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Original paper

Effects of silver nanoparticles on nosocomial Pseudomonas aeruginosa strains – an alternative approach for antimicrobial therapy

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Abstract Nanotechnology is emerging as a new interdisciplinary field combining microbiology, chemistry, physics, and material science. Nanaoparticles (NPs) act as excellent antimicrobial agents with potential clinical applications. Sliver NPs (AgNPs) have been successfully used in a wide range of applications including wound dressing, protective clothing, antibacterial surfaces, food preservation, and cosmetics as biocide and disinfecting agents. The aim of the study was the evaluation of the antimicrobial activities of AgNPs against *Pseudomonas (P.) aeruginosa* clinical isolates, identification of the morphological changes of *P. aeruginosa* cells after treatment with AgNPs by ecanning electron microscopy (SEM), and the impact of the AgNPs on the viability and virulence (azurin gene expression) of *P. aeruginosa* isolates.

Keywords Silver nanoparticles, antimicrobial activity, *Pseudomonas aeruginosa,* Azurin gene

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Introduction

The term nanotechnology is widely employed in various fields like engineering, medicine, biology, and electronics. Nanoparticles (NPs) are fine structures with size composition within the range of nanometer scale (Godasu, [1]). Nanotechnology plays a central role in the recent technological advances in the areas of the disease diagnosis, drug design and delivery. The nano technological applications for disease treatment, diagnosis, monitoring, and for the control of biological systems are referred to as nanomedicine (Moghimi & al., [2]; Vamanu & al., [3]). In nanotechnology, NPs provide several solutions to the technological and climate challenges in the areas of solar energy transformation, catalysis and medicine. Silver NPs (AgNPs) harbor many advantages due to their bacteriostatic and bactericidal activity against many pathogens. Currently, AgNPs are typically applied to antibacterial/antifungal products in biotechnology and are also used in bioengineering, textile engineering, drinking water treatment, silver-based consumer products or services. Their applications were recently extended to household products and solutions including electronic devices such as vacuum cleaners, the air cleaners, purifiers, room or space sanitizers and also water purification units. Due to their potent anti-bacterial activities, AgNPs are employed in numerous textiles but as well as coatings on certain implants. Further, AgNPs are used for the treatment of injuries and also burns or contraceptives (Wijnhoven & al., [4]). Their physical properties, particularly their size, shape andsurface area are particularly important to identify their mechanisms of interaction. It is known that small materials have a larger surface area and therefore, get a greater probability to be toxic (Johnston & al., [5]; Rycenga & al., [6]). The interaction between nanoparticles and bacterial surfaces cause changes in the membrane properties. The small size and large surface area of AgNPs lead to adhesion on the bacterial surface and to a better antimicrobial activity (Wei & al., [7]; (Antony & al., [8]; Reidy & al., [9]).

Pseudomonas (P.) aeruginosa is a Gram negative nonfermenting opportunistic bacterium found in water, moist environments (Staradumskyt & al. [10])]) and hospital settings, exhibiting resistance to numerous classes of antibiotics (multidrug resistance) like β-lactams (penicillins, carbapenems), aminoglicosides, quinolones and ciprofloxacin (Merezeanu & al., [11]) (Moore & Flaws, [12]; Lyczak & al., [13]). Azurin is a small, blue, copper-containing protein involved in electron transport during respiration of several microorganisms. Azurin is a 128-amino-acid periplasmic protein produced by the *P. aeruginosa* with antimicrobial activity (Amdursky & al., [14]).

The aim of the study was the characterization of AgNPs in terms of their effectiveness on *P. aeruginosa* isolates and also the investigation of the antimicrobial mechanism action of AgNPs.

Material and methods

1. Bacterial isolates

Forty *P. aeruginosa* strains were isolated from patients with different infections (data not shown). The strain isolation was made on MacConkey Agar, CLED Agar and Cetrimide Agar medium. The identification of the isolates was performed using the VITEK II automatic analyzer (BioMerieux). The *P. aeruginosa* ATCC 27853 were used as reference strain(Atlas & al.[15])

2. Characterizations of AgNPs

2.1. Scanning microscopy technique (SEM)

SEM was used to identify the morphological changes of AgNPs. AgNPs were centrifuged at 6000 r.p.m, for 5 minutes, washed 3 times with PBS (50mM, pH=7.3). From that point forward, a thin smear of the suspensions was spread on a silicon wafer slide and dried at room temperature; subsequently the silicon wafer slides were fixed with 1 ml of fixing buffer (sodium cacodylate with 2.5 wt% glutaraldehyde and 0.1M sucrose) and incubated at 37ºC for 1.5 h. The fixed samples on SEM stubs were sputtered with a 20 nm gold layer (for 5 min) to permit SEM perception (Kockro & al., [16]).

