

Synthesis of silver nanoparticles and its synergistic effects in combination with imipenem and two biocides against biofilm producing *Acinetobacter baumannii*

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ABSTRACT:

Objectives: Biofilms are communities of bacteria attached to surfaces through an external polymeric substances matrix. In the meantime, *Acinetobacter baumannii* is the predominant species related to nosocomial infections. In the present study, the effect of silver nanoparticles alone and in combination with biocides and imipenem against planktonic and biofilms of *A. baumannii* was assessed.

Materials and Methods: Minimum inhibitory concentrations (MICs) of 75 planktonic isolates of *A. baumannii* were determined by using the microdilution method as described via clinical and laboratory standards institute (CLSI). Among all strains, 10 isolates which formed strong biofilms were selected and exposed to silver nanoparticles alone and in combination with imipenem, bismuth ethandithiol (BisEDT) and bismuth propanedithiol (BisPDT) to determine minimum biofilm inhibitory concentrations (MBIC). Subsequently, minimum biofilm eradication concentrations (MBECs) of silver nanoparticles alone and in combination with imipenem against mature biofilm of the isolates were evaluated.

Results: Results showed that 29.3% of isolates were susceptible to silver nanoparticles and could inhibit the growth and eradicate biofilms produced by the isolates. For this reason, “FIC”, “FBIC” and “FBEC d” 0.05 were reported which shows synergism between silver nanoparticles and imipenem against not only planktonic cells but also inhibition and eradication of biofilms. The results of “FBIC >2” indicated antagonistic impacts between silver nanoparticles and BisEDT/BisPDT against biofilms.


Conclusion: It can be concluded that silver nanoparticles alone can inhibit biofilm formation but in combination with imipenem are more effective against *A. baumannii* in planktonic and biofilm forms.

Keywords: *Acinetobacter baumannii*, Biofilm, Bismuth ethandithiol (BisEDT), Bismuth propanedithiol (BisPDT), Imipenem, Silver nanoparticles (AgNPs)

INTRODUCTION

Silver is a metal known for its broad-spectrum of antimicrobial activities against Gram-positive and Gram-negative bacteria, fungi, protozoa and certain viruses. In addition, it was reported that using silver compounds in medical products such as dental fillings, catheters, bandages and implants decreases the chance of bacterial infections [1]. The emergence and spread

of antibiotic resistant pathogen is a distressing concern in clinical practices. There is a need of a cheap broad spectrum agent that can be used against variety of pathogens. The silver nanoparticles (AgNPs) have been found to be effective against various viruses and bacteria [2]. During the last decades, introducing of AgNPs has exhibited enhanced antimicrobial properties [3-5]. AgNPs anchor to the cell wall and penetrate into of the Gram-negative bacteria cell membrane, increasing the cell permeability, leading to an uncontrolled transport through the cytoplasmic membrane and finally cell death [4]. Furthermore, interaction of AgNPs

