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Original Research

Synthesizing Colloidal Zinc Oxide Nanoparticles for Effective Disinfection; Impact on the Inhibitory Growth of *Pseudomonas aeruginosa* on the Surface of an Infectious Unit

Mahsa Omrani, Ebrahim Fataei*

Department of Environmental Sciences, Ardabil Branch, Islamic Azad University, Ardabil, Iran

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Abstract

Pseudomonas aeruginosa has innate characteristics of developing resistance. Therefore, it is obligatory to find the new antipseudomonal agents: Zno colloidal nanoparticles (NPs) synthesized via chemical deposition method. Then, TEM, SEM, DLS, and UV-visible were done. Sampling was achieved from not the same sections of infectious unit and then *Pseudomonas aeruginosa* was isolated from hospital and its antibiotic resistance pattern was determined. Disc diffusion, cavity, MIC, and MBC tests were done. Absorption of UV-visible occurred at about 350 nm. The mid-range of hydrodynamic diameter and the average size of the ZnO NPs were 1.48 um and 5 nm, respectively. Isolated *Pseudomonas aeruginosa* was resistant to *Trimethoprim*, *Ampicillin*, and *Nitrofurantoin*. The disc diffusion and cavity test of antibiotic-resistant *Pseudomonas aeruginosa* showed respectively the least sensitivity to ZnO (DIZ = 8 mm and 5 mm) in comparison of standard strain of *Pseudomonas aeruginosa* (DIZ = 10 mm and 8 mm). According to the results, ZnO NPs could kill all antibiotic-resistant bacteria at a ratio of 1:16 (MBC = 7.5 ppm). However, it was able to eliminate the standard strain of *Pseudomonas aeruginosa* at a ratio of 1:64 (MBC = 0.937 ppm). This study demonstrated that ZnO NPs have high potential for disinfection of infectious units of hospitals against nosocomial infection – especially by *Pseudomonas aeruginosa.*

Keywords: zinc oxide, nanoparticles, *Pseudomonas aeruginosa*, disinfection, infectious unit

Introduction

Nosocomial infections are a major problem that nursing and medical staffs and also patients face daily [1]. *Pseudomonas* is a rod-shaped, aerobic, Gram-negative bacterium belonging to the family *Pseudomonadaceae* [2]. Risk factors for the growth of infections caused by *Pseudomonas* include neutropenia, cystic fibrosis, severe burns, and foreign device installations [3-4]. *Pseudomonas* can be diffused in hospitals by nursing staff, medical equipment, sinks, disinfectants, and food. *Pseudomonas*

*e-mail: ebfataei@iauardabil.ac.ir, ebfataei@gmail.com

infections are a serious and important problem in hospitals for two reasons. First, patients who are critically ill can die from *pneumonia* caused by *Pseudomonas*. Second, the elimination of *Pseudomonas aeruginosa* in patients with infections is very difficult because of its resistance to a variety of antibiotics [5]. *Pseudomonas aeruginosa* currently shows resistance to the following antibiotics: *penicillin G*; *aminopenicillin*, including those combined with *beta-lactamase* inhibitors; first- and second-generation *cephalosporins*; *piperacillin*; *piperacillin* and *tazobactam*; *cefepime*; *ceftazidime*; *aminoglycosides*; the *quinolones*; and the *carbapenems*; as well as *colistin* and *fosfomycin* [6]. The increasing resistance of *Pseudomonas aeruginosa* to numerous antibiotics, as a result of excessive antibiotic administration, is now leading to the accumulation of antibiotic resistance and cross-resistance between antibiotics and the appearance of multidrug-resistant (MDR) forms of *Pseudomonas aeruginosa* [7-8].

