Received: 2016-07-26
Revised: 2016-08-14
Accepted: 2016-09-03

Antimicrobial Effect of Silver Nanoparticles on *Staphylococcus Aureus*

Mohammad Soleimani, Maziar Habibi-Pirkoohi✉

Department of Microbiology, Faculty of Medicine, AJA University of Medical Sciences, Tehran, Iran

Abstract

Background: Development of new antimicrobial drugs targeting virulence factors of pathogenic bacteria is an effective strategy to address increasing emergence of resistant bacterial strains. Considering the clinical importance of *Staphylococcus aureus* and global emergence of antibiotic-resistant strains of this pathogen, the present study was carried out to investigate the inhibitory effects of silver nanoparticles (SNPs) on growth and capsule formation as a virulence factor of *S. aureus*. **Materials and Methods:** The SNPs were biologically synthesized by unicellular microalgae *Chlorella vulgaris* and its inhibitory effect on the growth of *S. aureus* and expression of capsule encoding gene (*cap8*) was quantitatively evaluated by serial microdilution and Real-Time PCR; respectively. The growth rate of *S. aureus* under nanoparticles treatment was monitored in a six-hour interval. **Results:** The results obtained in this research indicated the formation of monodisperse spherical SNPs that inhibited *S. aureus* growth at a concentration of 50µg/ml (minimum inhibitory concentration (MIC)=50µg/ml). The growth kinetic analysis showed that *S. aureus* growth was significantly diminished immediately after treatment. Moreover, the SNPs decreased expression of type 8 capsule (*cap8*) gene even at concentrations below MIC value. **Conclusion:** The results of this experiment suggest that biologically synthesized SNPs are fairly ideal candidates for the development of new antimicrobial drugs against *S. aureus*. [GMJ.2016;5(4):200-7]

Keywords: *Chlorella Vulgaris*; *Cap8*; Silver Nanoparticle; *Staphylococcus Aureus*.

Introduction

Silver nanoparticles (SNPs) are currently used in a large number of medical applications ranging from diagnosis and drug delivery to medical device dressing and, more notably, development of new generation of antibiotics [1]. Furthermore, potent antibacterial, antifungal and antiviral properties of SNPs have nominated them as a promising alternative to commercially available antibiotics [2].

Due to the emergence and global spread of bacterial strains resistant to multiple anti-

otics, and the continuing emphasis on health-care costs, many researchers have tried to develop a new generation of antimicrobial medicines free of resistance and cost [3]. Nanoparticles are of great interest due to their slight size, and a large surface to volume ratio, which provides them with unique mechanical, catalytic and biological properties [4]. The preparation of nanoparticle-based medicines with a unique size, shape and chemical properties is great potential in the formulation of new pharmaceutical products [5]. It is well established that medical formulations in the form of nanoparticles can be used as effective

GMJ

©2016 Galen Medical Journal
Tel/Fax: +98 71 36474503
PO Box 7193616563
Email: info@gmj.ir

✉ **Correspondence to:**
Maziar Habibi-Pirkoohi, Department of Microbiology,
Faculty of Medicine, AJA University of Medical
Sciences, Tehran, Iran
Telephone Number: +98 9359930388
Email address: Maziar.habibi.p@gmail.com

antimicrobial compounds. Therefore, preparation, characterization, modification, and functionalization of metal nanoparticles pave the road for the formulation of new bactericidal medicines [6]. Antimicrobial properties of SNPs against a large number of infectious bacteria explain the wide application of these particles in various fields of medicine, different industries, animal husbandry, packaging, accessories, cosmetics, health, and military [3, 6, 7].

