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# THE IN VITRO EFFECTS OF SILVER NANOPARTICLES ON BACTERIAL BIOFILMS

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**ABSTRACT**

Biofilm formation is one of the most important bacterial virulence factors that plays a key role in infections. In the present study, effects of silver nanoparticles were evaluated *in vitro* against bacterial biofilm. Ninety bacterial isolates were selected for study. The Congo Red agar, tube and microtitre assays were used for the detection of biofilm. Antimicrobial effects of silver nanoparticles were determined by the Kirby-Bauer and microdilution methods. The microtitre assay was used to study the biofilm inhibition activity. The most common biofilm producing bacteria was *Staphylococcus aureus.* The power of biofilm production is different among bacteria, and the effect of silver nanoparticles against *Escherichia coli* was less than *Staphylococcus aureus* and *Pseudomonas aeruginosa.* The silver nanoparticles are effective against planctonic and biofilm forms. Because of the importance of biofilm in infectious diseases and the development of drug resistance, silver nanoparticles may be an appropriate way for the control and the prevention of biofilm.

**Keywords:** Bacteria, Biofilm, Silver nanoparticles

## INTRODUCTION

Biofilms are complex and organized communities of bacteria that grow on animate and inanimate surfaces (**Høiby et al., 2014; Southey-Pillig *et al.*,2005**). Biofilm formation is one of the most important bacterial virulence factors that play a key role in serious infections **(Hassan *et al.*, 2011; Joseph, 2003; Odeyemi *et al.*, 2012**). Microbial biofilms can cause skin, wound and teeth infections, are a serious risk factor for patients using artificial biomedical devices such as contact lenses, central venous catheters, urinary catheters, artificial heart valves and intrauterine devices, and can cause serious problems in immune-compromised hosts **(Czaczyk and Myszka, 2007; Mah and O`Toole, 2001)**. It is estimated that about 65% of human infections are related to the biofilm **(Mah and O`Toole, 2001)**.

Considering the importance of biofilm in infectious diseases and increasing drug resistance, scientists are searching for appropriate ways to control and prevent biofilm. In general, therapy with a combination of antibiotics, novel cephalosporin, metals chelating agents, quorum sensing inhibitors, halogens, phage therapy and nanoparticles are used as antibiofilm agents (**Czaczyk and Myszka, 2007**). The diameter of the nanoparticles is about 1 to 100nm and possess sole physicochemical, optical and biological properties (**Whitesides, 2003**). Nanoparticles have wide applications in the medical field, including targeted drug delivery, imaging, artificial implants and are also included in the antimicrobial performance to destroy the wide range of pathogens and drug-resistant organisms (**Samia *et al.*, 2006**). Numerous natural and engineered nanoparticles which have strong antimicrobial properties are silver, gold, magnesium, zinc, copper, aluminum, platinum, palladium, and titanium (**Ravishankar and Jamuna, 2011)**. Silver is a metallic element about the atomic number 47 and silver compounds are used in the treatment of wounds, burns and infectious diseases (**Dunn and Edvards-Jones., 2004**). Silver nanoparticles have been used as a medium to delivery antibiotics and synthetic compounds used in antiseptic filters and coating materials (**Kim *et al.*, 2007**). The objective of our study was to determine *in vitro* effects of silver nanoparticles against bacterial biofilms.

## MATERIALS AND METHODS

### Identification and biofilm detection

Bacteria were isolated from various samples including ulcer, throat, mucus and urine, and were identified by standard tests (**Mahon, 2014**). Ninety bacterial isolates were selected which included *Staphylococcus aureus* (*S. aureus*) (n=30), *Escherichia coli* (*E.coli*) (n=30) and *Pseudomonas aeruginosa* (*P. aeruginosa* ) (n=30). In this study, we used three methods for the detection of biofilm-forming isolates including Congo red agar(CRA), tube method (TM) and microtitre assay (MA) (**Hassan *et al*,2011**).

### Congo- Red Agar method

Biofilm production was evaluated using cultivation isolates on Congo Red Agar (CRA), comprising 0.8g of Congo red (Sigma, the USA) and 36g of saccharose (Sigma, the USA) to one litter of brain heart infusion agar (Merck, Germany). Inoculated CRA dishes were incubated at 37°C for 24h followed by storage at room temperature for 48h. The production of rough black colonies by biofilm producing isolates was used to distinguish them from non-biofilm producing strains.