In recent decades, nanotechnology has opened a new chapter in human life. Zinc oxide (ZnO) is listed as "generally recognized as safe" (GRAS) by the U.S. Food and Drug Administration. Nano-sized particles of ZnO have more obvious antimicrobial activities than large particles, since the small size and high surface-to-volume ratio of NPs allow for better interaction with bacteria [9]. Researchers have suggested the use of nano-metal oxides, especially zinc oxide nanoparticles (ZnO NPs) as superior disinfectants and antimicrobial agents for nosocomial infections microorganisms [10]. In other words, they report on the toxicity of ZnO NPs to gram-negative and gram-positive bacterial systems, *Escherichia coli, Staphylococcus aureus*, and primary human immune cells. Those results show that ZnO NPs may potentially prove useful as antimicrobial agents at selective therapeutic dosing regimens [9]. Also, Jayesh assumed that a combination of metal oxide NPs may give rise to more complete bactericidal effects against mixed bacterial populations [11]. It is obvious that the bactericidal effect of metal NPs has been attributed to their small size, photo-catalytic activity, and high surface-to-volume ratio, which allows them to interact closely with microbial membranes and is not merely due to the release of metal ions in solution [12]. In this study, colloidal ZnO NPs were synthetized and antibacterial effects of ZnO NPs against antibiotic resistance *Pseudomonas aeruginosa* isolated from the infection unit of Rasoul-e-Akram Hospital was investigated.

Experimental

Materials and Methods

Synthesis of Colloidal Zinc Oxide NPs

Colloidal ZnO NPs were synthetized using chemical analysis. First, 0.2 M of zinc acetate dehydrate ($C_4H_6O_4Zn$, Merck, Germany) was added to 25 ml of methanol (CH_3OH, Merck, Germany). In addition, 1.2 M of sodium hydroxide (NaOH, Merck, Germany) was added to 25 ml of methanol. Then, the zinc acetate dehydrate solution was poured drop by drop to sodium hydroxide solution. The final solution was placed on a magnetic stirrer for about 3 hours. The solution was centrifuged for 10 min at 4,000 rpm. Next, nano-colloidal particles were dried at room temperature. The powder of ZnO NPs was added to 50 ml of deionized distilled water and then sonicated.

Characterization

The characterization of ZnO NPs was confirmed with UV-visible (BioTek, microplate reader, U.S), dynamic light scattering (DLS) (A-ONE Enc., Korea), inductively coupled plasma mass spectrometry (ICP-MS), scanning electron microscopy (SEM) (Hitachi, Japan), and Transmission Electron Microscopy (*TEM*) (Philips, Germany). In addition, dilutions of ZnO NPs were prepared according to Table 1.

Sampling

This study was conducted in the infection unit of medium-sized Rasoul-e-Akram Hospital in the city of Tehran. Also, sampling was conducted for a month, from 22 September to 22 October 2016. The research considered contaminated surfaces that are routinely and collectively handled by professionals from this service. Randomly, sampling was done from different sections of the infectious unit, especially in burn patient rooms, such as workbenches, beds, doorknobs, and walls. Swabs were quickly sent to the antimicrobial resistance research center (ARRC) laboratory. Note that just one sample of isolated *Pseudomonas aeruginosa* was chosen for this research.

Bacteriological Analysis

All swabs were placed into the enrichment medium – BHI broth (Merck, Germany) for 24 h at 37ºC.

Table 1. The approximate concentration of ZnO NPs in different dilutions in a final volume of one ml.

	Dilution								
ZnO	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
	~30 ppm	~15 ppm	~7.5 ppm	~3.75 ppm	~1.875 ppm	~0.937 ppm	~0.468 ppm	~0.234 ppm	~0.117 ppm

