J Appl Biotechnol Rep. 2018;5(4):162-165

doi 10.29252/JABR.05.04.05

Journal of
Applied Biotechnology
Reports

Original Article

In Vitro Evaluation of Antibacterial Properties of Zinc Oxide Nanoparticles on Pathogenic Prokaryotes

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Received August 10, 2018; **Accepted** October 28, 2018; **Online Published** December 20, 2018

Abstract

Introduction: Recent increases in microbial resistance to multiple antibiotics have led to the emergence of more economical methods for producing nanoparticles with physical, chemical effects and limited resistance. The aim of this research was to study zinc oxide (ZnO) nanoparticles synthesis and antibacterial properties against some gram-negative and gram-positive bacteria.

Material and Methods: In this study, ZnO nanoparticle was synthesized using ultrasonic method and bioassayed on 10 human pathogenic bacteria by agar well diffusion method. In addition, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined as well. Antibiotic resistance pattern of bacteria was determined to 9 antibiotics: gentamicin, ampicillin, nalidixic acid, amoxicillin, amikacin, ciprofloxacin, co-trimoxazole, norfloxacin and cephalexin by disc diffusion assay.

Results: The nanoparticles were synthesized with suitable morphology and distribution. All gram-positive and gram-negative bacteria were inhibited at the low concentration of ZnO nanoparticles most bacteria had resistance to antibiotics.

Conclusions: The findings suggest that the ZnO nanoparticles have potential applications as antibacterial compounds and their mechanism of action is dependent upon composition and surface modifications.

Keywords: ZnO Nanoparticle, Antibacterial Properties, Ultrasonic Method, Antibiotic

Citation: C. In vitro evaluation of antibacterial properties of zinc oxide nanoparticles on pathogenic prokaryotes. J Appl Biotechnol Rep. 2018;5(4):162-165. doi:10.29252/JABR.05.04.05.

Introduction

Bacterial resistance to antibiotics has become a global problem nowadays.[1] In examining different options to solve this problem, nanomaterials, such as metal oxide nanoparticles, have appeared to be promising candidates during the last few years. As a result, the science of nanotechnology has significantly advanced due to its wide application.[2] Recent research achievements make it possible to produce new types of surface-coated nanoparticles for advanced applications.[3] Characterization of these nanoparticles can be extremely effective in their antibacterial and cytotoxic effects. This is due to the fact that if the size of the nanoparticles becomes smaller, then as a result antibacterial effects will increase.[4] Nanoparticles are defined as particles with sizes ranging from 1 to 100 nm at least in one of the three possible dimensions.[5,6] Nanoparticles possess an enormous surface area per unit volume, a high proportion of atoms in the surface and near surface layers, and have the ability to exhibit quantum effects because of their small size.[5] They exist in several different morphologies such as spheres, cylinders, platelets, tubes and can be generated via a number of synthetic routes based on liquid, gas or solid phase methods.[7] As potential new antibacterial agents, metal oxide nanoparticles such as zinc oxide (ZnO), CuO, Fe3O4 and TiO2, are being thoroughly studied.[8] Among the various metal oxides studied for their antimicrobial effects, ZnO nanoparticles have been found to have selective toxicity to bacteria and only exhibit the fewest side effects on human cells, which recommend their prospective uses in food industries and agricultural.[9,10] However, there are several methods to produce the nanoparticle, hence; this project was designed to synthesize and characterize ZnO nanoparticles by the ultrasonic method and its antibacterial properties on the growth of some human pathogenic bacteria.

Materials and Methods

Preparation of ZnO Nanoparticles and SEM Imaging

In this investigation, ZnO nanoparticle was synthesized using ultrasonic method technique. Accordingly, in a typical experiment, 1 g of the obtained Zn $(OAc)_2$ nanostructures was loaded into a beaker that was later put in an ultrasonic devise. The sample was heated in air at 50°C at 60 W for 60 minutes. After the thermal treatment, the system was allowed to cool to room temperature naturally, and the obtained precipitations

were collected. The synthesized product was characterized by X-ray diffraction (XRD) and scanning electron microscopy. The scanning electron microscopy (SEM) imaging was performed by SEM (LEO 1455VP, London). The working distance was adjusted to 3 mm and the accelerating voltage was set to 10 kV and the contrast and brightness of the image adjusted to optimal values so that particles could be identified from the background.

