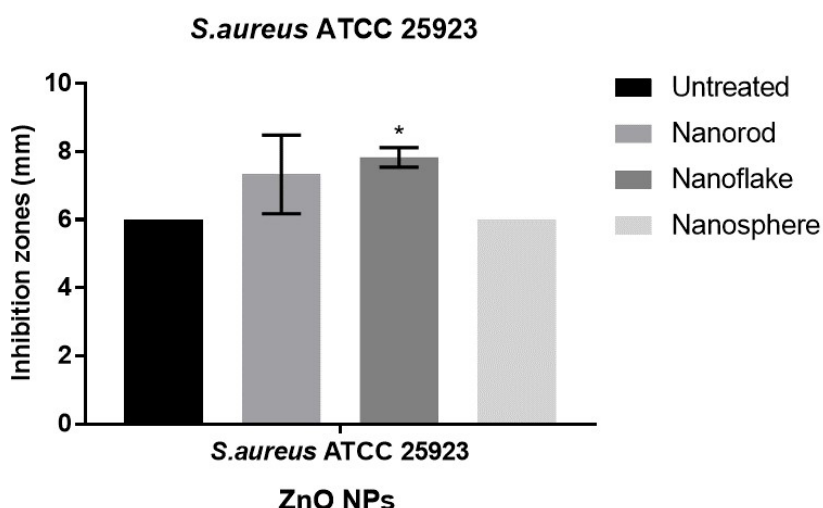
Figure 2: Effect of different shapes ZnO NPs on the growth of *S.aureus* ATCC 25923 pathogen. Inhibition zones were measured after 72 hours after exposure with light for 2 hours prior incubation treatment. Values are expressed as means (n =3) and error bars represent standard deviation. Asterisks (*) indicate a statistical significant difference (p < 0.05) between the untreated LDPE and ZnO NPs-LDPE thin films.

Table I:.Bacterial reduction percentage results of different shapes ZnO NPs on the growth of both Gram positive (*S.aureus* ATCC 25923)

Time (Hour)	ZnO NPs					
	Nanorods		Nanoflakes		Nanospheres	
	Inoculum (CFU/mL)	Bacterial reduction (%)	Inoculum (CFU/mL)	Bacterial reduction (%)	Inoculum (CFU/mL)	Bacterial reduction (%)
0	$(2.67 \pm 1.15) \times 10^2$	-	$(4.0 \pm 1.73) \times 10^2$	-	$(3.67 \pm 1.15) \times 10^2$	-
1	$(0.33 \pm 0.58) \times 10^2$	87.64	0.0	100	0.0	100
3	$(0.66 \pm 0.58) \times 10^2$	75.28	0.0	100	0.0	100
6	0.0	100	0.0	100	0.0	100

Colony were counted and reduction % was measured after 1, 3 and 6 hours after exposure with visible light.

(15-17). The antibacterial activities of ZnO NPs are influenced by several factors like particle size and shape as well as presence of light. This study has evaluated the antibacterial activities of various shapes of ZnO NPs (that have been incorporated into LDPE polymers) on a Gram-positive commensal HAI-related pathogen – S. aureus.

Antibacterial effectiveness was determined by the size of the growth inhibition zones and number of bacterial colonies following exposure to ZnO NPs for certain incubation periods. As an initial approach, the agar disc diffusion method was used as a screening test (18). During visual inspections of the formation of inhibition zones around the samples, only ZnO nanoflakes had significant zones of inhibition compared with the untreated thin films. Conversely, ZnO nanorods and nanospheres showed non-significant differences against the said controls (p > 0.05). The cultures that were exposed to ZnO nanospheres did not give rise to any inhibition zones around the samples, hence indicating that they did not possess any antibacterial properties. This could have been due to inadequate contact of

samples to water, thereby limiting the release of Zn2+ ions during incubation that was otherwise responsible for the inhibition of S. aureus growth. Moreover, this static condition did not result in adequate exposure of the surface area of both sides of ZnO nanospheres for contact with the surface layers of bacteria. After obtaining these screening results, we have confirmed the antibacterial activities by using further assays (which involved dynamic contact in aqueous solution and were guided by ASTM E-2149) under continuous light treatment for 6 hours. This method was chosen in order to overcome the limitations of conventional disk diffusion methods, apart from ensuring proper contact of the samples with the inoculums. Bacteriostatic and bactericidal effects were considered if the reduction in total original bacterial count exceeded a certain percentage. If the percentage reduction was more than 99.9% CFU/mL, the agent would be considered as bactericidal. Meanwhile, if the percentage reduction was between 90% and 99.9% CFU/mL, the agent would be categorized as bacteriostatic (19,20). All assayed samples showed high antibacterial activity, as per Table I. ZnO NPs tended to release Zn2+ ions when in contact

with water. This followed a change in the surface property, like free energy (21).