Table 2. Lists of antibiotic discs used on isolated *Pseudomonas aeruginosa.*

Antimicrobial agent	Symbol and count	Made in
Cefazolin	CEF10	PADTAN TEB Co. Iran
Gentamycin	GM10	PADTAN TEB Co. Iran
Amikacin	An	PADTAN TEB Co. Iran
Cefepime	FEP30	PADTAN TEB Co. Iran
Cefotaxime	CTX30	PADTAN TEB Co. Iran
Ciprofloxacin	CP10	PADTAN TEB Co. Iran
Trimethoprim sulfa methoxazole	TMP5	PADTAN TEB Co. Iran
Meropenem	MEN10	PADTAN TEB Co. Iran
Ceftazidime	CAZ30	PADTAN TEB Co. Iran
Nitrofurantoin	FM300	PADTAN TEB Co. Iran
Piperacillin Tazobactam	PTZ100/10	PADTAN TEB Co. Iran
Ampicillin	AM30	PADTAN TEB Co. Iran
Imipenem	IPM10	MAST Co. UK
Colistine	CL10	MAST Co. UK
Aztreonam	AZ15	PADTAN TEB Co. Iran

The swabs were cultured on blood agar (Merck, Germany) and MacConkey Agar (Merck, Germany) for 24 h at 37ºC. After that, samples were tested for the presence of suspicious β-hemolytic colonies of Pseudomonas aeruginosa using gram staining and biochemical tests such as oxidase and catalase, oxidation and fermentation, methyl red, and Voges-Proskauer reactions. Indole, SH2, motility in SIM medium (Merck, Germany), pigmentation, lactose fermentation, sugar fermentation in TSI medium (Merck, Germany) were also analyzed and, finally, *Pseudomonas aeruginosa* was approved.

Identifying and Determining Antibiotic Resistance Patterns of *Pseudomonas aeruginosa*

Biochemical tests were used to confirm the identification of antibiotic resistance of *Pseudomonas aeruginosa*. We used the disk diffusion method (Kirby-Bauer) in accordance with the standard clinical and laboratory standards institute (CLSI) and the National Committee for Clinical Laboratory Standards (NCCLS) to determine antibiotic resistance patterns. Antibiotic discs for isolated *Pseudomonas aeruginosa* are listed in Table 2 [13].

Disk Diffusion and Cavity Tests

First, one ml of colloidal ZnO NPs (60 ppm) were poured into the sterile tubes, and after that blank discs were placed in the tube. Then it was sonicated at room temperature at a frequency of 28 KHz (Fisher Scientific, U.S.) for 10 min. Next, discs were placed in desiccators for one hour at room temperature. Subsequently, two or three colonies of freshly antibiotic resistance of isolated *Pseudomonas aeruginosa* and standard strain of *Pseudomonas aeruginosa* (*ATCC 27853*) were injected into a test tube containing 10 ml of sterile normal saline (Mahban Darou. Iran) and equivalent to 0.5 McFarland. Then, 100 µl of bacterial suspension was cultured on Muler Hinton agar (MHB) (Merck, Germany). A disc impregnated with ZnO NPs was polluted on MHB and incubated at 37ºC for at least 18 hours. In order to perform a cavity test, we drilled several cavities on MHB and poured 100 µl of ZnO NPs into them. All tests were replicated three times.

MIC and MBC Tests

The serial dilution method was used to determine the minimum inhibitory concentration (MIC) of the colloidal ZnO NPs [12, 14]. We used 24 micro-plate wells for this test. In this way, 12 wells were filled with one ml of the liquid Muller Hinton broth (MHB) medium. Then, one ml of ZnO NPs was sonicated and added to No. 1 well. Subsequently, serial dilution was done. The 12^{th} well was continuously dedicated as a positive control. In total, microbial suspensions of isolated antibiotic resistance of *Pseudomonas aeruginosa* and standard strain of *Pseudomonas aeruginosa* (ATCC27853) containing 1.5×10^{8} $CFU.ml^{-1}$ were added to wells and incubated at 37ºC for 24 hours. For measuring the minimum bactericidal concentration (MBC), a loop-full from each well was inoculated on Muller Hinton agar (MHA) medium and incubated at 37ºC for 24 hours. The ZnO NP concentration illustrating bactericidal effect was picked out based on the absence of colonies

