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Antibacterial activity of silver-nanoparticles against *Staphylococcus aureus*

Mahsa Ydollahi[1], Hamed Ahari[1]* and Amir Ali Anvar[2]

[1]Department of Food Science and Technology, Tehran Science and Research Branch, Islamic Azad University, Tehran, Iran.
[2]Department of Food Hygiene, Tehran Science and Research Branch, Islamic Azad University, Tehran, Iran.

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In this study, the antibacterial activity of silver nanoparticles (Ag-NPs) as Ag-nano disc was assessed with respect to Gram positive bacteria. Scanning electron microscope (ESM) was used for *Staphylococcus aureus* by measuring the diameter of inhibition zones in culture media and Ag-NPS. Eventually, gauzes containing strain were further impregnated between 2-layer nanofilms at 1, 3, 5 and 7 ppm sized 2 × 2 cm and placed inside the sterile Petri dish for each. The mean diameter (mm) of the inhibition zones surrounding the discs were significantly different (*p*= 0.000, F=5971.57) and varied from 2.41 to 6.19 mm and the bacteria *S. aureus* was sensitive to all Ag-NPs concentrations. The inhibited bacterial growth for 1 ppm of Ag nanodisk (2.51±0.01 mm) was less than 3 (3.21±0.02), 5 (4.27±0.01) as well as 7 ppm (6.00±0.02 mm). It was concluded that the best concentration of Ag nanodisc that can inhibit the growth of *S. aureus* is 7 ppm at the size of 80-120 mm for about 24 h after inoculation.

Keywords: *Staphylococcus aureus*, silver nanoparticles, antibacterial activity.

INTRODUCTION

With the occurrence and increase in bacterial resistance to most antibiotics and with emphasis on health cost, many investigators focus on low cost or free resistance of effective antimicrobial compounds (Jones et al., 2004). Such constraints have led health strategist to the renaissance in the use of Ag-based composites. The historian, Herodotus reported that Cyrus, the Great King of Persia from 559 to 530 B.C, had water drawn from a watercourse, had it boiled and carried in silver containers placed on numerous four-wheeled carriages drawn by mules that followed the king wherever he visited at any time (USEPA, 2012). Nanotechnology, an enabling technology involving the characterization and utilization of constitution or materials that are clusters of silver atoms ranging in diameter from 1 to 100 mm (Neethirajan and Jayas, 2011), is used as antibacterial in medical applications (Ozcalik and Tihminlioglu, 2013). Owing to inhibiting bacterial growth by foods with longer conservation, Ag-NPs (Ag-NPs) has been incorporated into a range of food contact equipment such as, keeping bags and fruit packages, but the medical industry has been slow to develop the property of Ag-NPs in infection

*Corresponding author. E-mail: Dr.h.ahari@gmail.com.

prophylaxis. However, it is currently used in an increasing number of consumer and medical applications (Chaloupka et al., 2010). Nanoparticles usually have been known to inhibit the bacterial growth and show better performance than the whole materials of the same elements in a major health problem. Silver ion has long been known to possess inhibitory and bactericidal activity on approximately 15 species containing bacteria (Feng et al., 2000). This potential could be due to the interaction between silver ion and the thiol group (sulphydryl-SH) of the bacterial cell membrane (Liau et al., 1997). The size of nanoparticles is very crucial in its bactericidal effect. The smaller the Ag-NPs size, the more the antibacterial activity increases. Small nanoparticles with a great contact area to volume ratio supply sufficient performance for the bactericidal property even at low concentration (Rai et al., 2009; Wijnhoven et al., 2009).

Silver sulfadiazine and silver nitrate have been widely used in superficial and deep dermal burns of wounds and for the removal of warts (Li et al., 2006; Wijnhoven et al., 2009). Silver is a more toxic metal to bacteria than many elements such as Ag >Hg >Cu>Cd>Cr >Pb> Co>Au>Zn>Fe (Zhao and Stevens, 1998).

Nanosilver shows a new generation of bactericidal and kills both Gram-negative and Gram-positive bacteria including *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis*, (Sondi and Salopek-Sondi, 2004; Cho et al., 2005; Kim et al., 2011). It is also shown that chitosan nanoparticles and films have antimicrobial effects on *Listeria monocytogenes* and *S. aureus* (Rezaei and Kasra kermanshahi, 2013). *S. aureus* known as a facultative anaerobic gram-positive coccal bacterium is also an important cause of community and hospital-acquired infection. Methicillin-resistant infections caused by *S. aureus* are mainly nosocomial and are increasingly reported from many countries worldwide (Lowy, 1998). It is frequently part of the skin flora found in the nose and on the skin, and in this manner about 20% of the human population are long-term carriers of *S. aureus* (Ossowski et al., 2006). *S. aureus* is an important pathogen in the healthcare sector that has not been omitted from the hospital nor community environment. In humans, *S. aureus* causes superficial wounds in the skin and localized blisters, septicemia, septic arthritis, invasive endocarditis, and pneumonia (Ayala-Núñez et al., 2009).