Bacterial Isolates

Antibacterial activity of ZnO nanoparticles was performed on 10 bacteria obtained from clinical specimens including *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Serratia marcescens, Salmonella typhi, Acinetobacter baumannii, Citrobacter freundii, Proteus mirabilis, Staphylococcus aureus* and *Bacillus cereus*. The Pure cultures were propagated on Nutrient Agar (Merck, Germany) at 37°C and maintained at 4°C for subsequent studies.

Assay for Antibacterial Activity of ZnO Nanoparticles

Antibacterial activity of ZnO nanoparticles were assayed by Agar well diffusion.[11,12] In brief, the bacterial isolates were cultured on nutrient broth (Merck, Germany) at 37°C overnight. Following incubation, a standard inoculum of each bacterial isolate was prepared in sterile normal saline to a concentration of 1.5×10^8 CFU/mL and compared with a 0.5 McFarland standard solution. A sterile swab was dipped into the suspension and then inoculated on Muller-Hinton agar (Merck, Germany) plate in order to provide a uniform coverage of bacteria on the surface of the plate.[13-15] Different concentration of synthesized ZnO nanoparticles in dimethyl sulfoxide (DMSO): Methanol (1:1V/V) (Merck, Germany) was prepared. Wells in 6 mm diameters were punctured in the media using sterile cork borers and were filled with 20 µL of the ZnO nanoparticles suspension. The plates were then incubated at 37°C for 24 hours. Following incubation, antibacterial activity was determined by measuring the inhibition zones around the wells in mm. DMSO: methanol (1:1 V/V) solvent is considered as negative control. To determine MIC, 2 folds of dilution series (160, 80, 40, 20, 10, 5, 2.5, 1.25, 0.62, 0.31, 0.15 and 0.075 mg/mL) of ZnO nanoparticles suspension were prepared and bio-assayed as mentioned above.

Antibiotic Resistance Pattern Determination

Antibiogram test was done by agar disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI).[16] Bacterial cultures were prepared to 0.5 McFarland Standard ($1.5\times1o^8$ CFU/mL) in sterile distilled water prior to the assay and were inoculated on Mueller-Hinton agar medium (Merck, Germany). Antibiotic concentrations in applied discs (Padtan Teb, Tehran, Iran)) were as follows: gentamicin (10 µg/disc), ampicillin (10 µg/disc), nalidixic acid (30 µg/disc), amoxicillin (30 µg/disc), amikacin(30 µg/disc), ciprofloxacin (5 µg/disc), co-trimoxazole, (25 µg/disc), norfloxacin (10 µg/disc) and cephalexin (30 µg/disc) representing different families of antibiotics which were tested on all isolated organisms. The plates were incubated for 24 hours at 37°C and, then the results were recorded.

Results

Characterization of the Synthesized ZnO Nanoparticles

Characterization of the synthesized ZnO nanoparticles were performed and the XRD pattern and also the SEM images showed that the nanoparticles have been synthesized with suitable morphology and distribution. The XRD patterns of the structures were made between ($10<2\Theta<80$) (Figure 1). Two reflection peaks of the XRD pattern for ZnO nanoparticles are well indexed with calculated cell parameters. The crystalline size and diameter (Dc) of nanofibers can be determined about 121-128 nm from the diffraction patterns from the full width of the half maximum with the Debay-Scherer equation (Eq. 1):

$$D_{xrd}=0.9\lambda\ (57.3)\ /W_{size}\cos\Theta \quad \text{(Eq. 1)}$$

The microscopic morphology of nanostructures was visualized. Scanning Electron Microscopy (SEM) with a secondary electron detector can visualize crystal shape, surface morphology, dispersed and agglomerated nanoparticles, and surface functionalizations. The SEM image of the ZnO nanoparticles and the formation of nanoparticles with an average size of 50 nm has been shown in Figure 2. The results showed that the nanoparticle surface has a relatively good dispersion.

