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Original Research

Inhibitory effect of zinc oxide nanoparticles on *pseudomonas aeruginosa* biofilm formation

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Abstract

Objective(s): Bacterial biofilm formation causes many persistent and chronic infections. The matrix protects biofilm bacteria from exposure to innate immune defenses and antibiotic treatments. The purpose of this study was to evaluate the biofilm formation of clinical isolates of *Pseudomonas aeruginosa* and the activity of zinc oxide nanoparticles (ZnO NPs) on biofilm.

Materials and Methods: After collecting bacteria from clinical samples of hospitalized patients, the ability of organisms were evaluated to create biofilm by tissue culture plate (TCP) assay. ZnO NPs were synthesized by sol gel method and the efficacy of different concentrations (50- 350 µg/ml) of ZnO NPs was assessed on biofilm formation and also elimination of pre-formed biofilm by using TCP method.

Results: The average diameter of synthesized ZnO NPs was 20 nm. The minimum inhibitory concentration of nanoparticles was 150- 158 µg/ml and the minimum bactericidal concentration was higher (325 µg/ml). All 15 clinical isolates of *P. aeruginosa* were able to produce biofilm. Treating the organisms with nanoparticles at concentrations of 350 µg/ml resulted in more than 94% inhibition in OD reduction%. Molecular analysis showed that the presence of mRNA *of pslA* gene after treating bacteria with ZnO NPs for 30 minutes.

Conclusion: The results showed that ZnO NPs can inhibit the establishment of *P. aeruginosa* biofilms and have less effective in removing pre-formed biofilm. However the tested nanoparticles exhibited anti-biofilm effect, but mRNA of *pslA* gene could be still detected in the medium by RT-PCR technique after 30 minutes treatment with ZnO.

Keywords: Biofilm, *Pseudomonas aeruginosa*, *pslA* gene, ZnO nanoparticles

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Introduction

Metal nanoparticles are known to have unique features such as surface plasmon absorption and enhanced catalytic activity due to their quantum size confinements and extremely large surface areas [1-3].

Zinc oxide nanoparticles (ZnO NPs) are reported to possess anti-microbial activity. These particles significantly can reduce the skin infection, bacterial load and inflammation in mice, and also improve infected skin architecture [4].

Many microorganisms in the natural environment are organized in biofilm structures [5]. Biofilms can be defined as multicellular communities of bacteria, immobilized by an extracellular polymeric matrix produced by the bacteria, which can be attached to various biotic and abiotic surfaces [6, 7]. This three-dimensional biofilm structure is made up in 85% by the extracellular matrix which comprises polysaccharides, proteins, enzymes, DNA, bacterial glycolipids, water, and in 15% by aggregates of microorganism cells [5].

The matrix protects biofilm bacteria from exposure to innate immune defenses and antibiotic treatments [8]. Biofilm forming can promote the spread of drug resistance markers and other virulence factors [9]. As a result, pathogen persistence in biofilm establishes chronic and hard to treat infection such as upper respiratory infections, cystic fibrosis by *Pseudomonas aeruginosa* [10], urinary tract infections [11], periodontitis [12], catheter-induced and other device-associated infections [13]. Especially in immunocompromised patients, the manifestation of infections by opportunistic biofilm-forming pathogens can be devastating, leading to severe symptoms and, in many instances, death [14].

P. aeruginosa is a Gram negative bacterium that can cause a wide range of severe opportunistic infections in patients with serious underlying medical conditions. These infections damage host tissues and often exhibit resistance to antibiotics leading to mortality. Treatment of persistent infections is additionally hampered by adaptive resistance, due to the growth state of the bacterium in the patient and the ability of organism to grow as a biofilm [15]. Due to the reduced susceptibility of biofilm forming microorganisms to antibiotics and some disinfectants [16], introducing new prevention and treatment procedures are necessary to reduce the risk of infection associated with these microorganisms. Therefore we tried to evaluate the anti-biofilm activity of synthesized ZnO NPS on biofilm formation *P. aeruginosa* via microbiology and molecular tests**.**

Materials and Methods

Bacteria

Fifteen clinical isolates and one reference strain of *P. aeruginosa* (American Type Culture Collection: 9027) were used in this study. Among eighteen bacteria isolated from hospitalized patients, fifteen isolates were able to form an effective biofilm that were selected for further experiments. Bacteria were identified by biochemical differentiation tests including growth on cetrimide agar, oxidase and catalase tests, motility, growth at 42°C, growth in oxidation fermentation (OF) medium, TSI agar and Simon's citrate [17]. All bacteria were maintained in brain heart infusion broth containing 15% glycerol at -75°C during the study period.