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with DNA of bacteria inhibit DNA replication, and ultimately inducing cell death [6]. It has been also proposed that the antibacterial mechanism of AgNPs is correlated to the formation of free radicals and later on, the free radical-induced membrane damages [7]. The properties of nanoparticles can be affected by their size and shape, chemistry of the affected surface, solubility and aggregation of particles [8]. Moreover, AgNPs as an effective agent against broad spectrum of Gram-negative and Gram-positive bacteria was reported through the preventing of biofilm formation [3]. It was investigated that silver chelates or silver ions are more effective as therapeutic agents. To examine their mode of action and therapeutic activities against Gram positive bacteria is very difficult with AgNPs alone, although increasing percentage of effectiveness are the same for ampicillin in combination with AgNPs against Gram positive and Gram negative bacteria. It seems ampicillin molecules come into contact with AgNPs, wherein silver nano core which is surrounded by ampicillin molecules [9]. Likewise, it was observed that the antibacterial activity of penicillin G, amoxicillin, erythromycin, clindamycin, and vancomycin increased in the presence of AgNPs against *Staphylococcus aureus* and *Escherichia coli* [10]. There are evidences of antibacterial effects of AgNPs and synergetic capacity on planktonic cells and biofilm structures. These results suggest that AgNPs have potential activity to use as an adjuvant components to treat the infectious diseases [11]. *Acinetobacter baumannii* is a significant worldwide nosocomial pathogen with ability to extend antimicrobial resistance and cause nosocomial outbreaks of infection [12]. This organism commonly causes infections related to medical devices, e.g., vascular catheters, cerebrospinal fluid shunts or Foley catheters [13]. Biofilm formation is a well-known pathogenic mechanism in *Acinetobacter* infections [14]. The ability of *A. baumannii* to form biofilms could explicate its antibiotic resistance and survival properties [15]. Biofilm formation occurs as a sequence of procedures: microbial surface attachment, cell proliferation, matrix production and detachment [16]. Biofilms create an environment that increases antimicrobial resistance [17]. Imipenem is a β -lactamase-resistant carbapenem with broad and high antibacterial activities against bacteria. This antibiotic has superior affinity for the PBP2 of Gram-negative bacteria, thus prevents cell division and resulting the cell death [18].

Bismuththiols (BTs) are a new class of biocides with antibiofilm activity against bacteria. The thiol component acts as a lipophilic carrier that increases bismuth uptake by the bacteria, therefore enhances the effects of bismuth up to 1000-fold. Inside the cells, bismuth acts as a metabolic poison, resulting in the growth inhibition and the cell death. At sub-inhibitory concentrations, BTs inactivate bacterial respiratory enzymes, suppress exopolysaccharide expression and subsequently inhibit biofilm formation of bacteria [19]. Due to the clinical importance of *A. baumannii* as well its ability to form biofilm, inhibition and eradication of this structure was investigated in the presence of AgNPs alone and in combination with antimicrobial agents.

MATERIALS AND METHODS

Bacterial isolates

In this study, 65 isolates of *A. baumannii* were collected from previous investigations (55 isolates from burn wounds, Burn hospital, Tehran, Iran and 10 isolates from urinary catheters, Army hospital, Tehran, Iran) were used. Frozen stocks were stored in skim milk (Merck, Germany) containing 15% glycerol at -70°C . Isolates were transferred from the stock cultures into tryptic soy agar [TSA, (Merck, Germany)] and were aerobically incubated at 37°C for 24 h.

All tests were carried out in duplicate. Quality control as antibiotic susceptibility test were *Pseudomonas aeruginosa* ATCC 27853 and *E. coli* ATCC 27922.

Antimicrobial agents

The AgNPs were prepared via chemical reduction of silver ions (AgNO_3). For this reason, nanoparticles were prepared by mixing of an aqueous solutions of Ag ions (0.5 mL, 0.1 mM) with aqueous solution of sodium citrate (12 mL, 0.1 mM). Then, aqueous solution of NaBH_4 (0.5 mL, 0.1 M) was added into the above solution to form a stable colloidal solution of AgNPs. Imipenem (Exir, Iran) stock solution were prepared in 0.01 M phosphate buffer and diluted as described by CLSI protocol.

Bismuth ethandithiol (BisEDT) and Bismuth propanedithiol (BisPDT) were two biocides which was used in this study. For this reason, 4.58 mg of bismuth nitrate (BN) was dissolved in 1 mL of 1,2-propandiol. Either 100 μL of 1,2-ethanedithiol [EDT, (Merck, Germany)] or 100 μL of 1,2-propanedithiol [PDT, (Merck, Germany)] was dissolved in 900 μL of 1,2-

propandiol. Then, 1000 µg/mL working stock solution of each biocide was prepared by adding 50 µl of BN, 5.8 µl of HCl (12.1 M) and 4.2 µl of EDT or PDT to 940 µl of 1,2-propandiol [20].