### Tube method

Overnight fresh culture of bacterial isolates (equal to 1 McFarland) was prepared; 100µL of this suspension was inoculated into 3mL of Trypticase Soy Broth (Merck, Germany) with 2% glucose (Sigma, the USA) in a glass test tube. After overnight incubation at 37°C, the test tube was decanted and washed 3 times with phosphate buffer saline (pH 7.3), and dried. The adherent biofilm layer was stained with crystal violet 0.1% for 20min, and the excess stain was washed with deionized water. The tubes were dried in an inverted position. The strains which showed an adherent and visible biofilm layer on internal walls of the test tubes were considered as positive.

**Synthesis of silver nanoparticles**

Chemical reduction method was used for synthesis of silver nanoparticles by Sodium Boron hydride ($NaBH_4$). To stabilize the solution, 0.3% polyvinyl pyrolidine (PVP) was added to the solution to prevent the particles density. The size of nanoparticles in the silver nanoparticle suspension were determined by SALD2101. Suspension of silver nanoparticles became lyophilized powder by freeze-drying method and were kept in a closed container in the refrigerator at 4°C( **Guzmán *et al*., 2009**).

**Evaluation of anti-planctonic effects of silver nanoparticles**

Antimicrobial effects of silver nanoparticles against the planctonic form were determined using Kirby-Bauer method in Muller-Hinton Agar (Merck, Germany). The lyophilized powder of silver nanoparticles was used for the preparation of discs with concentrations of 5, 25, 100, and 400µg/disc. The petri dishes were evaluated after 24h incubation at 37°C by measuring the inhibition diameter of growth around the disks.
The microdilution assay was also used to determine the MIC of silver nanoparticles against 10 planctonic form of each bacterium( **Guzmán *et al*., 2009**). First, serial dilutions of silver nanoparticles (4.6, 9.3, 18.7, 37.5, 75, 150, and 300µg/ml) were prepared. Then, the wells containing silver nanoparticles and Muller-Hinton Broth (Merck, Germany) were inoculated with the $10^5$cfu/ml tested strains and were incubated at 37°C, and growth or no growth was evaluated after 24h. Additionally, the lowest concentration of nanoparticles that allows growth of less than 0.1% of the control culture is considered as minimal bactericidal concentration (MBC).

**Evaluation of biofilm inhibition activity of silver nanoparticles**

The microtitre assay was used to study the biofilm inhibition activity, and the second concentration of silver nanoparticles was used to obtain the biofilm inhibitory concentration (BIC). After preparing nanoparticles and inoculated $10^5$cfu/ml bacteria, the micro-plates were incubated at 37°C. The wells were washed with 200µl phosphate buffer saline two times after 48h. Then, 200µl of 0.1% crystal violet was added to the wells and incubated for 15 minutes; the wells were washed with water and allowed to dry at room temperature. Extra color attached to the surface was removed by ethanol 95%; finally optic density (OD) of stained biofilms was read by ELISA auto-reader at wave length 570nm. The percentage of biofilm inhibition was calculated by the following formula: {The percentage of biofilm inhibition = (OD Control – OD Treat) / OD Control × 100}(**Namasivayam *et al*.,2012**).

**Statical analysis**

In this study, all tests were repeated 3 times. Data was entered into the SPSS software version 16 and the results were analyzed by One-way ANOVA, LSD post hoc and Two-way correlated analysis of variance tests. In this study, Pv ≤ 0.05 was regarded statistically significant.

**RESULTS AND DISCUSSION**

**Biofilm detection**

Biofilms are communities of microbes that its function depends on a complex network of biological interactions (**Li and Tian, 2012**). Microorganisms associated with biofilms behave differently in growth rates. In the present study, three methods were used for biofilm detection, and the MA assay was more sensitive than Congo red agar and tube methods. According to a previous study, the MA is a gold standard assay for biofilm detection (**Mathur *et al*., 2006**). The most common biofilm producing bacteria was *S. aureus* (Chart 1), and biofilm-formation was different among the 3 bacteria (Pv=0.01). The biofilm formation probably is associated with type of clinical samples (Pv=0.05). All *S. aureus* producing biofilm were isolated from wound infections, *P. aeruginosa* producing biofilm were often isolated from urine and throat samples, and *E .coli* producing biofilm were isolated from urine samples.