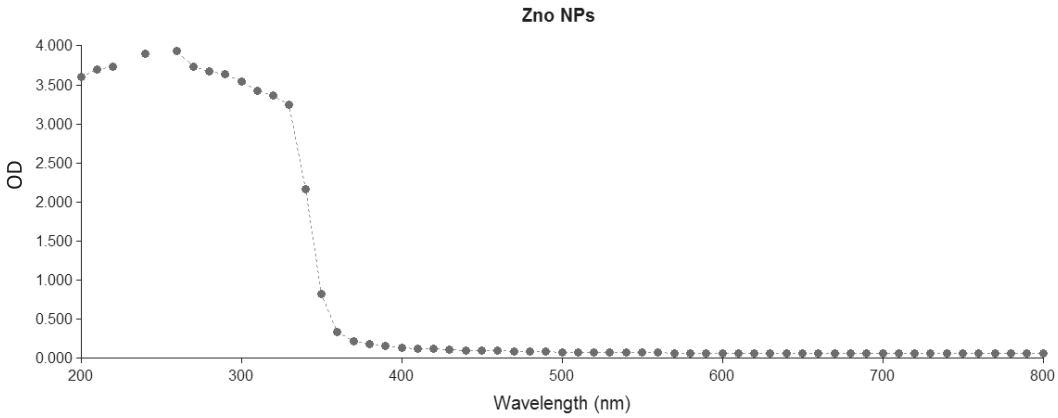

Fig. 1. UV-visible spectrum of zinc oxide nanoparticles

on the agar plate. All the experiments were replicated three times [12, 14].

Results

In the present study, the ZnO NPs were synthesized by chemical assay and planned for anti-bacterial activity against antibiotic resistance *Pseudomonas aeruginosa* isolated from a hospital infection unit. ZnO NPs in aqueous solution were proven via UV-visible spectrum. Absorption of ZnO NPs was done between 200 nm and 800 nm. Characteristic absorption of surface plasmon resonance band occurred at about 350 nm (Fig. 1) [15-16]. The size measured in DLS technique is bigger in comparison with macroscopic techniques. The mid-range hydrodynamic diameter of the ZnO NPs was 1.48 um [16-17]. Also, according to a previous study the Zeta potential of ZnO NPs was calculated at about 3.13 mV. On the other hand, inductively coupled plasma mass spectrometry (ICP-MS) proceedings shows that the concentration of ZnO NPs was around 55 ppm [18-20]. Fig. 2 shows the SEM image of ZnO NPs. The distribution of particle size was done with a 100 K magnification on a scale of 300 nm. The TEM images of ZnO NPs are shown in Fig. 3. The NPs were spherical in shape with a smooth surface morphology, as well as agglomerates. The TEM image also shows that the produced ZnO NPs is more or less uniform in size and shape[19]. The average size of ZnO NPs was estimated with (Digimizer software, v 4.1.1.0) less than 5 nm.

The biochemical tests of isolated *Pseudomonas aeruginosa* are presented in Table 3. Also, antibiotic resistance patterns of isolated *Pseudomonas aeruginosa* shows that it was sensitive to *Gentamicin*, *Imipenem*,

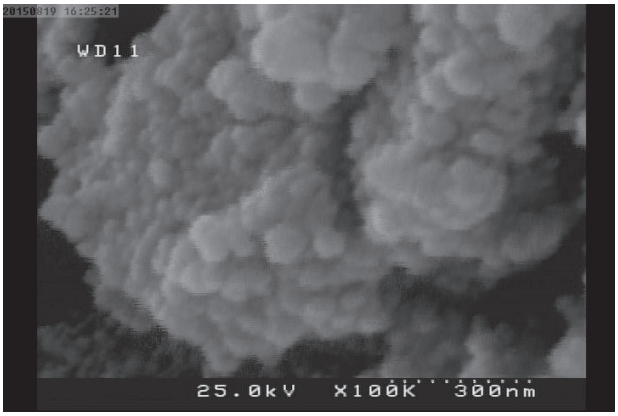

Fig. 2. SEM image of ZnO NPs 100 K magnification on a scale of 300 nm

Fig. 3. TEM images of ZnO NPs 160 K magnification at a scale of 100 nm

Table 3. Results of biochemical tests of *Pseudomonas aeruginosa.*

Gram stain	Motile	Oxidase	Lactose	TSI	SH_2	Catalase	Citrate	OF Aerobic	OF Anaerobic	β-hemolysis
Negative	Positive	Positive	Negative	ALK/ALK	Negative	Positive	Positive	Positive	Negative	Positive