MIC determination of silver nanoparticles on planktonic bacteria

To determine the minimum inhibitory concentration of AgNPs on planktonic bacteria, serial dilutions of AgNPs were prepared by diluting 15 times of 100 ppm solution of AgNPs to reach to the half concentration. Suspension of cells in Muller Hinton broth was adjusted to 0.5 McFarland tube and diluted 1:100 in Muller Hinton broth medium. Then, 100 µl of diluted AgNPs was added to each row of microtiter plate followed by addition of 100 µL of each isolates to the wells. Finally, microtiter plates were incubated at 37°C for 24 h [21]. Assessment of synergetic effect of AgNPs in combination with imipenem on planktonic bacteria was determined by using checkerboard microdilution technique. Stock solutions of imipenem and AgNPs were diluted and 50 µL of imipenem dilutions (0.25-1024 µg/mL) were added to each row of microtiter plate. Afterward, 50 µL of AgNPs (0.003-100 ppm) were added to each column of microtiter plates. Then, 100 µL of each bacterium was added to each well. Plates were incubated at 37°C for 24 h [22]. After that, the fractional inhibitory concentration (“FIC) for both antimicrobials was calculated according to European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (2000):

$$FIC (A) = \frac{MIC (A \text{ in the presence of } B)}{MIC (A \text{ alone})}$$

$$FIC (B) = \frac{MIC (B \text{ in the presence of } A)}{MIC (B \text{ alone})}$$

$$\Sigma FIC = FIC (A) + FIC (B)$$

“FIC d”0.5 is a sign of synergism, >0.5-1 sign of addition, >1 to 2 indifference and e”2 is a sign of antagonism.

MIC determination of silver nanoparticles against biofilm

For determination of minimum biofilm inhibitory concentration (MBIC) and minimum biofilm eradication concentration (MBEC) of nanoparticles, 10 isolates out of 75 isolates of *A. baumannii* were chosen. The criterion for theselection was the ability of these strains

to form strong biofilms as described in previously study[23].

To determine MBIC of silver nanoparticles, serially diluted nanoparticles were prepared in TSB. Subsequently, microtiter plates were filled and incubated by AgNPs and suspension of cells, respectively as described for planktonic cells. Subsequently, plates were emptied and washed 5 times with phosphate buffer saline (PBS) and were left to dry. This step was followed by adding 200 µL of 0.2 % crystal violet. After 5 minutes; the plates was washed for 5 times and destained by using 33 % (v/v) glacial acetic acid. Next, 100 µL of each well contents were transferred to the wells of a new microtiter plate and the plates were read by an ELISA plate reader [24]. The efficacy of biocides was calculated according to the following equation.

$$Percentage \text{ Reduction} = \frac{[(C - B) - (T - B)]}{(C - B)} \times 100$$

Where B denotes the average of absorbance for blank wells, C denotes the average of absorbance for control wells and T denotes the average of absorbance for treated wells [25].

To determine MBIC of AgNPs in combination with imipenem, Bismuth-ethandithiol (BisEDT) and Bismuth-propanedithiol (BisPDT) stock solutions of each antimicrobial were prepared in tryptic soy broth [TSB, (imipenem 0.125-1024 µg/mL, BisEDT and BisPDT 0.09-1000 µg/mL)] and synergetic effect of AgNPs as well fractional biofilm inhibitory concentration (“FBIC) were determined.

To determine minimum biofilm eradication concentration (MBEC), each microtiter plate was filled with given concentration of isolates and incubated for 24 h at 37°C to form biofilm. Next, plates were emptied and washed 5 times to remove non-biofilm forming cells [25]. To determine of MBEC of AgNPs, serially diluted concentrations of AgNPs were added to each well and incubated at 37 ° C for 20 minutes. Then plates were emptied and filled again and repeated to get the final time of 1h. Afterward, plates were emptied and washed 5 times and followed by adding up neutralizer for 5 minutes [25]. The sodium salt of XTT (2,3-Bis-(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide)(Sigma) was dissolved in phosphate buffer saline to the concentration of 1 mg/mL. In addition, menadione (Sigma) was dissolved in acetone to reach to 1 mM concentration. The XTT/menadione

reagent must be prepared fresh prior to each assay and contained 12.5 parts XTT/1 part menadione. Then, 100 μ L XTT-menadione were added to each well and incubated at 37°C in a shaker incubator at 150 r.p.m for 1h. Finally, absorbance at 505 nm were read by plate ELISA reader [26].The efficacy of biocide was calculated as described above.