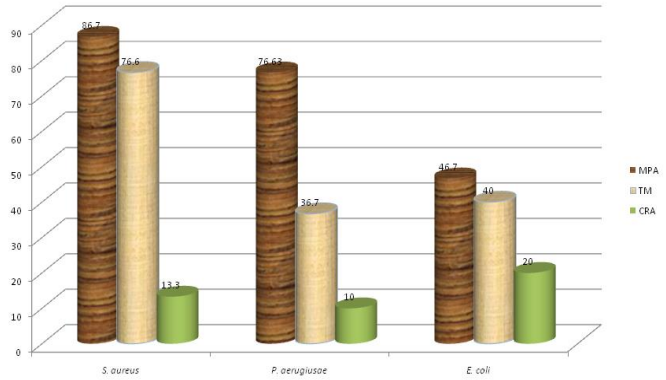


**Chart 1** The frequency of biofilm-formation by MA, TM and CRA methods in tested bacteria

**Evaluation of anti-planctonic effects of silver nanoparticles**

Antimicrobial effects of silver nanoparticles were tested by disk diffusion agar and microdilution methods, and according to the disk diffusion agar results, increasing the concentration of nanoparticles in the discs will be increase the diameter of the inhibition zone (Table 1). Additionally, the post hoc LSD test confirmed this result. Based on the diameter of the growth inhibitory, *P. aeruginosa* isolates showed more sensitivity to nanoparticles in comparison to *S. aureus* isolates and is also more sensitive than *E. coli* isolates.

**Table 1**The antimicrobial effects of silver nanoparticles against planctonic form by disk diffusion agar method.

| | 400µg/disc | 100µg/disc | 25µg/disc | 5µg/disc |
|---|---|---|---|---|
| *S. aureus* | 14.2* | 12.4 | 6.9 | 6 |
| *E. coli* | 11.6 | 9.8 | 6.5 | 6 |
| *P. aeruginosa* | 16.6 | 13.6 | 10.11 | 7.9 |

* The mean growth inhibition size (millimeter) of bacteria with different concentration of silver nanoparticles.

The MIC and MBC results of silver nanoparticles against bacteria are shown in Table 2. One-way ANOVA test showed that the MIC and MBC of nanoparticles were significantly different in various bacteria. The effects of silver nanoparticles against *E. coli* was less than *S. aureus* and *P. aeruginosa*. According to the results of the post hoc LSD test, there are significant differences between isolates of *S. aureus* and *P. aeruginosa* and *E. coli* in terms of MIC and MBC (Pv=0.05), but there were no significant differences between isolates of *S. aureus* and *P. aeruginosa* (Pv=0.741). In this study, based on the results of MIC values, silver nanoparticles had almost the same effect on Gram-positive bacteria (*S. aureus*) and Gram-negative bacteria (*P. aeruginosa*), whilst MIC in 70% of *E. coli* was doubled in comparison to *S. aureus* and *P. aeruginosa*. There are controversy about the effects of silver nanoparticles on Gram-positive bacteria and Gram-negative bacteria. Shrivastava *et al*. (**2007**) reported that silver nanoparticles are generally more active on Gram-negative bacteria than Gram-positive bacteria. The resistance of Gram postive bacteria to silver nanoparticles may be due to the cell wall and the thick peptidoglycan layer of Gram-positive bacteria (**Feng *et al*., 2001**). Similar to our data, Doudi *et al*. **(2011)** and Ruparelia *et al*. **(2008)** reported that *E. coli* had a higher resistance to silver nanoparticles than *S. aureus*. Some researcher believe that lipopolysaccharide of Gram-negative bacteria trap positively charged silver nanoparticles and lead to the blocking of nanoparticles. As a result, antibacterial activity of silver nanoparticles needs to reach the cell membrane. In fact, the silver nanoparticles are attached to the surface of cell membranes and can disrupted the performance of the membrane, penetrate the cell and release silver ions.