Synthesis of nanoparticles

ZnO NPs (purity over 99.7%) with an average size of ~20 nm were prepared by using sol-gel method in gelatin media and the samples were crystallized in single phase wurtzite structure. The diameter of ZnO NPs was measured using transmission electron microscope and particle size method.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Broth dilution method was used to determine the MIC. For this purpose, a

stock of suspension of ZnO NPs was prepared at a concentration of 1µg/µl (1 mg of NPs were mixed in 1 ml of double distilled water). Brain heart infusion (BHI) broth (Merck, Germany) was provided with twice concentration and media with different concentrations of NPs were prepared according to table 1. A tube without nanoparticles was used as positive control. At last, 10 µl of bacterial suspension with turbidity equivalent to 0.5 Mc-Farland was added to the tubes contained different concentration of nanoparticles (0 to 400 µg/ml). MIC was reported as the lowest concentration of the nanoparticles that inhibited visible bacterial growth.

The concentration of ZnO NPs that inhibited 50% and 90% of the isolates, was measured as MIC_{50} and MIC_{90}. The minimum bactericidal concentration (MBC) was established by the lack of growth after re-inoculation from ZnO NPs-treated media to agar medium without nanoparticles. All experiments were carried out three times.

Table 1. Different concentrations of nanoparticles prepared in BHI broth to determine the MIC.

Tube No.	1	2	3	4	5	6	7	8	9	10
BHI broth* (µl)	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000
ZnO NPs stock (µl)	200	250	300	350	400	450	500	550	600	650
DDW** (µl)	800	750	700	650	600	550	500	450	400	350
Final concentration (lµg/ml)	100	125	150	175	200	225	250	300	350	400

* Brain heart infusion broth with twice concentration
** Double distilled water

The ability of isolates for biofilm formation

Determination of biofilm formation was carried out for all isolates by tissue culture plate method as described by Christensen et al with suitable modification (18).

Anti-biofilm formation assay

Different concentrations (100, 200, 300, 400, 500, 600 and 700 mg/100 ml) of ZnO NPs were prepared by suspending the nanoparticles in double-distilled water. Individual wells of sterile, polystyrene, 96-well-flat bottom tissue culture plates (TCP) were filled with 180 µl of BHI broth and inoculated with 10 µl of overnight culture (OD_{620}=0.01).

Imme-diately after vigorous vortex mixing, 10 µl of ZnO NPs were added from the stocks to the wells, so that final concentrations were made between 50 and 350 µg/ml. Final volume in every well was 200 µl. The tissue culture plates were incubated for 24 h at 37ºC. After incubation, content of each well was gently removed. The wells were washed four times with phosphate buffered saline solution (pH = 7- 7.2) to remove free-floating planktonic bacteria.

Biofilms formed by bacteria were fixed with ethanol (95%) and stained with crystal violet (0.1%, w/v). Excess stain was rinsed off by several times washing with deionized water and plates were kept for drying. 0.225 ml of glacial acetic acid (33%) was added to the wells and after 15 minutes the optical densities (OD) of stained adherent bacteria were determined with a micro plate reader (model CS, Biotec) at 590 nm.These OD values were considered as an index of bacteria adhering to surface and forming biofilms. Experiments were performed in triplicate, the data was then averaged.

The efficacy of ZnO NPs on formed biofilm

To evaluate the effect of ZnO NPs on elimination of pre-formed biofilm,

individual wells of TCP were filled with 180 µl of BHI broth and inoculated with 10 µl of bacterial suspension (OD_{620} = 0.01). The tissue culture plates were incubated for 24 h at 37ºC. After incubation, 10 µl of ZnO NPs dilutions were added to each well. So that final concentration of ZnO in each well became 50 to 350 µg/ml. A well containing 0.2 ml of BHI broth inoculated with bacteria was considered as positive control. After 2 h, the content of the micro plate was gently removed and as described earlier, OD of stained adherent bacteria in wells were read at 590 nm.

