



Evaluation of Anti biofilm and Antibiotic Potentiation Activities of Silver Nanoparticles against Some Nosocomial Pathogens

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Abstract

Nowadays silver nanoparticles (AgNPs) are used as antimicrobial due to its well known physical, chemical, and biological properties. A large collection of bacterial cells adhering to a surface is called bacterial biofilm. Exposure to silver nano particles (AgNPs) may prevent colonization of new bacteria onto the biofilm. In the present work, we have investigated whether the biofilm formation of some hospital isolates of pathogenic bacteria could be influenced by AgNPs. Also we examined the susceptibility of the isolates to some antibiotics in combination with AgNPs. Standard strains of *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Enterococcus faecalis* and *Staphylococcus aureus*, in addition to nine hospital isolates for each species were used. Tube double serial dilution method for determination of MIC of AgNPs against isolates was performed. Biofilm formation was evaluated by end smooth 96 cells micro titer plates. A disc diffusion method was used to assay the various antibiotics and combinations for bactericidal activity against the isolates. Mean MICs of AgNPs for *Ps. aeruginosa*, *A. baumannii*, *E. faecalis* and *S. aureus* were 3.125, 6.25, 6.25 and 12.5 ug/mL, respectively. AgNPs exhibited more than % 90 inhibitory effect on biofilm formation of the examined species. The results indicated that AgNPs have strong anti bacterial and anti biofilm activity against the examined pathogens. Synergistic effects of AgNPs in combination with gentamicin, kanamycine, cephalosporin and penicillin were observed in different cases. *Ps. aeruginosa* and *S. aureus* showed more sensitivity (increase in fold) to examined antibiotics plus AgNPs compared to *A. baumannii* and *E. faecalis*. Our results demonstrated that AgNPs showing promising anti biofilm activity on hospital isolates of *Ps. aeruginosa*, *A. baumannii*, *E. faecalis* and *S. aureus*. The study also demonstrated a possible combination of examined antibiotics with AgNPs which concluded as synergism.

Key words: Silver nano particles, Biofilm, Antibiotics, Pseudomonas, Acinetobacter, Enterococcus, Staphylococcus

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1. Introduction

Bacterial biofilm is a large collection of bacterial cells live as communities adhered to a solid surface. Biofilms estimated to be associated with 65% of nosocomial infections, [1] and are considered as the major problem in medication because they protect pathogenic bacteria against toxic materials such as antibiotics. Biofilms are supposed to be one of the main causes leading to chronic infections [2].

Increasing reports regarding emerging new resistant bacterial strains to antimicrobials has claimed for the application of novel bactericides to use in combination with antibiotics for more decisive treatments when required. The anti microbial activities of silver nano particles (AgNPs) has lead to a new generation of products and medicines that reduce bacterial growth [3].

Exposure to AgNPs may prevent colonization of new bacteria onto the biofilm and decrease the development and succession of the biofilm [4]. The reports showed the efficacy of the nano particles in reducing biofilm formation, disrupting biofilm structure, and retaining the anti bacterial property even after aging [5].

The mode of toxic action of AgNPs is not yet fully defined but both the release of silver ions and the nano particles (NPs) characteristics may have roles for its anti bacterial properties [6]. Besides the intrinsic anti bacterial effects of NPs, it is suggested that NPs enhance antibiotic effectiveness when mixed together to overcome bacterial infections.

The antibiotic resistance organisms, demonstrate more susceptibilities to antibiotics in combination with AgNPs [6]. In the present work, we have investigated the effects of AgNPs at minimum inhibitory concentration (MIC) and sub minimal inhibitory concentrations (sub MICs) on biofilm formation of some nosocomial pathogens. Also we examined the susceptibilities of the hospital isolates to AgNPs and antibiotic combinations.

2. Materials and Methods

2.1. Bacterial Isolates

The studied microorganisms included the reference strains of *Pseudomonas aeruginosa* ATCC 9027 and *Acinetobacter baumannii*, NCTC 13305 kindly delivered by Dr B. Zamanzad and Dr A. Gholipour (Dept. of microbiology, Shahrekord Medical school), *E. Faecalis*, RTCC 1923, and *S. aureus*, RTCC 2465, collected from our lab. bacterial collection. In parallel to each standard strain nine hospital isolates for each species (other than *S. aureus*) collected from Shahrekord and Esfahan hospitals were used. Nine clinical isolates of *S. aureus* isolated from human skin

wound infections in our earlier work [7], also added to our examined isolates. Early morphological and biochemical tests were done to confirm the genera and species of the received isolates. The methods for isolation and identification of all isolates were based on James et al. guidelines [8].