Table 4. Determination of antibiotic resistance patterns of *Pseudomonas aeruginosa.*

Antimicrobial Agent	Symbol and count	Antibiotic resistance
Cefazolin	CEF	Resistance
Gentamycin	GM10	Sensitive
Amikacin	An	Sensitive
Cefepime	FEP30	Sensitive
Cefotaxime	CTX30	Sensitive
Ciprofloxacin	CP10	Sensitive
Trimethoprim sulfa methoxazole	TMP5	Resistance
Meropenem	MEN10	Sensitive
Ceftazidime	CAZ30	Sensitive
Nitrofurantoin	FM300	Sensitive
Piperacillin Tazobactam	PTZ100/10	Sensitive
Ampicillin	AM30	Resistance
Imipenem	IPM10	Sensitive
Colistine	CL10	Sensitive
Aztreonam	AZ	Sensitive
Ceftriaxone	CRO30	Sensitive
Cefixime	CFM5	Sensitive
Nitrofurantoin	FM300	Sensitive

Colistin, *Ciprofloxacin*, *Meropenem*, *Amikacin*, *Ceftazidime*, *Cefepime,* and *Cefotaxime*. Likewise, it was resistant to *Trimethoprim*, *Ampicillin*, and *Nitrofurantoin* (Table 4). Disc diffusion and cavity tests show that ZnO NPs have an antibacterial effect against the standard strain of *Pseudomonas aeruginosa* and isolated *Pseudomonas aeruginosa* from the infectious unit. According to the results, ZnO NPs could kill all antibiotic-resistant bacteria at a ratio of 1:16 (MBC = ~7.5 ppm). However, it was able to eliminate the standard strain of *Pseudomonas aeruginosa* at a ratio of 1:64 (MBC = ~0.937 ppm; Table 5). Consequently, however, colloidal nanoparticles could inhibit growth of *Pseudomonas aeruginosa* resistance and standard strain of *Pseudomonas aeruginosa* at ratios of 1:16 (MIC = ~3.75 ppm) and 1:128 (MIC = ~0.468 ppm), respectively. As observed, the standard strain of *Pseudomonas aeruginosa* shows the highest sensitivity to colloidal ZnO NPs (Table 5).

Discussion

One of the interesting characteristics of metal oxide NPs is their optical properties, which vary according to size and shape. Optical characteristics will vary with the size, shape, and spacing from each other and the refractive index of the surrounding NPs [21]. In this study, DLS presented the size and dispersion of ZnO NPs. The size measured in DLS technique is the hydrodynamic diameter of the theoretical sphere that diffuses with the same speed as the measured NPs. This size not only depends on the metallic core of the NPs, but it is also prejudiced with all substances adsorbed on the surface of the NPs and the thickness of the electrical double layer, moving along with the particle. Recently, Jafari and collegues synthesized colloidal Zno NPs using chemical analysis and measured its DLS, ICP-MS, SEM, and TEM images and UV-visible spectrum [22]. The characterizations of ZnO NPs were confirmed in accordance with a previous study [22]. TEM images of ZnO NPs showed that synthesis of NPs using chemical analysis could be present in very small sized NPs. Also, SEM images of ZnO NPs demonstrated agglomeration of particles in aquatic solution, just like the investigation of Jafari et al. [22].