2.2. Atomic Force Microscope (AFM)

AgNPs were air-dried and their size, morphology and Ag clustering were visualized by AFM (Model-Nanosurf easyscan 2 AFM, Switzerland). AFM images were taken with silicon cantilevers with a force constant 0.02–0.77 N/m, tip height 10–15 nm (Logeswari & al., [17]). De-ionized water was used as blank.

2.3. The AgNPs activity on *P. aeruginosa*

2.3.1. Antimicrobial activity of AgNPs

Antimicrobial activity of AgNPs against *P. aeruginosa* strains was analyzed on Mueller Hinton (MH) agar plates using a disk diffusion method. Fresh bacterial strains were adjusted to a density corresponding to 0.5 McFarland (Saviuc & al., [18]). For this purpose, 5 µL from a stock solution of the tested product, containing different concentrations of NPs (25, 50, 75, and 100 µg/ml), as well as the control used at the same concentration, were distributed on a blank paper disk. Dimethyl sulfoxide (DMSO) was used as solvent was and comparatively tested for its potential antimicrobial activity. All the experiments were performed in triplicate. The plates were incubated for 24h at 37°C. The antimicrobial activity was quantified by measuring the bacterial growth inhibition zones around the spots.

The antimicrobial activity of AgNPs was evaluated also by fluorescent microscopy. The bacterial strains were cultured at 37°C for 24h on MH agar plates. One bacterial

colony was used to inoculate 10 ml of nutrient broth medium then the tubes were incubated for 24h at 37°C. After incubation 1ml of *P. aeruginosa* was added to 50 ml of nutrient broth culture media with Silver nanoparticles at concentration 50µg/ml then incubated at 37°C for another 24hr with shaking. The bacterial growth was estimated by reading optical density at 600nm using spectrophotometer.

In order to detect the impact of the AgNPs on the viability of *P. aeruginosa* cells were stained with Acridine orange / Ethidium Bromide (AO/EtBr). For this, 20 µl of bacterial suspension and 5 µl of AO/EtBr were incubated for 10 minutes and then the sample were centrifuged 15 min at 1500 r.p.m and washed with PBS three times. After that 5 µl of each sample were plated on a slide and were analyzed by fluorescence microscopy (Jabir & et al., [19]).

2.3.2. Effect of AgNPs on expression of azurin gene

QuantitativeReal-Time PCR (RT-PCR) wasused to evaluate the effect of AgNPs on *P. aeruginosa* azurin gene expression. The primers used for amplification of azurin gene were F:5'-ATGCTACGTAAACTCGCTG-3', R:5'-CACTTCAGGGTCAGGGTG-3'. RNA was extracted from *P. aeruginosa* using a commercial purification system (Abcam ExCellenCT Lysis Kit). The concentration of RNA had been between (40 and 50) ng/ µl. The 16s rRNA gene was used as housekeeping gene. Detection of gene expression have been performed using Abm's One-Step BrightGreen qRT-PCR Kit (Dumas & al., [20]).

2.3.3. Statistical analysis

The obtained data were statically analyzed using unpaired T-test with GraphPad Prism 6. The values were presented as the Mean ± S.E of the three replicate of each experiments.

Results and Conclusion

1. Characterization of AgNPs by SEM

The SEM images revealed that the investigated nanoparticles had spherical shape and the average size diameter of AgNPs ranged between 20-30 nm. AgNPs have uniform distribution (fig. 1A).

2. Characterization of AgNPs by AFM

AFM showed the topo-graphic image of well-dispersed AgNPs (fig. 1B) and revealed the formation of spherical AgNPs. The AFM image showed the Ag nanoparticles as crystals on a nanometer scale.