To determine of MBEC of AgNPs in combination with imipenem, diluted antimicrobials were added to plates via checkerboard method and fractional biofilm eradication concentration (“FBEC) were then determined.

Scanning electron microscopy was applied to determine effectiveness of antimicrobial agents to eradicate *A.baumannii* were placed in the wells of a 12-well sterile microtiter plate. The steps was followed by adding 3 mL of bacterial suspension and medium in the first well, 1.5 mL bacterial suspension and 1.5 mL AgNPs in the second well and 1.5 mL bacterial suspension and 1.5 mL AgNPs and imipenem into the third well. The plate was incubated at 37°C for 24 h. After that, slides were washed by sterile distilled water and placed in Glutaraldehyde 4% for 2 h to fix.

Then, to dehydrate all slides, they were put for 15 min in the serial concentrations of 40 %, 60 %, 80 %, 90 % and 100 % of absolute ethanol (Merck, Germany), respectively. Finally, biofilms on the surface of the glasses were lyophilized. Electron microscopic micrographs were taken using Phillips XL 30.

Statistical analysis

Results were analyzed with SPSS software using one way ANOVA. All experiments were performed in triplicate. P values of d” 0.05 were regarded as significant.

RESULTS

In this study, MIC of AgNPs against planktonic bacteria was determined. Only 22 isolates (29.3%) showed susceptibility to 100 ppm and remaining isolates were resistant to all AgNPs concentrations. Also, MBIC of AgNPs alone and in combination with imipenem, BisEDT and BisPDT was determined (Fig. 1). The concentrations of AgNPs, imipenem, BisEDT and BisPDT for all isolates has been shown in Table 1. As showed in scanning electron microscopy micrographs, AgNPs alone could reduce biofilm of *A.*

baumannii isolates, but in combination with imipenem, biofilm producing isolates could not attach to the surface of glass and form the biofilm (Fig. 2).

In the other hand, in comparison with AgNPs alone, AgNPs in combination with imipenem and biocides could inhibit biofilm formation of *A. baumannii* at lower concentrations.

Moreover, results indicated synergistic activity of AgNPs and imipenem. However, antagonistic impact of AgNPs with BisEDT and BisPDT was clearly observed.

MBEC of AgNPs alone and in combination with imipenem were also determined and presented in Fig. 3. Since generation time of *Acinetobacter* isolates was reported 20 minutes in previous[27], it seems after induction of cell division, AgNPs can inhibit this process resulting in cell death.

As it was shown in the Table 1, synergistic impact between AgNPs and imipenem to eradicate biofilms was observed.

Furthermore, statistical analysis showed significant differences between AgNPs alone and in combination with antimicrobials data with P values of d” 0.05.

Finally, the combination of AgNPs and imipenem at lower concentrations could be inhibited biofilm formation of *A.baumannii*.

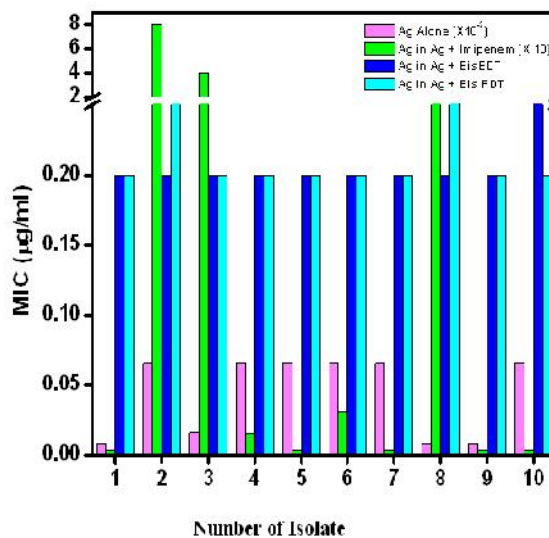


Fig. 1. MBIC of AgNPs alone and in combination with imipenem, BisEDT and BisPDT was shown