**Table 2** The MIC and MBC results of silver nanoparticles against 10 planctonic form of each bacterium

| | Number (%) | MIC (µg/mL) | MBC (µg/mL) |
|---|---|---|---|
| *S .aureus* | 8 (80) | 75 | 150 |
| | 2 (20) | 37.5 | 75 |
| *E. coli* | 6 (60) | 150 | 300 |
| | 4 (40) | 75 | 150 |
| *P. aeruginosa* | 9 (90) | 75 | 150 |
| | 1 (10) | 37.5 | 75 |

**Evaluation of biofilm inhibition activity of silver nanoparticles**

The average percentage of biofilm inhibition in *S. aureus*, *P. aeruginosa* and *E. coli* isolates by silver nanoparticles at a second concentrations were 58%, 56% and 44%, respectively. However, the potency of silver nanoparticle biofilm

inhibition was different and the percentage of biofilm inhibition by silver nanoparticles in *E. coli* was less than *P. aeruginosa* and *S. aureus*. Two-way correlated analysis of variance results showed that silver nanoparticles were effective against bacterial biofilm (Pv=0.01). The amount of biofilm inhibition is significantly different between diverse doses of silver nanoparticles. Statistical analysis post hoc LSD tests showed that the percentage of biofilm inhibition at high doses was more than low doses.

In general, the ability of resistance to antimicrobial agents in biofilm is 10 to 1000 times higher than planctonic cells (**Czaczyk and Myszka**, **2007; Taylor and Webster**, **2009**; **Monroe**, **2007**). In this study, silver nanoparticles had potent anti-biofilm effects. Antimicrobial effects of silver nanoparticles have been previously studied (**Taylor and Webster, 2009; Li and Tian, 2012; Velázquez-Velázquez et al., 2015**), but there are a few studies on effects of silver nanoparticles against bacterial biofilm (**Guzmán *et al*., 2009; Mathur *et al*.,2006**). A study from India reported that the production of biofilms in *E. coli*, *S. aureus*, *Salmonella typhi* and *Vibrio cholerae* were inhibited by silver nanoparticles (**Kumar *et al.*, 2012)**. Namasivayam *et al.* **(2012)** studied the effects of alone silver nanoparticles and also in combination with several antibiotics, and they concluded that silver nanoparticles made a complete inhibition of biofilm within 24 hours, as well as a good compatibility with combination of silver nanoparticles and antibiotics to inhibit biofilm.

The range of silver nanoparticles size was 50 to 150nm and average particle size was 92nm (Chart 2). The high surface to volume ratio of nanoparticles plays an important role in inhibiting the growth of bacteria. Our study showed that bactericidal effects of nanoparticles is influenced by the particle diameter. Therefore, the choice of synthesis method is effective for controlling the size of silver nanoparticles (**Guzmán *et al*., 2009**). The small particles were more antibacterial and had more antibiofilm activity than large particles, as well as, the triangular-shaped nanoparticles antimicrobial activity was more than spherical particles. In the past studies it was also reported that antimicrobial activity depends on the size of the nanoparticles (**Martinez-Castanon *et al* 2008; Pal *et al*., 2007**).

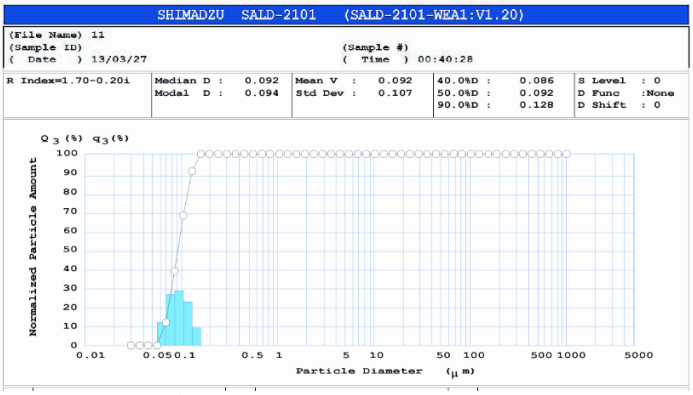


**Chart 2** The range of silver nanoparticles size

## CONCLUSION

The biofilm formation is associated with type of clinical specimens. The small particles of silver nanoparticles are more antibiofilm activity, and antibacterial activity depends on concentration. This research shows that silver nanoparticles have strong antibacterial and antibiofilm activity. The antibiofilm effectes of silver nanoparticles against bacteria is different and *P. aeruginosa* isolates is more sensitive to nanoparticles. The silver nanoparticles can be used to inhibit bacterial biofilms, and may be useful for treatment of infectious diseases due to biofilm. We recommend conducting more studies concerning this issue and particularly conducting *in vivo* and clinical trial searches before the administration of silver nanoparticles in the treatment of infections due to biofilms.

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