

The effect of silver nanoparticles on *Staphylococcus epidermidis* biofilm biomass and cell viability

Bahman Khameneh¹, Hamed Zarei², Bibi Sedigheh Fazly Bazzaz^{3*}

¹Students Research Committee, Ph.D. Candidate, Department of Food and Drug Control, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran

²Students Research Committee, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran

³Biotechnology Research Center, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran

Abstract

Objective(s): Bacterial biofilm has been considered responsible for many deaths and high health costs worldwide. Their better protection against antibacterial agents compared to free living cells leads to poor treatment efficiency. Nanotechnology is promising approach to combat biofilm infections. The aim of the present study was to eradicate *Staphylococcus epidermidis* biofilm with silver nanoparticles (SNPs).

Materials and Methods: SNPs were used at different concentrations (two fold dilutions) and incubation times (24, 48, 72 h). The crystal violet staining and pour plate assays were used to assess biofilm biomass and bacterial viability, respectively. The ability of SNPs on biofilm matrix eradication was assessed through optical density ratio (ODr). Positive control was defined as an ODr =1.0.

Results: The crystal violet assay indicated that the biofilm matrixes were intact at different concentrations of SNOs and incubation times. There were no significant differences between these parameters ($P > 0.05$). Bacterial enumeration studies revealed that higher concentrations of SNPs were more effective in killing bacteria than lower ones. Although, longer incubation times led to enhancement of anti-biofilm activity of SNPs.

Conclusion: The anti-biofilm activity of SNPs was concentration- and time-dependent. The results of this study highlighted that SNPs were effective against cell viability; however they were ineffective against biomass.

Keywords: Biofilm, Biomass, Silver nanoparticles, *Staphylococcus epidermidis*, Viability

*Corresponding Author: Bibi Sedigheh Fazly Bazzaz, PhD, Biotechnology Research Center, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran
Tel: +98511-8823255, Email: fazlis@mums.ac.ir

Introduction

It has been shown that Gram-positive cocci such as *Staphylococcus epidermidis* are responsible for device related infections (1). The ability of *S. epidermidis* to form a mucoid biofilm on medical devices is the reason of the bacterial pathogenicity (2).

Bacterial biofilm formation is because of accumulation of adherent microorganisms on a non-living surface. Biofilm communities are surrounded by an exopolysaccharide matrix (EPS) (3). These structures are one of the distinguishing characteristics of biofilms and also protect the embedded microorganisms against antimicrobial agents. Delayed penetration of antibiotics into the biofilm or reduction of metabolism in microorganisms is suggested as protective mechanisms of biofilm (4).

These extremely robust defense mechanisms of biofilms are lead to investigate approaches to treat biofilm infections. Important strategies are combination therapy, application of antibacterial enzyme and employing nanotechnology (5).

In the recent years, there has been considerable interest in combating antibiotic resistance through development of nanotechnology (6-7). Nanoparticles such as solid lipid nanoparticles, liposomes and nano-emulsions have been developed for bacterial eradication. Metallic nanoparticles are considered as efficient antibacterial agents (8-10). They have been widely used for microbial eradication and eliminating bacterial resistant species (11-12). They have potential advantages such as easily prepared to the nanometer scale and wide range of biomedical applications (13-16). Silver nanoparticles (SNPs) have been used as antibacterial agent for many years (17-18). The antibacterial activity of SNP is related to the inhibition of replication by interfering with DNA and RNA, disruption of the cell membrane, interference with cell respiration, and inactivation and

alteration of enzyme conformation (19). It was demonstrated that SNP coating or even impregnated on the medical device can impede biofilm formation. In this scenario, in present study the anti-biofilm activity of SNP on biofilm producing *S. epidermidis* was studied.

Materials and Methods

Materials

SNP and 2, 3, 5-triphenyltetrazolium chloride (TTC) were purchased from Sigma (USA). Trypticase soy broth (TSB) was provided by Merck (Germany). Muller Hinton broth (MHB) was purchased from Hi media (India).

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

The MIC was determined by tetrazolium based colorimetric method (20). Bacterial growth changes the colorless TTC to a red color.

The *S. epidermidis* strain DSMZ3270 (DSMZ Cloning, Germany) was used as a microbial strain in this study.