Table 1. Concentrations ($\mu\text{g}/\text{mL}$) of AgNPs, imipenem.BisEDT and BisPDT of MBIC were presented

	isolate	1	2	3	4	5	6	7	8	9	10
AgNPs	AgNPs	0.8	6.5	1.6	6.5	6.5	6.5	6.5	0.8	0.8	6.5
AgNPs + IM ¹	AgNPs	0.0003	0.8	0.4	0.0015	0.0003	0.0031	0.0003	0.2	0.0003	0.0003
	IM	0.25	32	32	8	0.125	32	1	16	16	16
AgNPs + BisEDT	AgNPs	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.4
	BisEDT	16	8	32	32	32	16	16	32	16	16
AgNPs + BisPDT	AgNPs	0.2	0.4	0.2	0.2	0.2	0.2	0.2	0.4	0.2	0.2
	BisPDT	16	16	32	16	16	16	16	16	16	32

¹IM = imipenem

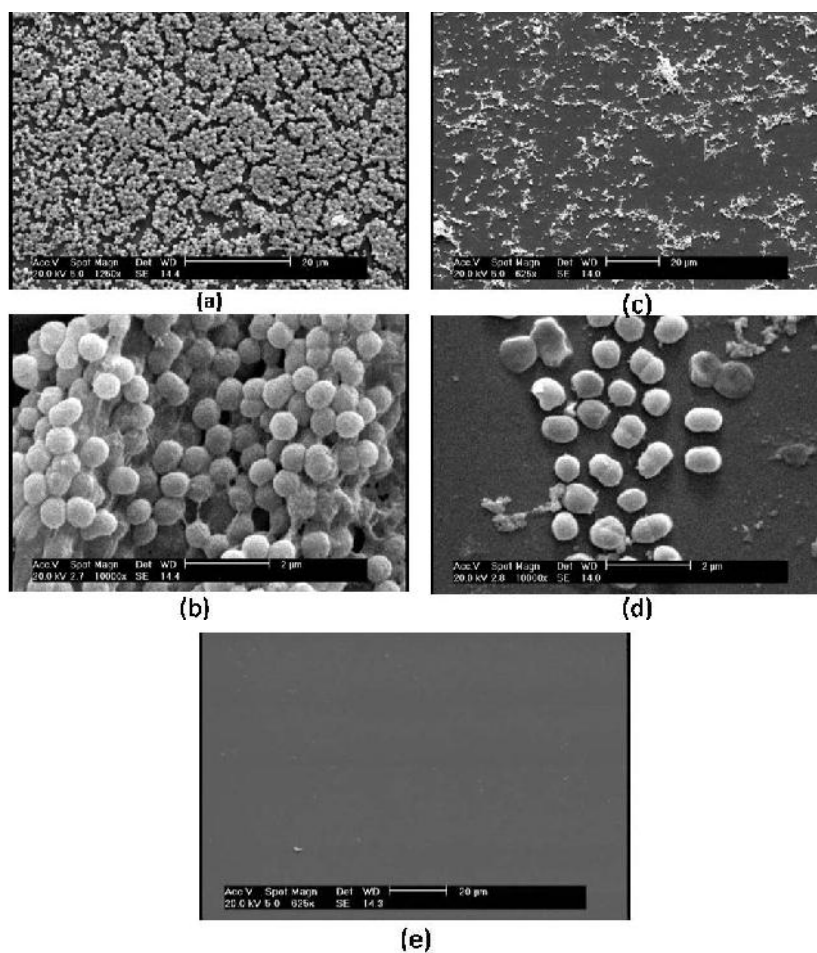


Fig. 2. Scanning electron micrographs of (a) and (b) positive control with different magnification, (c) and (d) treated the isolates with AgNPs alone with different magnification, (e) mixed AgNPs and imipenem treatment against the same isolate number 10