RT-PCR

RNA extraction and cDNA synthesis were necessary to perform RT-PCR. For this purpose, pure bacteria were inoculated in two tubes containing 2 ml BHI broth. One of the tubes contained no nanoparticles (as positive control) and the other had ZnO NPs with concentration of twice as MIC. The ZnO concentration used herein was determined based on MIC and MBC results so that was less than MBC concentration (19). Tubes were incubated at 37°C, shaking 200 rpm for 30 minutes (19). Bacterial RNA was extracted using RNeasy Protect Bacteria Mini Kit (Qiagen, Germany) according to the procedure instructions and cDNA was synthesized using Quanti tect Reverse transcription kit (Qiagen, Germany) and specific primers of *pslA* gene according to the instructions of kit. In the next step, DNA was increased by polymerase chain reaction (PCR) using specific designed primer by AlleleID 6.0 software. Detection of *pslA* gene was conducted with a pair of primer pslA-F (5'-AGGAGGCGGTCAGCGAATACAG-3') and pslA-R (5'-GCGACGGCGTTCATC-AGTAGAC-3') which amplified a 234 bp fragment. PCR was performed in 50 µl mixture of 5 µl 10X buffer, 4 µl of 20 mM $MgCl_2$, 0.5 µl of 5 U/µl Taq DNA polymerase (Fermentas-Lituania), 1 µl of 10 mM of each deoxynucleaotide triphosphate, 1 µl of 10 µM of each primer and 1.5 µl of cDNA in a thermal cycler (Kyratec, Korea).

The amplification was performed according to the conditions which are shown in Table 1.

The PCR products were electrophoresed on 1% gel and stained with ethidium bromide. A 100 bp ladder standard (Fermentas, Lithuania) was used as molecular weight ladder.

Table 2. PCR conditions for amplifying *pslA* gene by polymerase chain reaction.

Stage	Temperature (˚C)	Time	No. cycle
Hot start	94	5 min	1
Denaturation	94	45 sec	
Annealing	52	45 sec	30
Extension	72	90 sec	
Final extension	72	5 min	1

Statistical analysis

Statistical analysis was performed using SPSS (version 20).

One-way analysis of variance (ANOVA) was used for analyzing differences between OD values.

A value of $p < 0.01$ was considered statistically significant.

Results

The results of various biochemical tests revealed that all the isolated bacteria were *P. aeruginosa*. Scanning electron microscopy (SEM) and particle size analysis for ZnO NPs showed that spherical shape and a mean size of about 20 nm. Minimum inhibitory and minimum bactericidal conc-

entrations of ZnO NPs were determined 158 and 325 µg/ml for 15 clinical isolates of *P. aeruginosa* and 150 and 325 µg/ml for the reference strain, respectively. MIC_{50} and MIC_{90} of isolates were 150 µg/ml and 175 µg/ml. The MBC of nanoparticles was higher than the MIC that indicates ZnO NPs can kill bacteria at higher concentrations.

The results showed that fifteen isolates of *P. aeruginosa* bacteria were able to produce biofilm by using TCP test. ZnO NPs showed anti-biofilm activity on the all bacteria and the anti-biofilm activity increased with the rising concentration of nanoparticles.

Treating these organisms with ZnO NPS at concentrations of 250 and 350 µg/ml resulted in more than 75% and 94% OD reduction%, respectively (Figure 1).