The overnight subculture of *S. epidermidis* strain was cultivated in MHB media supplemented with 0.25% glucose to reach and match the 0.5 point of a MacFarland standard. The stock suspension was approximately 10^8 CFU/ml. SNP was prepared from serial two-fold dilutions in the MHB media. The inoculum was prepared by pipetting 0.5 ml of the stock suspension into 9.5 ml of MHB media. 250 μ l of SNP was added to each well of a microtiter plate. This was followed by the addition of 25 μ l of inoculum. The inoculated microplates were incubated at 37 °C for 24 h. Subsequently, TTC (50 μ l, 5 mg/ml) was added to each well and microplate was incubated for 30 minutes at 37 °C. The MBC was defined as the lowest concentration reducing the initial inoculum

by $\geq 99.9\%$. MBC was assessed by sub-culturing 10 μ l of the test dilutions from MIC wells on to fresh Mueller-Hinton agar

plates. Plates were incubated for 18-24 h at 37 °C. MBC was defined as the lowest concentration of antibacterial agents that showed no single bacterial colony on TSA plates.

Crystal violet assay

This test was used to assess the efficacy of SNP for biofilm biomass reduction. Bacterial suspension was prepared in TSB (containing 0.25% glucose) from overnight culture of *S. epidermidis*. Each well of a microtiter plate was filled with 200 µl of bacterial suspension (final concentration 5×10^5 CFU/ml). The inoculated microplates were incubated at 37 °C for 24 h. After incubation, bacterial biofilm was attached to the bottom of a 96-well polystyrene microtiter plate. For removing of unattached bacteria, each well was rinsed three times with PBS (200 µl).

Serial dilution of SNP was prepared in the media from stock solution. Then, they were added to each well and microplates were incubated for different time periods (24, 48 and 72 hours). After an incubation process, biofilms were rinsed three times with 200 µl PBS and were then stained with crystal violet (0.3% for 5 min). In order to solubilize the bounded crystal violet, 200 µl of ethanol (96%) was added in each well. The optical density (OD) at 540 nm was determined using a microplate reader (Awareness, UK). Each experiment was performed at least in three replicates.

The quantitative measurement of OD ratio (OD_r) was calculated by dividing OD of each well containing SNP to OD value of positive control (native biofilm). The control was obtained by adding TSB (containing 0.25% glucose) without SNP to the biofilm cultures. This measurement was related to the ability of formulation on biofilm removal (21).

Susceptibility of biofilm to SNP

Biofilm was prepared in 96-well microtitre plates, as described above. To each well containing the biofilm, SNP solution (200 µl) was added. After the periods of 24, 48

and 72 h of growth, the non-adherent bacteria were removed and the biofilm was washed twice by PBS (200 µl). The wells were thoroughly scraped, until >93% of the biofilm was removed (as determined by crystal violet spectrophotometric readings), and resuspended in 1 ml of PBS, followed by centrifugation for 8 min at 9000 g. This process was repeated twice to obtain bacterial cultures free from biofilm and followed by preparing 10-fold serial dilutions of them. The viable bacteria were determined by plating 100 µl of the dilutions in triplicate on TSA plates and incubation of plates for 20 h at 37 °C (22).

Statistical analysis

A one-way analysis of variance (ANOVA) was used for analyzing differences between OD_r and bacterial population of treated biofilm. Differences between mean were statistically significant if the p-value was less than 0.05.

Results

Determination of MIC and MBC

The MIC and MBC values of SNP against planktonic bacteria were 8 and 32 ppm, respectively.

The results of this assay were represented according to the OD_r values (Figure 1). Positive control was defined as an OD_r =1.0. Decrease in OD_r values implicated the efficacy of SNPs in biofilm biomass reduction. These results indicated that the high concentrations of SNPs were less effective than lower ones (Figure 1.A).

There were significant differences between OD_r of concentrations (P <0.05). The effect of incubation time was shown in Figure 1.B. Statistical analysis indicated a time-dependent correlation between the incubation time of SNPs and the bacterial biofilm reduction (P <0.05). The best results were observed at 24 hours, whereas, 48 and 72 hours showed insufficient results.