3. Morphological analysis

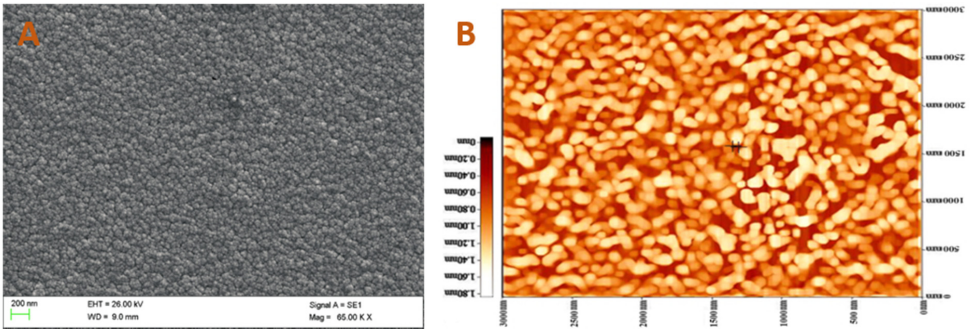

Figure 1. Morphological analysis (A-SEM, B-AFM) for AgNPs

Antimicrobial activity of AgNPs

Cell impermeability is particularly important for the antimicrobial resistance mechanisms in bacteria. The cell wall structure of Gram negative bacteria, and particularly the presence of an outer membrane, is often responsible for the impermeability of antimicrobial agents, (Escárcega-González & al., [21]). Consequently, the uSE of AgNPs should increases drug susceptibility of bacterial strains by inducing changes in the permeability of the cell membrane (Moore & Flaws, [12]; Kim & al., [22]).

The results concerning the antimicrobial activity of AgNPs against *P. aeruginosa* isolates are shown in fig. 2. The susceptibility of the *P. aeruginosa* increases with concentration of AgNPs. At 25 µg/ml concentration AgNPs produces an inhibition area with 13 mm in diameter while at 50 µg/ml the inhibition zone was 20 mm. The largest inhibition zone was 32 mm at 100 µg/ml (fig. 2).

Based on obtained results we can confirm the role of AgNPs in inhibiting the growth of *P. aeruginosa* strains andwe can suggest that AgNPs can represent a new generation of antimicrobial compounds which could be effective against multidrug resistant microorganisms.

4. Bacterial morphology after exposure to AgNPs

Interaction of bacterial cell with AgNPs can lead to cell membrane damage (Ivask & al., [23]). SEM analysis revealed the differences between the bacterial cells treated with AgNPs and non-treated bacterial cells. The bacterial cells that have not been exposed to AgNPs were bacillus-shaped, with an intact cell surface, while the bacterial cells treated with AgNPs at concentration 50µg/ml underwent considerablestructural changes (fig. 3).

Previous studies have shown that expousure of bacterial strains to AgNPs can produce structural changes in outer cell membrane leading to cell death (Sondi & Salopek-Sondi, [24]; Ramalingam & al., [25]). AgNPs cause cells damage by their ability to bind cell surface proteins and lipopolysaccharides. The disorganization of cell membrane leads to an increased osmotic imbalance produced by leakage outside the cell (Cohen, [26]).

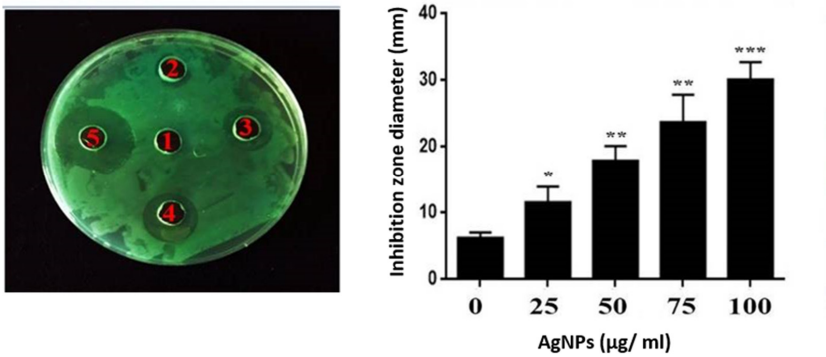

Figure 2. Antimicrobial activity of AgNPs against *P. aeruginosa*. 0- negative control, 1- AgNPs concentration 25µg/ml, 2- AgNPs concentration 50µg/ml, 3- AgNPs concentration 75µg/ ml, 4- AgNPs concentration 100µg/ml. The values are shown as the mean ± SD *p<0.05, **p<0.01, ***p<0.001.