Although inhibitory effects of SNPs have been widely studied, the majority of the studies have been focused mainly on the inhibitory effect of SNPs on the growth of bacteria and hence, there is a significant gap about antagonistic effects of SNPs on virulence factors of the pathogenic microorganisms. The capsule is a common virulence factor among many pathogenic bacteria including *Staphylococcus aureus*. Bacteria that cause clinical disease, produce extracellular capsular structures [8]. Capsules enhance microbial virulence by rendering the bacterium resistant to immune responses of the host. Type 8 capsular polysaccharide (CP8) is the most prevalent capsule type in clinical isolates of *S. aureus* [9]. The *S. aureus* is a Gram-positive human pathogen that causes serious infections and diseases associated with significant morbidity and mortality. The occurrence of new drug-resistant strains of this pathogen- as a result of unharnessed use of antibiotics- is currently a major challenge for both patients and medical communities [9].

Biosynthesis of metal nanoparticles by different classes of plants is considered as a cost-effective, environmentally benign and time-saving approach for eco-friendly production of various types of metal nanoparticles [10]. Microalgae constitute a diverse group of unicellular plants that growth fast and produce a large volume of biomass in a fairly short time. These properties make them an ideal choice for biosynthesis of metal nanoparticles [11]. The main goal of the present study was to investigate the utility of SNPs as a new generation of antibiotic against *S. aureus*. For this purpose, two objectives were sought: 1- to evaluate the utility of green microalgae *Chlorella vulgaris* in the biosynthesis of SNPs as a quick and cost-effective system; 2- to investi-

gate the inhibitory effect of biologically synthesized SNPs on the growth of *S. aureus* and expression of the type 8 capsule (*cap8*) gene as the main virulence factor of this infectious bacterium.

Materials and Methods

Biosynthesis of SNPs

To prepare silver nitrate stock, 3.39gr of AgNO₃ was dissolved in 100ml sterile water to give a 200mM solution. A healthy culture of *C. vulgaris* was harvested in logarithmic phase and centrifuged at 5000 rpm for 15 min at 4°C. After discarding the supernatant, the biomass was washed with sterile water for three times to remove culture medium ingredients. The biomass was re-suspended in 47.5 ml of distilled water. Then, 2.5ml of the 200mM AgNO₃ solution was added to get a final 10mM concentration. The bioreduction medium was incubated at 25°C for 48h. A 50ml suspension of *C. vulgaris* free of AgNO₃ was used as a control.

Characterization

Bioreduction of silver ions to SNPs was monitored by measuring the UV-Vis spectra of the solution over the range between 300 to 500nm using Spectrophotometer. Morphology of the SNPs was studied using transmission electron microscopy (TEM) using Leo 912 AB high-resolution TEM operating at an accelerating voltage of 120 kV. A sample of the aqueous biomass solution was placed on the carbon-coated copper grid and dried prior to microscopy. For X-ray diffractometer (XRD) measurement, a sample of bioreduction solution was spread in a petri dish and oven dried. The dried sample was taken for XRD analysis using PHILIPS PW1480 X-ray diffractometer (Netherlands). The concentration of biosynthesized SNPs was measured by high-resolution ICP-OES spectrometer (SPECTRO ARCOS, Germany).

Antimicrobial Assay

The *S. aureus* (PTTC 1431) was provided by Mashhad University of Medical Science, Iran. Minimum inhibitory concentration (MIC) of the biologically synthesized SNPs was determined using broth microdilution method in a

96-well standard ELISA plate. Luria Bertani (LB) broth containing 10^5 CFU/ml of *S. aureus* cells was used as a culture medium. The final concentrations of SNPs used in this assay were 0, 12.5, 25, 50, and 100 µg/ml. No SNP was added to negative control well. The lowest concentration of SNPs inhibiting bacterial growth was assigned as MIC. For further evaluation of the influence of SNPs on *S. aureus*, the growth kinetics of *S. aureus* in a 6h period was monitored. For this purpose, bacterial growth curve at 0, 12.5, 25, 50, and 100 µg/ml of SNPs was plotted by measuring optical density (600nm) against three 2h time intervals. The growth curve was drawn using Excel software.