Susceptibility of biofilm to SNPs

Figure 2 showed the mean logarithmic reduction of bacterial viability after

Anti-biofilm activity of SNP

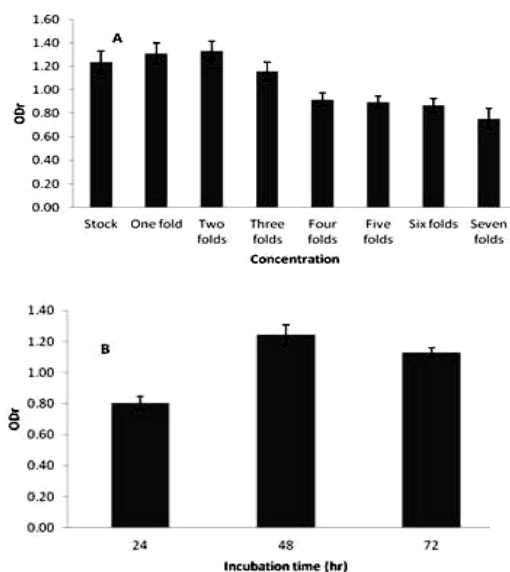


Figure 1. The effect silver nanoparticles (SNPs) on biomass reduction of biofilm producing by *Staphylococcus epidermidis* DSMZ 3270. A) The effect of different SNP concentrations on biomass reduction. B) The effect of different incubation times on biomass reduction. ODr values obtained by dividing OD of each antimicrobial agent to OD of positive control (native biofilm). Positive control was defined as an ODr = 1.0.

exposure of biofilms to SNPs. According to the statistical data, biofilms were more susceptible to higher concentrations of SNP ($P < 0.05$). The effect of time on bacterial viability was illustrated in Figure 2.B. These results implicated that biofilm cells were eradicated in a time-dependent manner ($P < 0.05$).

Discussion

In the recent years, various approaches have been developed for combating biofilms. Nanotechnology provides a useful approach in biofilm control. SNPs elicit bactericidal effects due to release of Ag^+ ions from their surfaces. SNPs have also had remarkable therapeutic efficacy in killing biofilm-based microorganisms (18). In this study, the effect of SNPs on biofilm control was investigated.

The MIC and MBC values indicated that SNP was effective against planktonic bacteria. These findings were consistent with the results of a previous study in which the effect of silver ions and nanosilver on planktonic and biofilm

bacterial cell were evaluated (23). It was demonstrated that biofilms were about 4-fold more resistant to nanosilver inhibition than planktonic bacteria.

The anti-biofilm activity of SNPs was evaluated by two different methods. The biomass reduction was assessed by crystal violet assay (Figure 1). These results indicated that SNP was ineffective in biomass reduction. According to these data, increasing the amount of SNP could not eradicate biofilm matrix. It was reported that upon attachment of nanosilver to biofilm the size of nanosilver increased and subsequently the anti-biofilm activity reduced (23).

Longer incubation time did not produce better results. Taken together, the results of crystal violet assay reflected that biofilm matrixes were intact during incubation with SNPs. These results might be due to the lack of efficiency of this assay for evaluating the anti-biofilm activity of SNPs. The other possible reason for reducing the SNPs anti-biofilm activity was attributed to the interaction of adsorbed SNPs on the biofilm matrix surface by crystal violet stain. The insufficient crystal violet results were observed previously (24). The susceptibility of bacterial cell embedded in biofilm was studied by plate count method. The results of this part of study were not consistent with crystal violet results. According to the plate count data (Figure 2), the SNPs were effective in bacterial biofilm killing. It is interesting to note that stock solution of SNPs was the most effective ones on bacterial death. The effectiveness of SNP on biofilm removal was previously described (23). However, our results indicated that decreasing SNPs concentration has led to decrease in the numbers of bacteria. The incubation time data showed that the incubation period had an effective role in biofilm eradication. These data were consistent with those reported in the previous study.