2.2. AgNPs and MIC Determination

Colloidal AgNPs with particle size ranges from 10- 20 nano meter (Sigma, CAS Number 7440-22-4) prepared from Kian Eksir Co. which imported AgNPs as powder and prepared its colloidal form. A dilution of 100 ug/mL. in Nutrient broth (Nb) prepared from the stock solution (1000 ug/mL.) after sonication. Nb was used for all the anti bacterial assays. For determination of AgNPs MIC against examined isolates, Tube double serial dilution method performed, according to guidelines of the Clinical and Laboratory Standards Institute [9]. The bacterial cultures (in tryptic soy broth, TSB, tubes) were incubated aerobically at 37 °C for 18- 24 h. The turbidity of the cultures adjusted to 0.5 McFarland (1.5×10^8 CFU/ml) and then diluted in saline solution so that to obtain an inoculum of 5×10^6 CFU/ tube. Positive and negative controls were also set up.

The inoculated tubes were aerobically incubated in shaking for about 18 h at 37 °C. The lowest concentration that inhibits visible growth after incubation was defined as MIC which was confirmed in next step.

2.3. Anti Biofilm Assays

Biofilm formation was evaluated by end smooth 96 cells micro plates as explained by Tendolkar et al. [10]. Briefly, isolates were grown at 37°C in TSB (Merck, Darmstadt, Germany). The bacterial cells were then pelleted at $6,000 \times g$ for 10 minutes, and the cell pellet was resuspended in 5mL of fresh medium. The optical densities (ODs) of the bacterial suspensions were measured by spectrophotometer (Jenway, OSA, UK) and normalized to an absorbance of 1.00 at 595 nm. The cultures were diluted 1:40 in fresh TSB and 200 µL of cells were dispensed into 12 wells in a single row of a sterile 96 well flat bottom polystyrene micro titer plate.

After incubation at 37°C for 24 hours, the planktonic cells were aspirated and the wells were washed three times with sterile phosphate buffered saline (PBS). The plates were inverted and allowed to dry for one hour at room temperature. For biofilms quantification, 200 µL of 0.2% aqueous crystal violet solution was added to each well, and the plates were allowed to stand for 15 minutes. The wells were subsequently washed three times with sterile PBS to wash off the excess crystal violet. Crystal violet bound to the biofilms was extracted with 200 µL of an 80:20 (vol/vol) mixture of ethyl alcohol and acetone, and the absorbance of the extracted crystal violet was measured at 595 nm.

For assaying AgNPs effects on biofilm formation of the isolates, each well was loaded with different concentrations of AgNPs (1.6 – 25 µg /ml). The plates were incubated in

shaking at 37°C for 24 h. The Control wells were also considered. The Growth was assayed using a microtiter enzyme linked immunosorbent assay (ELISA) reader (Stat Fax 4200) by monitoring absorbance at 600 nm. Further process was as above. All biofilm and anti biofilm assays were performed in triplicate.

Interpretation of biofilm production was done according to the criteria described by Stepanovic et al. [11]. Based on these criteria OD_c (optical density cut-off value) is defined as: average OD of negative control + 3 × SD (standard deviation) of negative control, and the biofilms producers are categorized as: no biofilm producer \leq OD_c, weak biofilm producer $OD_c < \sim \leq 2 \times OD_c$, moderate biofilm producer $2 \times OD_c < \sim \leq 4 \times OD_c$ and strong biofilm producer $> 4 \times OD_c$. Where "~" stands for the average of sample ODs. All bacterial isolates were examined for biofilm formation and nine isolates in each species that were strong or medium biofilm producers were selected to determine the MIC of AgNPs on biofilm formation. Percentage of biofilm inhibition calculated as explained by Guo-Xian et al., using the equation $(1 - A_{595} \text{ of the test} / A_{595} \text{ of non-treated control}) \times 100$ [12].

2.4. Assays for AgNPs and Antibiotics Combined Effects

Antibiotics were selected from three groups having different modes of action, including tetracyclin (tetracyclines), gentamicin, streptomycin, kanamycine (aminoglycoside), cephalosporin and penicillin (beta lactams). A

disc diffusion method was used to assay the various antibiotics for bactericidal activity against the isolates on Muller Hinton agar (MHA) plates. The standard antibiotic discs were purchased from Techno biolab Co.