Real-Time Polymerase Chain Reaction (PCR) Assay

Real-Time PCR assay was conducted to assess expression of *cap8* gene after two hours of SNPs treatment. RNA isolation and cDNA synthesis was carried out following general procedure. Primer pair sequences for Real-Time PCR of the virulence factor were designed by AllelID 6.0 software as follows: 5'AGCCGTTAATCCGCCTAATGTC3' (forward) and 5'ACTGGACCAACCCGCTTCG3' (reverse). The 16s RNA was used as house-keeping (reference) gene and internal control in Real-Time PCR assay. Forward and reverse sequences of the reference gene were 5'-CGTGCTACAATGGACAATACAAA-3' and 5'-ATCTACGATTACTAGCGATTCCA-3'; respectively [12]. Expression of the virulence gene (*cap8*) was quantitatively analyzed using a Real-Time PCR system (BioRad). Re-

al-Time PCR was carried out in a 20 µL reaction volume containing 0.5 µM of each primer and 10 µl of SYBR Green Real-time PCR Master mix (Genet Bio, South Korea). Quantitative Real-Time PCR experiments were performed in duplicate for each sample. The Real-Time RT-PCR data were analyzed by the ($\Delta\Delta C_t$) method as described by Xiang *et al.* [12].

Results

Characterization SNPs

A quick and traceable change was observed in bio reduction medium after addition of silver nitrate into *C. vulgaris* broth culture. In UV-Vis Spectroscopy, a surface plasmon resonance peak was observed at about 450nm which confirms the formation of SNPs (Figure-1). The TEM microscopy revealed that the SNPs are of spherical shape with size about 10nm (Figure-2). Moreover, TEM image showed monodispersity of the biosynthesized SNPs with only a few particles had different size. Energy Dispersive Spectrometry (EDS) assay indicated a sharp signal for Ag confirming biosynthesis of SNPs (Figure-3). An absorption peak at 3keV confirmed the presence of SNPs. The XRD results showed peaks corresponding to (111), (200), (220) and (322) Bragg reflections (Figure-4). This pattern coincides with unique peaks of silver; confirming the presence of SNPs in the sample. According to Joint Committee on Powder Diffraction Standards (JCPDS), four XRD peaks observed in this study indicate that the biosynthesized SNPs are pure crystalline sil-

Figure 1. UV-absorption spectra of *C. vulgaris* suspension incubated with AgNO₃. The absorption peak at 450 nm indicates the formation of SNPs (A). Gradual color change in *C. vulgaris* suspension is indicating bio reduction of silver ions into nanoparticles (B).

ver. The concentration of the biosynthesized SNPs was determined using inductively-coupled plasma (ICP) method. Results showed that the concentration of SNPs in a 25ml sample containing both SNPs and algal biomass was 2.934mg/l in average.

Antimicrobial Assay

A typical serial microdilution assay was used to evaluate the inhibitory effect of SNPs on the growth of *S. aureus*. Results of this test showed that SNPs at the concentration of 50µg/ml could inhibit the growth of the pathogen; the MIC of the SNPs was therefore de-

termined as 50µg/ml. Bacterial suspension showed normal growth below MIC value. Bacterial growth was also normal at negative control well.

The growth kinetics of *S. aureus* under treatment with different concentrations of SNPs is presented in Figure-5. The control group showed a rapid growth pattern which followed an ascending trend until the end of the assay. In contrast, at all SNP concentrations, bacterial growth followed a descending trend; so that at the end of the assay (six hours), bacterial growth in all treatments was nearly zero. The growth decreasing was more severe in the

Figure 2. The TEM micrograph of biologically synthesized SNP (original magnification: 50,000x).

Figure 3. The EDS results indicating sharp peak for silver (Ag^0)

sample treated with 100 µg/ml of SNPs, so that a drop of growth was observed only after 2 hours.

The SNPs Decreased the Expression of cap8 mRNA

Influence of SNPs on *cap8* – as a virulence factor of *S. aureus* – was studied via Real-Time

PCR. Results of Real-Time PCR are presented in Figure-6. As could be seen, a dose-dependent decrease in expression of *cap8* under treatment with various concentrations of SNPs was recorded. In the range of 0 to 100 µg/ml of SNPs, a nearly linear relation was observed between SNPs concentration and reduction of *cap8* expression.