It was shown that older formulation was significantly had higher activity ($P < 0.05$). The effect of shelf-life time on the efficacy of the

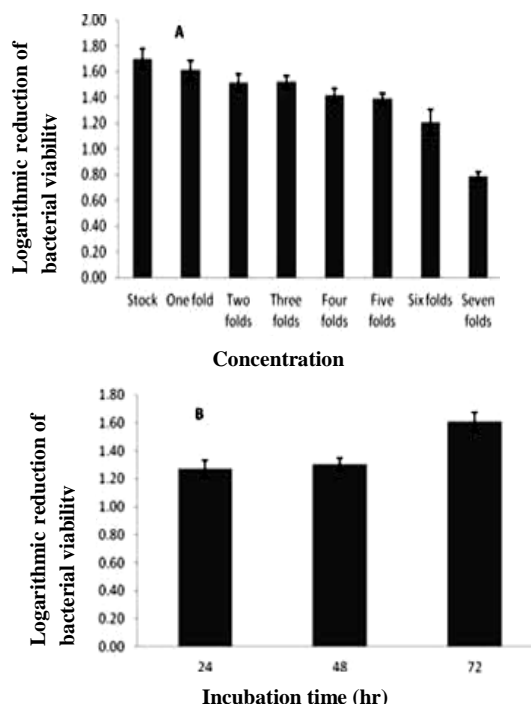


Figure 2. Mean fold reduction of bacterial viability after biofilm exposure to SNP. The y-axis indicates the difference in the log₁₀ cfu/mL between untreated biofilm (controls) and biofilm treated with SNP. A) The effect of different concentrations on bacterial viability. B) The effect of different incubation period times on bacterial viability.

formulation for biofilm eradication was previously described (24-26).

Conclusion

The results of this study confirmed the high efficacy of SNPs on biofilm eradication. SNPs did not disrupt the biofilm matrix, however, it was able to kill the embedded bacteria in biofilm structure. The results of concentrations and incubation time indicated that the best results observed when high concentrations with a longer incubation time were employed.

Acknowledgement

This project was financially supported by a grant from Vice Chancellor for Research, Mashhad University of Medical Sciences, Mashhad, Iran.

References

1. Eftekhari F, Dadaei T. Biofilm formation and detection of icaAB genes in clinical isolates of methicillin resistant

2. Staphylococcus aureus. Iran J Basic Med Sci. 2011; 14(2): 132-136.
3. Agostinho A, James G, Wazni O, Citron M, Wilkoff BD. Inhibition of *Staphylococcus aureus* biofilms by a novel antibacterial envelope for use with implantable cardiac devices. Clin Transl Sci. 2009; 2(3): 193-198.
4. Bose S, Ghosh AK. Biofilms A challenge to medical science. J Clin Diagn Res. 2011; 5(1): 127-130.
5. Campoccia D, Montanaro L, Ravaoli S, Pirini V, Cangini I, Arciola CR. Exopolysaccharide production by *Staphylococcus epidermidis* and its relationship with biofilm extracellular DNA. Int J Artif Organs. 2011; 34(9): 832-839.
6. Shunmugaperumal T. Microbial colonization of medical devices and novel preventive strategies. Recent Pat Drug Deliv Formul. 2010; 4(2): 153-173.
7. Chakraborty SP, Sahu SK, Pramanik P, Roy S. In vitro antimicrobial activity of nanoconjugated vancomycin against drug resistant *Staphylococcus aureus*. Int J Pharm. 2012; 436(1-2): 659-676.
8. Taylor EN, Webster TJ. Multifunctional magnetic nanoparticles for orthopedic and biofilm infections. Int J Nanotechnol. 2011; 8 (1-2): 21-35.
9. Kalishwaralal K, BarathManiKanth S, Pandian SRK, Deepak V, Gurnathan S. Silver nanoparticles impede the biofilm formation by *Pseudomonas aeruginosa* and *Staphylococcus epidermidis*. Colloids Surf B Biointerfaces. 2010; 79(2): 340-344.
10. Sawosz E, Chwalibog A, Szeliga J, Sawosz F, Grodzik M, Rupiewicz M, et al. Visualization of gold and platinum nanoparticles interacting with *Salmonella Enteritidis* and *Listeria monocytogenes*. Int J Nanomedicine. 2010; 5: 631-637.
11. Wei-Qin L, Jia-Lin Y, Guan-Xin L, Xiang-Na G, Xiu-Ju Z, Li-Ting L. Effect of silver nanoparticle on the structure of *Staphylococcus epidermidis* biofilm. Chinese Journal of Antibiotics. 2011; 36: 394-397.
12. Brandt YI, Armijo LM, Rivera AC, Plumley JB, Cook NC, Smolyakov GA, et al. Effectiveness of tobramycin conjugated to iron oxide nanoparticles in treating infection in cystic fibrosis. SPIE Proceedings. 2013; 8595. doi: 10.1117/12.2009549.
13. Cabal B, Cafini F, Esteban-Tejeda L, Alou L, Bartolomé JF, Sevillano D, et al.