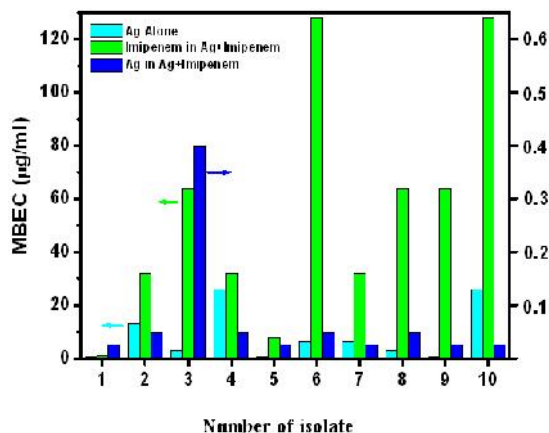


Fig. 3. MBEC of AgNPs alone and in combination with imipenem

DISCUSSION

It was suggested that the positively charged AgNPs can attach to the lipopolysaccharides of Gram-negative bacteria and to be effective via metal reduction[29], However, Hong et al. reported that negatively charged AgNPs can be electrostatically repulsed from the negatively charged (-20 mV) surfaces of bacteria [30]. This feature could be attributed to the long time exposure (for example 24 hours) of nanoparticles in which resistance mechanisms like efflux systems could be deactivated the antimicrobial activity of AgNPs. In this study, synergistic effect of AgNPs in combination with imipenem (“FIC d” 0.05) against all isolates was observed. According to results of MBIC, it could be suggested that the excretion of adhesive substances such as polysaccharides and proteins is crucial factor for the initial attachment of organisms as well to hold the bacteria together. It was reported that the AgNPs might be involved in preventing these adhesive substances and thereby resulting in prevention of biofilm formation[31]. Since *A. baumannii* biofilm matrix comprises of extracellular DNA (eDNA), it seems that reaction between positively charged AgNPs and negatively charged eDNA play a major role in the inhibition of biofilm formation in isolates. BTs are the new classes of anti-biofilm agents that has been shown to have remarkable activity against a broad spectrum of biofilm producing organisms through

inhibiting the slim expression in both Gram-positive and Gram-negative bacteria at non-toxic concentrations [32]. In this study, it was assumed that the combination of AgNPs and BisEDT or BisPDT have a synergistic impact. Although both biocides are exopolysaccharide inhibitors but they have antagonistic activity when used together. The synergism between AgNPs and imipenem could be due to the damage of the matrix and consequently improved penetration of antibiotic. Furthermore, antibiotic molecules have many active sites such as hydroxyl and amine groups which can interact with AgNPs and act as a chelating agent. Moreover, imipenem lyses the cell wall of bacteria leading to increase in penetration of AgNPs into the cells.

Thiols can chelate bismuth and other metals. It seems in the presence of AgNPs, thiols preferably bond to silver ions leading to easier penetration of Ag into the cells. Therefore, lower concentrations of AgNPs when mixed with thiols, can inhibit biofilm formation. However, higher concentrations of thiol would be required when there is antagonism (“FBIC > 2) between AgNPs and thiols.

CONCLUSION

This study demonstrated a synergistic effect of imipenem and AgNPs against planktonic cells as well biofilms, making it a promising candidate for a new treatment of infections caused by MDR *A. baumannii* strains. Further investigation should be carried out to be sure of effectiveness of this nanoparticle *in vivo* experiments and assay probable cytotoxicity of this material.

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CONFLICT OF INTEREST

No conflict of interest.

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