Up to now, few reports have been obtainable on the anti-bacterial activity of ZnO metal oxide nanoparticles against antibioticresistant *Pseudomonas aeruginosa* [23-26]. According to Vijaya Chaudhari's research on antibiotic resistance patterns of *Pseudomonas aeruginosa* in a tertiary care hospital, the highest resistance depended on *ciprofloxacin* while the lowest was *Meropenem* [27]. In 2004 Gan and his colleagues showed that metal oxide NPs were capable of inhibiting or destroying many pathogenic bacteria [28]. Also, Guogang and

Table 5. Disk diffusion, Cavity, MIC, and MBC tests of ZnO NPs against *Pseudomonas aeruginosa.*

	Disc Diffusion	Cavity	MIC	MBC
Pseudomonas aeruginosa (ATCC 27853)	10 mm	8 mm	1:128 (~0.468 ppm)	1:64 (~0.937 ppm)
Pseudomonas aeruginosa (Resistance)	8 mm	5 mm	1:16 (~3.75 ppm)	1:8 (~7.5 ppm)

Jayesh believed that dispersed suspension of metal oxide NPs with ultrasonic waves will increase antibacterial properties [29-30]. Currently, Dortet et al. found that "carba-NP test-II" was settled to identify *carbapenemase* production in *Enterobacteriaceae* and *Pseudomonas* spp., and to differentiate between the diverse types of *carbapenemases*. This is based on detection of the acidification resulting from *imipenem* hydrolysis, coupled with *tazobactam* and EDTA as inhibitors. In fact, it is a sensitivity and specificity technique for detecting not only *carbapenemase* activity but also *carbapenemase* types in *Enterobacteriaceae* and *Pseudomonas aeruginosa* [31]. Reddy in 2007 reported that ZnO NPs are not toxic against eukaryotic cells [9]. Also, Ling Yang confirmed that photo catalytic nanoparticles such as ZnO NPs increases its oxidation and reduction abilities while suppressing bacterial growth [32].

In 2016 Jafari studied the MIC and MBC of ZnO NPs against clinical antibiotic resistance *Pseudomonas aeruginosa*. Results showed that ZnO NPs were able to inhibit *Pseudomonas aeruginosa* at 512 $\mu g.ml^{-1}$, whereas MBC was ≥8,192 $\mu g.ml^{-1}$[10]. Also, they found that the lowest MIC in *Pseudomonas aeruginosa* was observed AgZnO NPs with 256 $\mu g.ml^{-1}$, which has the highest inhibitory effect on *Pseudomonas aeruginosa*. In fact, the greatest resistance to zinc oxide NPs was seen in *Pseudomonas aeruginosa*.

Although the antibacterial mechanism of ZnO NPs is still unknown, the possibilities of membrane damage caused by direct or electrostatic interaction between ZnO and cell surfaces, cellular internalization of ZnO NPs, and the production of active oxygen species such as H_2O_2 in cells due to metal oxides have been suggested [33-34]. A few studies have suggested that the primary cause of the antibacterial function might be from the disruption of cell membrane activity [35]. However, Jafari confirmed that gram-negative antibiotic-resistant bacteria such as *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Escherichia coli* have low sensitivity to colloidal ZnO NPs in comparison of gram-positive ones [10]. As mentioned before, another possibility is the induction of intercellular reactive oxygen species, including hydrogen peroxide, a strong oxidizing agent harmful to bacterial cells [36-37]. It has also been informed that ZnO NPs can be activated by UV and visible light to generate highly reactive oxygen species such as OH^-, H_2O_2, and O_2^{-2}. The negatively charged hydroxyl radicals and super-oxides cannot penetrate the cell membrane and are likely to remain on the cell surface, whereas H_2O_2 can penetrate bacterial cells [26].

Conclusions

Zinc oxide nanoparticles could be used as a high potential agent for disinfection of infectious units of hospitals against nosocomial infection – especially with *Pseudomonas aeruginosa*. The bottom line is that photo-catalyst metal oxide nanoparticles' instance of zinc oxide colloidal nanoparticles not only can be self-cleaning disinfection, but also be able to control contamination in hospitals.

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