Figure 4. The XRD pattern of SNPs biosynthesized by *C. vulgaris* biomass

Figure 5. The growth kinetics of *S. aureus* under treatment with different concentrations of biologically synthesized SNPs.

Figure 6. Dose-Dependent Reduction of *Cap8* Expression Under Treatment with Various.

Discussion

In the current research, the antimicrobial effect of SNPs on *S. aureus* growth and expression of the *cap8* gene was studied. Characterization assays indicated that circular SNPs with good monodispersity were produced by incubation of *C. vulgaris* with silver nitrate. This finding is agreement with previous studies that reported the efficacy of unicellular microalgae for biosynthesis of various types of metal nanoparticles [13, 14, 15]. Although the exact mechanism of nanoparticle production by microalgae is not fully understood [16], some potential processes have been proposed to explain this biological synthesis. The most probable mechanism is secretion of cellular reductases into growth medium by microalgal cells. These enzymes can efficiently reduce silver ions into SNPs [17, 18]. Moreover, metal ions can be trapped by the carboxylate groups residing on the surface of microalgal cells. The entrapped ions are then reduced by reductase enzymes which subsequently results in the formation of nanoparticles [19]. Monodisperse spherical nanoparticles with small size (~10nm) were generated in this experiment. The small size of SNPs provides a higher surface area that promotes their reactivity and hence, enhances their antibacterial potential. In fact, the size-dependent interaction of SNPs with pathogenic bacteria and

viruses has been reported by many authors [6, 20, 21, 22]. An absorption peak at 3keV in EDS study confirmed the presence of SNPs in the solution. The XRD pattern obtained in this study was in accordance with previously determined Bragg reflections associated with SNPs [14, 23]. Particularly, it has been reported that the reactivity of SNPs is favored by high-atom-density facets such as (111) [24]. Following the main goals of this experiment, we investigated inhibitory effects of the biologically synthesized SNPs on the growth of the highly infectious bacterium *S. aureus* and expression of *cap8* gene cluster as a major virulence factor of the bacterium. Results of microdilution assay showed that SNPs could fully inhibit bacterial growth at a concentration of 50µg/ml. This finding is in line with previous studies demonstrating antibacterial properties of SNPs [6, 20, 25, 26]. In contrast to many of previous studies limited to evaluation of the SNPs effect on bacterial growth, we extended our research by quantification of *cap8* gene expression under treatment with SNPs. The results obtained in Real-Time PCR assay showed that SNPs, even at an as low concentration as 12.5µg/ml, decrease *cap8* expression. Again, a dose-dependent behavior was observed for antagonistic effects of SNPs on the expression of this virulence gene. This finding is of great biomedical importance because it shows that SNPs not

only inhibit growth but also negatively affect the expression of a virulence factor of *S. aureus*. As mentioned by Ragle and Wardenburg, neutralization of the main virulence factors is an effective way to find effective preventative and therapeutic agents to combat staphylococcal infection [27].

Silver occupies a high position among inorganic antibacterial agents. Infectious bacteria are unlikely to develop resistance against silver because this metal has a negative impact on a broad range of targets in the bacteria, suggesting that pathogens have to develop a large number of mutations simultaneously to protect themselves [3]. Our results are also in line with those reported by Li *et al.* (2012) that found out that dressing endotracheal tube coated with silver is highly effective in preventing ventilator-associated pneumonia among adults [28]. Moreover, compared to bulk metal silver, SNPs are safe and nontoxic; thus they can be widely used dressing agents in manufacturing various medical devices ranging from dental bandages, catheters, and bone cement [1] to cardiovascular implants

[29]. Particularly, regarding inhibitory effects of SNPs on growth and virulence properties of *S. aureus*, these nanoparticles can be effectively used in the development of wound dressings for curing staphylococcal skin infections.