Antibacterial activity of ZnO Nanoparticles

Compared to other bacteria, ZnO nanoparticles showed proper antibacterial activities. The inhibition of growth was observed in a concentration-dependent manner (Figure 3). For all bacteria, ZnO nanoparticles showed a high growth-inhibitory effect even in low concentration, and there was statistically significant inhibitory effect compared with the control (general antibiotics) in this condition. Also, there was no antibacterial activity in solution DMSO: methanol (1:1 v/v) devoid of ZnO nanoparticles used as a vehicle control, reflecting that antibacterial effect was directly related to the ZnO nanoparticles. The minimum inhibitory concentration

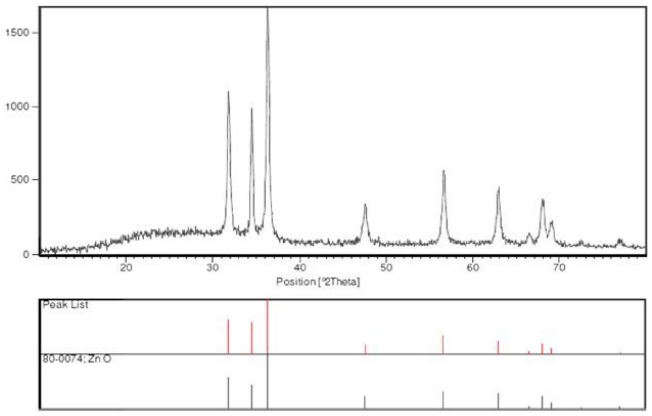

Figure 1. XRD Patterns of ZnO Nanostructure Sublimated at 50°C. As it is illustrated in the figure, the ZnO nanoparticles were synthesized properly.

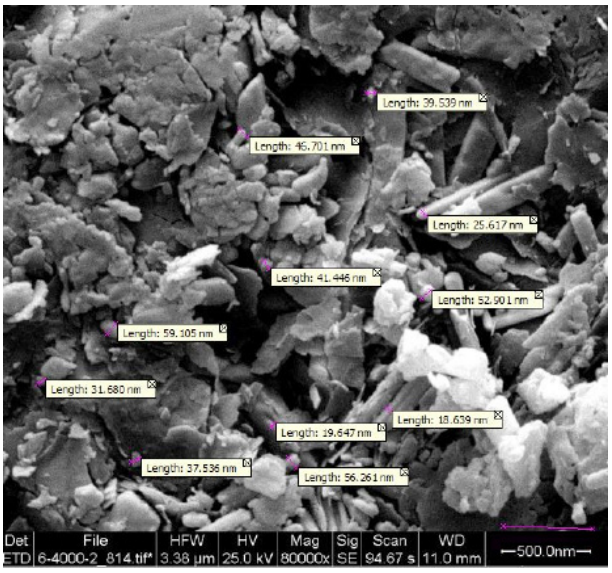

Figure 2. SEM Images of ZnO Nanoparticles. Due to the figure, it appears that the nanoparticle surface has a relatively good dispersion.

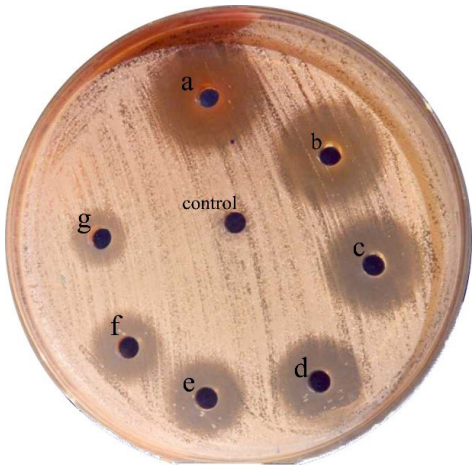

Figure 3. Dose Response of ZnO Nanoparticles Against *S. aureus*. a: 160 mg/mL, b: 80 mg/mL, c: 40 mg/mL, d: 20 mg/mL, e: 10 mg/mL, f: 5 mg/mL, g: 2.5 mg/mL.