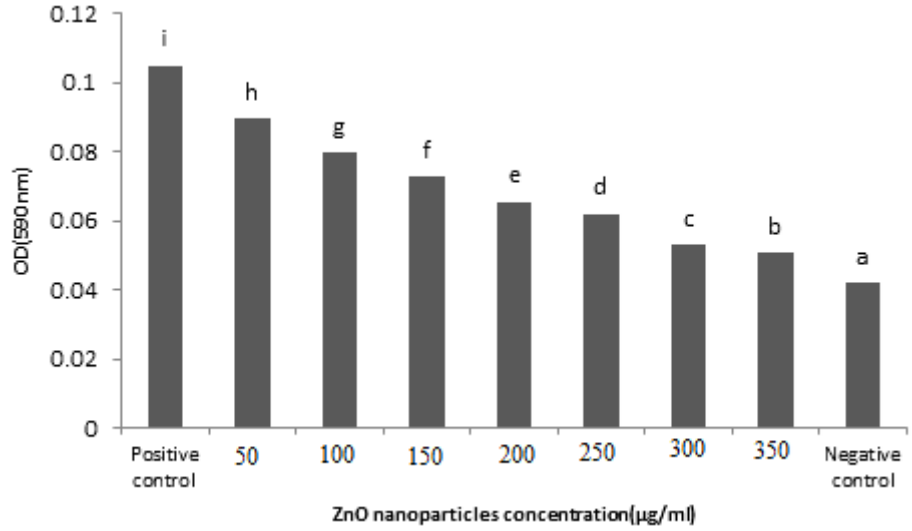

Figure 1. Anti-biofilm effect of different concentrations of ZNO NPs on biofilm formation of *P. aeruginosa* tested bacteria with the positive and negative control according to OD values after 24 h of incubation (Letters above the columns indicate significant differences among isolates and at least one letter in common shows the lack of significance).

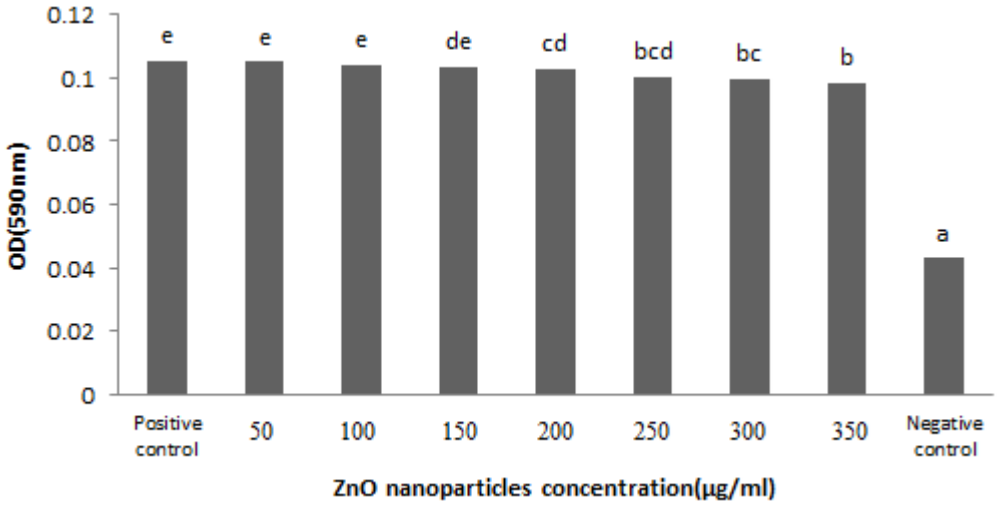

Figure 2. Anti-biofilm activity of different concentrations of ZnO NPs on pre-formed biofilm by *P. aeruginosa* bacteria according to OD values after 24 h of incubation (Letters above the columns indicate significant differences among isolates and at least one letter in common shows the lack of significance)**.**

Figure 2 displays the effect of ZnO NPs on elimination of pre- formed biofilm. As the figure indicates, ZnO NPs have fewer potential to remove pre-formed biofilm at

the same concentrations. According to the statistical data, the effect of ZnO NPs on biofilm formation was significantly more than the effect of ZnO NPs on pre-formed biofilm at all concentrations ($p < 0.01$).Molecular analysis showed that the concentration of the cDNA of *pslA* gene. So mRNA of the gene has been existed among extracted mRNAs from treated *P. aeruginosa* bacteria with ZnO NPs after 30 minutes. Figure 3 shows the PCR products.