Conclusion

The results of this research revealed the potential utility of SNPs in formulating new antimicrobial drugs addressing the problem of occurrence of resistant bacterial strains. Medical implications of these results may include the development of silver-based creams and ointments for curing staphylococcal lesions, new types of disinfecting solutions, silver-containing dressings in treating burns, dressing endotracheal tubes and urinary catheters, and dental instruments and bandages.

Conflict of Interest

There is no conflict of interest.

References

1. Ge L, Li Q, Wang M, Ouyang J, Li X, Xing MM. Nanosilver particles in medical applications: synthesis, performance, and toxicity. *Int J Nanomedicine*. 2014; 9:2399.
2. Sadeghi B, Garमारoudi FS, Hashemi M, Nezhad HR, Nasrollahi A, Ardalan S, Ardalan S. Comparison of the antibacterial activity on the nanosilver shapes: nanoparticles, nanorods and nanoplates. *Adv. Powder Technol.* 2012; 23(1):22-6.
3. Iravani S. Green synthesis of metal nanoparticles using plants. *Green Chem.* 2011;13(10):2638-50.
4. Manikprabhu D, Cheng J, Chen W, Sunkara AK, Mane SB, Kumar R, Hozzein WN, Duan YQ, Li WJ. Sunlight mediated synthesis of silver nanoparticles by a novel actinobacterium (*Sinomonas mesophila* MPKL 26) and its antimicrobial activity against multi drug resistant *Staphylococcus aureus*. *J. Photochem. Photobiol. B: Biology* 2016; 31:158:202-5.
5. Prabha G, Raj V. Formation and characterization of β -cyclodextrin (β -CD)-polyethyleneglycol (PEG)-polyethyleneimine (PEI) coated Fe₃O₄ nanoparticles for loading and releasing 5-Fluorouracil drug. *Biomed. Pharmacother.* 2016; 31:173-82.
6. Rao NH, Lakshmidēvi N, Pammi SV, Kollu P, Ganapaty S, Lakshmi P. Green synthesis of silver nanoparticles using methanolic root extracts of *Diospyros paniculata* and their antimicrobial activities. *Mater. Sci. Eng., C.* 2016; 62:553-7.
7. Durán N, Durán M, de Jesus MB, Seabra AB, Fávaro WJ, Nakazato G. Silver nanoparticles: A new view on mechanistic aspects on antimicrobial activity. *Nanomed Nanotech Biol Med.* 2016; 12(3):789-99.
8. Li W, Ulm H, Rausch M, Li X, O'Riordan K, Lee JC, Schneider T, Müller CE. Analysis of the *Staphylococcus aureus* capsule biosynthesis pathway in vitro: characterization of the UDP-GlcNAc C6 dehydratases CapD and CapE and identification of enzyme inhibitors. *Int. J. Med. Microbiol.* 2014; 304(8):958-69.