To determine their combined effects, each antibiotic disc was impregnated with 20 ul solution (1 MIC concentration for each isolate) of AgNPs. Similar experiments were carried out with antibiotic discs alone. A single colony of each strain was grown overnight in TSB medium at 37 °C. The inocula were then applied to the plates along with the standard and prepared discs. After incubation at 37°C for 24 h, the zone of inhibition was measured. The assays were performed in triplicate.

The increase in fold area was assessed by calculating the mean surface area of the inhibition zone of each tested antibiotic. The fold increase area of different antibiotics for examined isolates was calculated by the equation $b^2 - a^2 / a^2$, where a and b are zones of inhibitions for antibiotic (a) and (b) antibiotic + Ag-NPs [6].

3. Results and Discussion

Mean MICs of AgNPs for *Ps. aeruginosa*, *A. baumannii*, *E. faecalis* and *S. aureus* were 3.1, 12.5, 3.1, and 6.25 ug/mL, respectively. All strains selected based on strong to medium biofilm formation. Percentages of biofilm formation inhibition of the 40 isolates in the presence of different concentrations of AgNPs are presented in table 1.

AgNPs showed higher antibacterial activities against *Ps. aeruginosa* and *E.*

faecalis (3.125 ug/mL.) compared to other tested isolates. Least activity was recorded against *A. baumannii* (12.5 ug/mL.). In the present work, using the disc diffusion method, the combined effects of AgNPs and antibiotics (gentamicin, tetracycline, streptomycin, cephalosporin, kanamycine and penicillin) was studied against 40 hospital pathogenic isolates (*Ps. aeruginosa*, *A. baumannii*, *E. faecalis* and *S. aureus*) and compared with antibiotics alone. The diameters of inhibition zones (in mm) around the different discs with and without AgNPs against isolates are shown in Table 2.

Ps. aeruginosa and *S. aureus* showed more sensitivity (increase in fold) to combined antibiotics plus AgNPs compared to other strains. For *A. baumannii* only tetracyclin and gentamicin and for *E. faecalis* only tetracyclin plus AgNPs showed fold increasing. Maximum increase in fold area reported for penicillin, cephalosprin and kanamycine, (16, 8 and 2.36 ug/mL. respectively) against *Ps. aeruginosa*.

The mean MICs of AgNPs recorded here for examined isolates are comparable with other reports regarding examined species [13]. Thus, we can conclude that the AgNPs

inhibited growth of the examined isolates although for *A. baumannii* our data don't match with this conclusion (table 1).

The major findings can be summarized as (I) AgNPs showed antibacterial and anti biofilm formation activity. The anti biofilm activities doesn't seem to be concentration dependent (Table 1); AgNPs exert higher antibacterial activities against *Ps. aeruginosa* and *E. faecalis*. (II) *Ps. aeruginosa* and *S. aureus* showed more sensitivity (increase in fold) to combined antibiotics and AgNPs compared to *A. baumannii* and *E. faecalis*.

Maximum increase in fold area was for penicillin, cephalosporin, and kanamycine (16, 8, and 2.36 ug/mL., respectively) against *Ps. aeruginosa* (Table 2). The data are in agree with Singh *et al* that reported an enhancement of the antibacterial effect of penicillin and amoxicillin in the presence of AgNPs against *P. aeruginosa*,

In their report highest overall synergistic activity was recorded for Vancomycin plus AgNPs against *Enterobacter aerogenes* [14]. In a just recent study by Helmlinger *et al.* [13] the biological effect of the different shapes of silver nano particles on prokaryotic and eukaryotic cells was analysed. They reported

Table 1. MICs (ug/mL.) and percentages of biofilm inhibition by nano silver particles in 40 standard and hospital isolates of pathogenic bacteria.

Bacteria	Mean MICs	AgNPs	concentrations (*)	
<i>Ps. aeruginosa</i>	3.1	6.25 (93)	3.1 (88)	1.6 (84)
<i>A. baumannii</i>	12.5	25 (64)	12.5 (63)	6.25 (63)
<i>E. faecalis</i>	3.1	6.25 (96)	3.1 (94)	1.6 (93)
<i>S. aureus</i>	6.25	12.5 (96)	6.25 (96)	3.1 (92)

*- Numbers in parentheses are mean percentages of biofilm inhibition for different concentrations of silver nano particles against 10 isolates of each bacterial species.

Table 2. The mean zone of inhibition (mm) of different antibiotics against *Ps. aeruginosa*, *A. baumannii*, *E. faecalis* and *S. aureus* in the absence and presence of silver nano particles (AgNPs) *.