Anti-biofilm activity of SNP

- Inhibitory effect on in vitro *Streptococcus oralis* biofilm of a soda-lime glass containing silver nanoparticles coating on Titanium Alloy. PLoS ONE. 2012; 7. doi: 10.1371/journal.pone.0042393.
13. Chen X, Schluesener HJ. Nanosilver: A nanoparticle in medical application. Toxicol Lett. 2008; 176(1): 1-12.
 14. Hajipour MJ, Fromm KM, Akbar Ashkarran A, Jimenez de Aberasturi D, Larramendi IRD, Rojo T, et al. Antibacterial properties of nanoparticles. Trends Biotechnol. 2012; 30(10): 499-511.
 15. Hamouda IM. Current perspectives of nanoparticles in medical and dental biomaterials. J Biomed Res. 2012; 26(3): 143-151.
 16. Fazly Bazzaz BS, Khameneh B, Jalili-Behabadi MM, Malaekheh-Nikouei B, Mohajeri SA. Preparation, characterization and antimicrobial study of a hydrogel (soft contact lens) material impregnated with silver nanoparticles. Cont Lens Anterior Eye. 2014; 37(3): 149-152.
 17. Babapour A, Yang B, Bahang S, Cao W. Low-temperature sol-gel-derived nanosilver-embedded silane coating as biofilm inhibitor. Nanotechnology. 2011; 22(15): 155602. doi: 10.1088/0957-4484/22/15/155602.
 18. Fabrega J, Zhang R, Renshaw JC, Liu WT, Lead JR. Impact of silver nanoparticles on natural marine biofilm bacteria. Chemosphere. 2011; 85(6): 961-966.
 19. Ahamed M, AlSalhi MS, Siddiqui M. Silver nanoparticle applications and human health. Clin Chim Acta. 2010; 411(23-24): 1841-1848.
 20. CA. Biofilm formation by fluconazole-resistant *Candida albicans* strains is inhibited by fluconazole. J Antimicrob Chemother. 2007; 59(3): 441-450.
 21. Wu WS, Chen CC, Chuang YC, Su BA, Chiu YH, Hsu HJ, et al. Efficacy of combination oral antimicrobial agents against biofilm-embedded methicillin-resistant *Staphylococcus aureus*. J Microbiol Immunol Infect. 2013; 46(2): 89-95.
 22. Cerca N, Martins S, Cerca F, Jefferson KK, Pier GB, Oliveira R, et al. Comparative assessment of antibiotic susceptibility of coagulase-negative staphylococci in biofilm versus planktonic culture as assessed by bacterial enumeration or rapid XTT colorimetry. J Antimicrob Chemother. 2005; 56(2): 331-336.
 23. Choi O, Yu CP, Esteban Fernández G, Hu Z. Interactions of nanosilver with *Escherichia coli* cells in planktonic and biofilm cultures. Water Res. 2010; 44(20): 6095-6103.
 24. Moghadas-Sharif N, Fazly Bazzaz BS, Khameneh B, Malaekheh-Nikouei B. The effect of nanoliposomal formulations on *Staphylococcus epidermidis* biofilm. Drug Dev Ind Pharm. 2014. Early Online: 1-6 DOI: 10.3109/03639045.2013.877483
 25. Jones MN, Kaszuba M, Reboiras MD, Lyle IG, Hill KJ, Song YH, et al. The targeting of phospholipid liposomes to bacteria. Biochim Biophys Acta. 1994; 1196(1): 57-64.
 26. Kim HJ, Gias ELM, Jones MN. The adsorption of cationic liposomes to *Staphylococcus aureus* biofilms. Colloids Surf A Physicochem Eng Asp. 1999; 149(1-3): 561-57