9. Luong TT, Ouyang S, Bush K, Lee CY. Type 1 capsule genes of *Staphylococcus aureus* are carried in a staphylococcal cassette chromosome genetic element. *J. Bacteriol.* 2002;184(13):3623-9.
10. Rajeshkumar S, Malarkodi C, Vanaja M, Annadurai G. Anticancer and enhanced antimicrobial activity of biosynthesized silver nanoparticles against clinical pathogens. *J. Mol. Struct.* 2016;1116:165-73.
11. Mahdih M, Zolanvari A, Azimee AS. Green biosynthesis of silver nanoparticles by *Spirulina platensis*. *Sci. Iran.* 2012; 19(3):926-9.
12. Xiang H, Qiu JZ, Wang DC, Jiang YS, Xia LJ, Deng XM. Influence of Magnolol on the Secretion of α -Toxin by *Staphylococcus aureus*. *Molecules.* 2010; 15(3):1679-89.
13. Mandal D, Bolander ME, Mukhopadhyay D, Sarkar G, Mukherjee P. The use of microorganisms for the formation of metal nanoparticles and their application. *Appl Microbiol Biotechnol.* 2006; 69(5):485-92.
14. Prasad TN, Kambala VS, Naidu R. Phyconanotechnology: synthesis of silver nanoparticles using brown marine algae *Cystophora moniliformis* and their characterisation. *J Appl Phycol.* 2013; 25(1):177-82.
15. Jena J, Pradhan N, Dash BP, Panda PK, Mishra BK. Pigment mediated biogenic synthesis of silver nanoparticles using diatom *Amphora* sp. and its antimicrobial activity. *J. Saudi Chem. Soc.* 2015; 19(6):661-6.
16. Fenfen LÜ, Yixian GA, HUANG J, Daohua SU, Qingbiao LI. Roles of biomolecules in the biosynthesis of silver nanoparticles: case of *Gardenia jasminoides* extract. *Chin. J. Chem. Eng.* 2014; 22(6):706-12.
17. Kannan N, Subbalaxmi, S. Biogenesis of nanoparticles-a current prospective. *Rev. Adv. Mater. Sci.* 2011; 27: 99–114
18. Ahmed S, Ahmad M, Swami BL, Ikram S. A review on plants extract mediated synthesis of silver nanoparticles for antimicrobial applications: A green expertise. *J. Adv. Res.* 2016 ;7(1):17-28.
19. Sharma B, Purkayastha DD, Hazra S, Gogoi L, Bhattacharjee CR, Ghosh NN, Rout J. Biosynthesis of gold nanoparticles using a freshwater green alga, *Prasiola crispa*. *Mater. Lett.* 2014;116:94-7.
20. Yasin S, Liu L, Yao J. Biosynthesis of silver nanoparticles by bamboo leaves extract and their antimicrobial activity . *J Fiber Bioeng Inform.* 2013; 6(1):77-84.
21. Matsumura Y, Yoshikata K, Kunisaki SI, Tsuchido T. Mode of bactericidal action of silver zeolite and its comparison with that of silver nitrate. *Appl. Environ. Microbiol.* 2003; 69(7):4278-81.
22. Morones JR, Elechiguerra JL, Camacho A, Holt K, Kouri JB, Ramírez JT, Yacaman MJ. The bactericidal effect of silver nanoparticles. *Nanotechnol.* 2005;16(10):2346.
23. Dhanalakshmi PK, Azeez R, Rekha R, Poonkodi S, Nallamuthu T. Synthesis of silver nanoparticles using green and brown seaweeds. *Phykos.* 2012; 42(2):39-45.
24. Pal S, Tak YK, Song JM. Does the antibacterial activity of silver nanoparticles depend on the shape of the nanoparticle? A study of the gram-negative bacterium *Escherichia coli*. *Appl. Environ. Microbiol.* 2007;73(6):1712-20.
25. MubarakAli D, Thajuddin N, Jeganathan K, Gunasekaran M. Plant extract mediated synthesis of silver and gold nanoparticles and its antibacterial activity against clinically isolated pathogens. *Colloids Surf B Biointerfaces.* 2011;85(2):360-5.
26. Sadeghi B. Synthesis of silver nanoparticles using leaves aqueous extract of *Nasturtium Officinale* (NO) and its antibacterial activity. *Int. J. Mol. Clin. Microbiol.* 2014; 2: 428-434
27. Ragle BE, Wardenburg JB. Anti-alpha-hemolysin monoclonal antibodies mediate protection against *Staphylococcus aureus* pneumonia. *Infect Immun.* 2009;77(7):2712-8.
28. Li X, Yuan Q, Wang L, Du L, Deng L. Silver-coated endotracheal tube versus non-coated endotracheal tube for preventing ventilator-associated pneumonia among adults: A systematic review of randomized controlled trials. *J. evid. based med.* 2012 ;5(1):25-30.
29. Andara M, Agarwal A, Scholvin D, Gerhardt RA, Doraiswamy A, Jin C, Narayan RJ, Shih CC, Shih CM, Lin SJ, Su YY. Hemocompatibility of diamondlike carbon-metal composite thin films. *Diam Relat Mater.* 2006;15(11):1941-8.