	<i>Ps. aeruginosa</i>			<i>A. baumannii</i>			<i>E. faecalis</i>			<i>S. aureus</i>		
	Ab	Ab + AgNPs	†	Ab	Ab + AgNPs	†	Ab	Ab + gNPs	†	Ab	Ab + AgNPs	†
Gentamicin	20	25	0.56	5	10	3	2	2	0	20	25	0.56
Streptomycin	20	20	0	3	3	0	10	10	0	17	22	0.67
Tetracyclin	15	20	0.77	10	15	1.25	4	7	2.06	20	22	0.21
Cephalosprin	10	30	8	2	2	0	3	3	0	3	35	1.35
Kanamycin	6	11	2.36	1	1	0	2	2	0	17	22	0.67
Penicillin	2	10	16	1	1	0	2	2	0	27	34	0.58

*1- Numbers are mean inhibition zones of 10 isolates for each species. 2- Ab denotes for antibiotic, † denotes for fold increases of different antibiotics against examined isolates that were calculated as $(b^2 - a^2)/a^2$, where a and b are the inhibition zones for antibiotic and antibiotic plus AgNPs, respectively.

silver nanoparticles (25 ug/mL.) showed an anti microbial effect on *S. aureus*. In addition to this effect, a particle shape dependent effect on antibacterial activity was reported that may explain differences in effective doses of AgNPs against various bacterial species in reports of this type.

In the mentioned report it has been shown that the particles with a higher specific surface area such as platelets dissolve faster than the particles with a smaller specific surface area such as cubes. They suggested that this can increase silver ion release from former particles and is the reason for higher anti microbial activity of platlet form of AgNPs.

It seems that the anti bacterial mechanism of AgNPs is only related to the silver ion release because bacteria are probably unable to ingest the used silver nanoparticles in contrast to eukaryotic cells [13]. Wang et al. demonstrated that gold nanospheres adhered well to the surface of *Salmonella typhimurium*, but were unable to get into the bacteria [14].

AgNPs with a concentration range of 3.1 – 12.5 ug/mL. exhibited more than % 90 inhibitory effect on biofilm formation of the examined species (Table 1). Generally, the inhibition of the biofilm formation relates to the inhibition of bacterial growth. These results are well comparable with Mohanty *et al.* report that tested anti biofilm activities of varying concentrations of AgNPs against *P. aeruginosa* and *S. aureus* and reported a 65% to 88% reduction in biofilm formation by the tested bacteria [15].

Gurunathan et al. also reported anti bacterial and anti biofilm activity of antibiotics or AgNPs, or combinations of both against *P. aeruginosa*, *Shigella flexneri*, *S. aureus*, and *Streptococcus pneumonia* [16]. They suggested that combining AgNPs with antibiotics could be a possible alternative strategy against bacterial infections. There are reports that show the applications of nano silver as coating catheters, could well prevent biofilm formation by both gram positive and gram negative bacteria [17, 18].

Hence, the effective anti biofilm concentration of AgNPs could be lower than that of anti bacterial, at the same concentration and an higher anti biofilm activity is expected (table 1). A recent study on *Staphylococcus epidermidis* shows that anti biofilm activity of AgNPs was concentration and time dependent [19]. The anti biofilm activity of AgNPs was also reported in other studies with emphasis on bacteria showing resistance to conventional antibiotics [20].

As appeared in table 2, the results clearly indicated that anti bacterial activity against *Ps. aeruginosa* and *S. aureus* was more when antibiotics plus AgNPs were used compared to antibiotics alone, as evidenced by increase in fold area. Fayaz *et al.* [19] suggested that the increase in synergistic effect may be caused by the bonding reaction between antibiotic and AgNPs.

This combination effect doesn't seem to be fully successful for examined *A. baumannii* and *E. faecalis* isolates, (Table 2). This finding challenges Gurranthan *et al.* [16] suggesting that irrespective of the antibiotics, the combination treatments resulted in significantly higher toxicity in bacterial cells that were treated with AgNPs or antibiotics alone. Interestingly such differences are not appeared for anti biofilm activity of AgNPs (Table 1).

4. Conclusion

Our results demonstrated that AgNPs show promising anti biofilm activity on *Ps. aeruginosa*, *A. baumannii*, *E. faecalis*, and *S.*

aureus hospital isolates. Also these particles can compete with commercial antibiotics used for the treatment of various infections and sometimes are even better.

Regarding examined *Ps. aeruginosa* and *S. aureus* isolates, the study also showed a possible combination of tetracyclin, gentamicin, streptomycin, kanamycine, cephalosporin, and penicillin with AgNPs which showed the enhanced antimicrobial effects and was concluded as synergism.

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