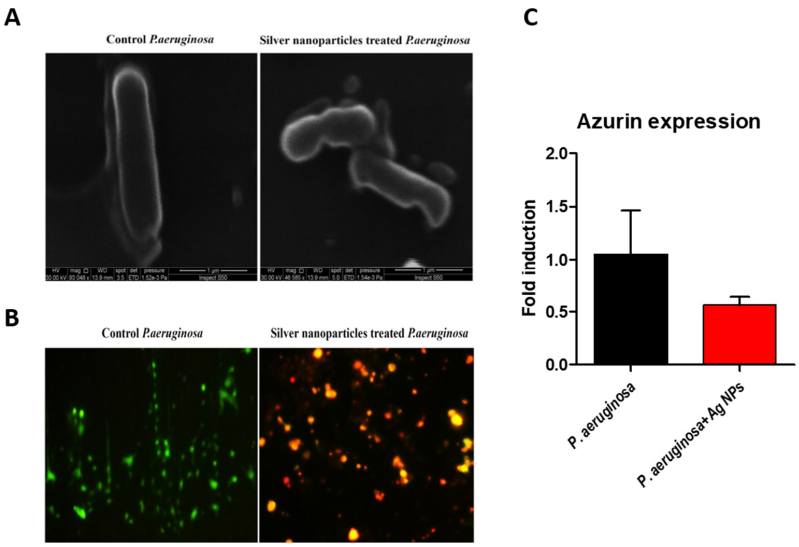

Figure. 3. The effect of AgNPs on *P. aeruginosa* morphology, viability and virulence. **A**. Visualization of *P. aeruginosa* treated with Silver nanoparticles using SEM, treated bacterial cells showing membrane damage. **B.** Fluorescence microscopic images of the green / red fluorescence stained *P. aeruginosa* in the absence / presence of AgNPs**. C.** Influence of AgNPs on azurin gene expression. The value is shown as the mean±SD.

5. AO/EtBr dual staining

The impact of tested AgNPs on the viability of *P. aeruginosa* isolates was investigated using fluorescent microscopy after AO/EtBr staining. After interaction with AgNPs, most bacterial cells lose their membrane integrity and are stained with ethidium bromide (shown in red, Figure 4).

DNA damages caused by NPs may be occur by several distinct mechanisms. NPs cause direct chemical damage to DNA, by binding and inducing double-strand breaks (Singh & al., [27]). There are studies reporting that some NPs interact with proteins which inhibit the ligase domain of topoisomerase enzymes, leaving the nuclease domains intact and thereby permitting the enzyme to cut DNA without re-ligation (Pizarro-Cerdá & Cossart, [28]). A commonly observed example of this is the production of reactive oxygen species (ROS) in response to manufacturing compounds and the incorporation of oxidized guanine residues into the genome (Vizcarra & al., [29]). The results from this study suggest that the effect on membrane proteins and the oxidative stress induced by AgNPs are the main mechanism responsible for the antimicrobial activity (Zhang & al., [30]). This study proposes possible mechanisms underlying AgNPs induced cytotoxicity for the bacterial cells. First, AgNPs induce oxidative stress, leading to cell membrane instability and increased membrane permeability by incorporation of AgNPs and forming permeable pits on the membrane, which leads to an osmotic collapse in the cells and a release of the intracellular materials.

6. Effect of AgNPs on expression of azurin gene

The influence of AgNPs on azurin gene expresion was evaluated through RT-PCR, using16s rRNA gene as a housekeping gene. As shown in fig. 5, the presence of AgNPs induce downregulation of azurin gene in *P. aeruginosa* strain comparing with non-treated cells (mean±SD 0.9800±0.09074) and treated (mean±SD 0.4100±0.04583).

Previous studies have indicated that azurin produced by *P. aeruginosa* strains function as an electron donor of the nitrite oxidase. A location of this enzyme on the periplasmic side of the plasma membrane or in the outer membrane would produce the requirement for the azurin to be present in the periplasmic space (Canters, [31]). Azurin is a redox protein initially believed only to serve as an electron donor to nitrite reductase during anaerobic respiration (Cutruzzolà & al., [32]). However, the periplasmic azurin of *P. aeruginosa* is not essential for denitrification, but is involved in protection from oxidative stress (Wu & al., [33]; Tomkinson, [34]). There are some studies which confirm that azurin represents a virulence factor of *P. aeruginosa* strains due to their cytotoxicity against phagocytic cells (Goto & al., [35]; Zaborina & al., [[36]).

The results obtained within this study highlight that AgNPs are remarkably well effective in inhibiting growth of bacterial isolates. These results confirmed that the bactericidal effect of AgNPs can be translated into important therapeutic and clinical options in the future, especially considering the shortage of new antimicrobials agents against the emerging multidrug resistant microorganisms, therefore AgNPs may act as a powerful tool against multidrug-resistant bacteria. AgNPs were also associated with attenuation of the virulence by inducing a decrease in expression of the gene coding for azurin in the *P. aeruginosa* strains.

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