NPs get attached to bacterial cell surfaces due to the electrostatic attraction of Zn2+ ions to the negatively-charged membranes (3). In addition, the sizes of the ZnO NPs also play a major role in the ionic interactions with the bacterial membranes. Specifically, a decrease in the size of the NPs leads to an exponential increase in their surface area to volume ratio, thereby increasing the area for Zn2+ ions to attach to the bacteria and damage the cell membranes' integrity. Ann et al. (2014), their study proved that reducing the size of ZnO enhanced the inhibition of S. aureus (22). The bactericidal effects of ZnO NPs involved the blocking of two main systems of bacterial survival: (i) food source disruption, as well as (ii) alterations in the the release and absorption of potassium (K+) and sodium (Na+) ions from the bacterial cells (23-26). The recurrent pitting of ZnO NPs on the cell walls of S. aureus may also lead to cytoplasmic leakage of intracellular constituents such as ions and proteins, ultimately causing cell death (27). Furthermore, the photoactivation of the samples via light treatment for 2 hours prior to incubation (in the agar disc diffusion assay) and continuous light treatment for 6 hours (in ASTM E-2149) was another mechanism of the bactericidal activities of ZnO NPs. ZnO NPs are well-known for their photocatalytic activities and wide band gaps. Thus, when the samples were irradiated by photons which were of higher energy than the band gaps, (e.g. visible light), reactive oxygen species (ROS) and free radicals were generated on the surfaces of ZnO NPs-LDPE. ROS like superoxide ions, hydroxyl ions, singlet oxygen, and peroxide are produced mainly via visible light irradiation (28). Some studies have reported that the negative ions like hydroxyl and superoxide ions were unable to penetrate the cell membrane. However, peroxide ions were able to do so due to the presence of electrostatic forces around the bacterial membranes (29). The mechanism of the bactericidal activities of ZnO NPs is illustrated in Fig. 3. This study has shown that
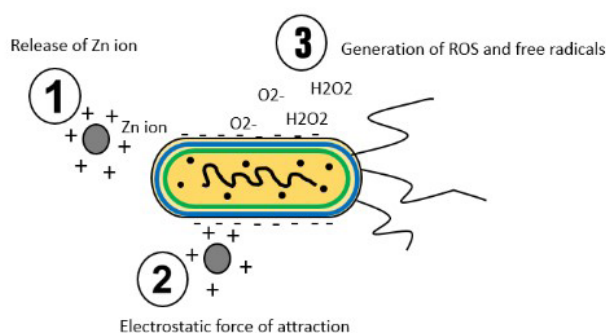
was possible to develop thin antibacterial films using ZnO NPs. Further studies are needed to investigate the cytotoxicity of these samples in order to further improve their future applications in the biomedical field.

CONCLUSION

In conclusion, ZnO nanoflakes and nanospheres provided better antibacterial activities against Gram-positive pathogens. The shape-dependent inhibition behavior was associated with a large surface area to volume ratio, which allowed direct attachment of antibacterial ions with the bacterial cell surfaces. ZnO NPs could be excited by light irradiation, following which they could react with molecular oxygen to generate ROS as well as effect cytotoxicity on the bacterial membranes. Additionally, ROS formation in aerobic respiration in bacteria also caused the accumulation of ROS with the formation of free radicals that led to oxidative stress. These resulted in damage to the nucleotides and membrane lipids of S. aureus. Furthermore, ROS could reduce the synthesis of catalase – one of the protective barriers in bacteria – thus causing serious damage to the intracellular components and leading to bacterial cell death. The findings presented here demonstrated the potential ZnO NPs to minimize the rates of HAI in the future.

Figure 3: Schematic diagram of possible mechanisms of bactericidal activity of ZnO NPs-LDPE thin films. Immersion of samples in aqueous solution did release the (1) antibacterial ion (Zn2+), which directly contact with the bacterial membrane. (2) Electrostatic forces between Zn2+ ions and negatively charged S.aureus cell walls also promote the attachments and (3) generation of ROS due to light irradiation leads to cytoplasmic leakages, thus causing cell death.

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