(MIC) of ZnO nanoparticles against *S. aureus*, *S. marcescens* and *E. coli* was estimated to be more than 2.5 mg/mL and MIC of other bacteria was 5.0 mg/mL. The minimum bactericidal concentration (MBC) of ZnO nanoparticles against *P. aeruginosa*, *A. baumannii, K. pneumoniae* and *S. aureus* was 10 mg/mL compared to other bacteria, which were 20 mg/mL.

Antibiotic Resistance Pattern of the Bacterial Isolates

In parallel with ZnO nanoparticles, antibiogram test was also performed against 10 pathogenic eukaryotes. Results of antibiogram test for bacterial isolates revealed that amoxicillin and co-trimoxazole were the most resistance antibiotics to all isolates. Gentamicin was the most effective antimicrobials against the strains except *S. aureus* and *E. coli*. *Cephalexin* and ampicillin were only effective against *A. baumannii* and *S. aureus*. Other antibiotics did not affect any of the bacteria. These results revealed that *most bacteria* had resistance to antibiotics.

Discussion

In recent years, inorganic antimicrobial agents have increasingly been used to control microorganisms in different statements.[17] Nanoparticles have attracted attention due to their distinctive features that are inaccessible in the conventional macroscopic materials.[18] In this research, nanostructures were prepared at first and then, after identification with XRD and SEM analysis, the antimicrobial activities of the structures were investigated. Antimicrobial activity of metal oxides, including MgO and ZnO, was first shown by Japanese researchers.[19] Nanotechnologists have become familiar with the new properties associated with nanoparticles, which may have a dramatic effect in the future. In the present study, ZnO nanoparticle dilutions readily prevented the growth of all examined bacteria. Susceptibility to ZnO nanoparticle dilutions depended on the concentration. All isolates were susceptible to more than MIC of ZnO nanoparticle compared to a high resistance to multiple classes of the routine antibiotics. Jiang et al, have documented the antimicrobial activity of ZnO nanoparticles against the food related bacteria *Bacillus subtilis, E. coli* and *Pseudomonas fluorescens*.[20] In another study, it has been reported that the ZnO nano-fluids had bacteriostatic activity against *E. coli* O157:H7 and the SEM analyses of the bacteria before and after treatment with ZnO nanofluids showed that the presence of ZnO nanoparticles are associated with damages to the cell membrane of the bacteria.[10].There are also other investigations confirming the effective antibacterial activity of ZnO nanoparticles wherein the nanoparticles could completely lyse *Salmonella typhimurium* and . *aureus* causing food poisoning.[21] In another study, ZnO nanoparticles (12 nm) showed antimicrobial activities against *L. monocytogenes* and *S. enteritidis* in liquid egg white and culture media. Their findings demonstrated several approaches (powder, PVP capped, film, and coating) for the incorporation of ZnO in food systems and for using ZnO nanoparticles in food safety.[22] The above findings and our results in the present study suggest that ZnO nanoparticles can be used in food systems and can be used to inhibit the growth of pathogenic bacteria. Several mechanisms for the antimicrobial properties of ZnO nanoparticles have been proposed. The generation of hydrogen peroxide and the release of Zn^{2+} ions can damage the cell membrane and interact with intracellular contents. On the other hand, with decreasing the particle size of nanoparticles and increasing of surface area, the antimicrobial properties of the ZnO will increase.[5] The use of inorganic nanoparticles has many advantages over the antimicrobial agents and current antibiotics. The most important problem with the current antimicrobial agents is the prevalence of microbial resistance.[23] Therefore, an alternative way to overcome the microbial resistance of many microorganisms is needed.

Conclusions

In conclusion, it has been demonstrated that ZnO nanoparticles display excellent antibacterial potential for

the gram-negative and the gram-positive bacteria. this investigation suggests that ZnO nanoparticles may represent useful candidates, but will require important development to ensure optimal bactericidal activity and low host toxicity.

Authors' Contributions

All authors equally contributed to the present study.

Conflict of Interest Disclosures

Authors have no conflict of interest to declare.

Acknowledgments

Authors would sincerely like to thank the members of the microbiology research laboratory of Islamic Azad University, Kerman Branch and Dr. Mehdi Ranjbar from the Pharmaceutics Research Center, Institute of Neuropharmacology, University of Medical Sciences, Kerman for their kind considerations.

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