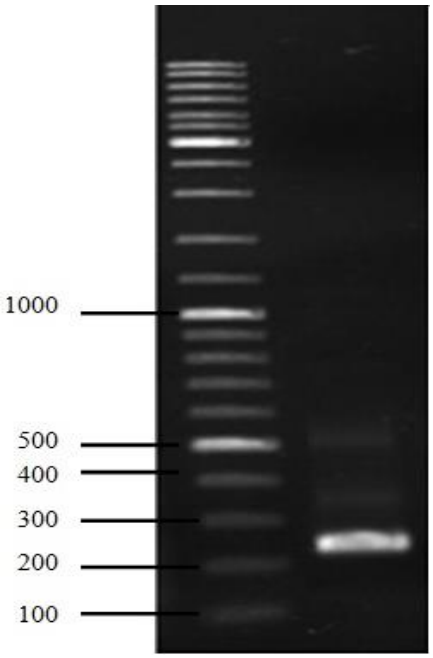

Figure 3. Gel electrophoresis of PCR product of sample treated with ZnO NPS for 30 minutes.

Discussion

P. aeruginosa is an important opportunistic bacterial pathogen which can colonize the surfaces and form biofilm. Clinically, biofilms are responsible for many problems related to persistent and chronic infections arising from reduction of immune response and antibacterial efficiency [20].

Research to find new compounds that have anti-biofilm effect may provide strategies for the control of infections and problems related to biofilm formation.

In our study we have studied about biofilm formation ability of *P. aeruginosa* bacteria isolated from hospitalized patients and the activity of ZnO NPs on the biofilm formation by *P. aeruginosa*.

The all tested bacteria in our study were able to form biofilm. ZnO NPs were found to effectively inhibit the growth of *P. aeruginosa* and restrict the biofilm formation. Anti-biofilm effect of nanoparticles gradually increased from concentration 50 to 350 µg/ ml and at concentration of 350 µg/ml biofilm formation was almost entirely inhibited. Also ZnO NPs at concentration of 100 to 350 µg/ml reduced pre-formed biofilm of *P. aeruginosa*. Overall, our results suggest that ZnO NPs could inhibit the establishment and development of biofilm, but its effect is less to remove pre-formed biofilm.

Some previous studies have showed the antibacterial activity of ZnO NPs. Saadat et al has studied the effect of ZnO NPs with the size of 30-90 nm against *P. aeruginosa* and reported the concentration of 300 µg/ml for the MIC, as the present study [21]. In another study which the antibacterial of ZnO NPs with the average of 20 nm was investigated against some bacteria, the MIC for *P. aeruginosa* was 156.25 µg/ml [22]. Similar results were found by Lee et al showed that ZnO NPs

inhibit *P. aeruginosa* biofilm formation and virulence factor production [23]. Applerot et al showed that ZnO nanoparticle-coated surfaces inhibit bacterial biofilm formation and increase antibiotic susceptibility. They showed hydroxyl radicals, originating from the coated surface had a main role in anti-biofilm activity [24]. The results of a study conducted by Khameneh et al confirmed a high efficacy of nanosilver particles (SNPs) on biofilm eradication by pour plate assay. The results indicated that higher concentrations of SNPs and longer incubation times were more effective in enhancement of anti-biofilm activity of SNPs. Although, they believed that crystal violet results were insufficient for evaluating the anti-biofilm activity of SNPs. SNPs were effective against cell viability and they were ineffective against biomass [25].

Antibacterial mechanistic studies by Pati et al showed that ZnO NPs can disrupt bacterial cell membrane integrity, reduce cell surface hydrophobicity and down-regulate the transcription of oxidative stress-resistance genes in bacteria [4].

Finally, the mRNA of *pslA* gene, a gene involved in biofilm formation, existed among extracted mRNAs from treated *P. aeruginosa* bacteria with ZnO NPs after 30 minutes. It is likely that the expression level of the gene has become low, but still pre- generated mRNAs of this gene were detectable in bacteria. Our experience was at first attempt to detect mRNAs of the gene associated with biofilm formation by RT-PCR. However the results were an onset to carry out further studies. Our recommendation is to investigate the mRNAs after longer than 30 minutes. Also more experiences are need for studies by Real-time PCR.

Conclusion

Overall, our findings showed that ZnO NPs could inhibit the biofilm formation of *P. aeruginosa* at examined concentrations in this study however these particles could not eliminate the pre-formed biofilm at the same concentrations. On the other hand the mRNAs of *pslA* gene were detectable by RT-PCR after 30 minutes of treatment with nanoparticles and we recommend detection of these mRNAs after longer time exposure with ZnO NPs.

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