The purpose of this study was to examine the antibacterial activity of different concentration of silver nanoparticles as antibiotic discs against *S. aureus* by measuring the diameter of inhibition zones in culture media.

MATERIALS AND METHODS

Silver nanofilms

Nanofilms as thin coating tangles infused with certain concentrations of 1, 3, 5 and 7 ppm silver compounds were produced after changing in the line of Nano Nasb Pars Company, Tehran, Iran. They were afterward transported to the laboratory and kept away from light for antimicrobial purposes.

Bacteria strain and measurement of colony-forming unit (CFU)

A Gram-positive bacteria, *S. aureus* with the concentration of 10° $CFU.mL^{-1}$ (ATCC 6538) was obtained from Iranian type Culture Collection to test the antibacterial activity of Ag nanocomposite film. The bacteria were kept at -80°C (stock solution), was cultured twice in Tryptic Soy Broth and incubated for 24-48 h at 37°C and consequently were sub-cultured on non-selective culture media, Tryptic Soy Agar and incubated for 24-48 h at 37°C to isolate the target colonies and suspended in saline solution (0.80% w/v). The bacterial suspension was attuned to the turbidity of McFarland standard solution 0.5, resulting in inoculums containing approximately 1×10^{8} CFU.mL.

A bacterial inoculum of *S. aureus* was sub-cultured in Baird Parker Agar (BP, Difco). Following this, discs (1 cm diameter) of each treatment for nanocomposite films were placed on the surface of the aforementioned inoculated agar culture media. Petri dishes with microorganism and discs of nanocomposite films were incubated at 37°C for 24 h. The antimicrobial activity of nanocomposite films was determined by measuring the inhibition zone around each disc of films (mm).

Disk diffusion assay

A disk diffusion method was used to assay the Ag-NPs for antibacterial activity against test strains. In the first stage, pure cultures of the bacteria were prepared in the liquid medium, buffered peptone water to ensure that the anti-bacterial property of nanofilms could be performed. The inoculates were prepared by diluting the overnight cultures with 0.9% NaCl to a 1 McFarland standard and were applied to the plates along with the standard. The sterile sample pieces of gauze with the definite size (2x2 cm) were prepared, allowed to soak into the liquid medium containing the bacteria on a rotary shaker (200 rpm) at 37°C and prepared disks containing differing amounts of Ag-NPs. Every piece of gauze soaked in pure culture and strain were further saturated between 2 layer nanofilms at 1, 3, 5 and 7 ppm sized 2x2 cm and each were placed inside the sterile Petri dish. Immediately, sterile swabs were carried out from the gauze samples and linearly culture was done on a nutrient agar medium. The same action (streaking) was carried out between 24 h intervals to 72 h. At that point they were incubated at 37°C for 24 h. Eventually, the results of Log_{10} CFU and the zones of inhibition (mm) were measured and recorded and the assays were performed in nine replicates (Lansdown, 2002).

Scanning electron microscope (SEM)

An SEM (Vega Tescan, USA) in which the reflected light determines the size of nano-particles that are invisible to the eye, was used in assaying the morphological measurement of nanoparticles. Master Batches or the constituent particles were dissolved in an acetonitrile solvent, to make a water base solution. These dissolved ingredients in the acetonitrile were put on two metal legs in a device called sputter coater. Nanoparticles were sputter coated with gold prior to the SEM examination (Thakur and SiṅGha, 2013).

Statistical analysis

All bacterial counts were expressed as log_{10} colony forming units per gram (log_{10} CFU). The mean log_{10} values were calculated on

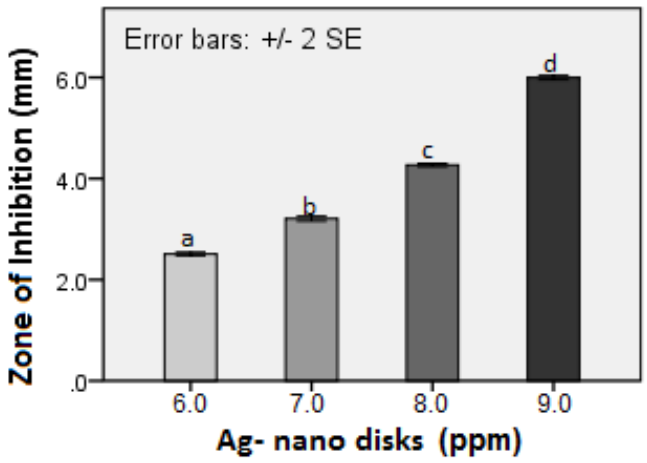

Figure 1. Zone of inhibition (mm) for antibacterial activity of different concentrations of Ag-nano discs against *S. aureus*.

the assumption of normal distribution. The effect of the nano-composite film at the different hours (Time × Nano) was analyzed with the GLM, Repeated Measurement ANOVA using SPSS 18. The greater and lower values of Pillai's Trace and Wilks' Lambda respectively showed more effectiveness in the independent factors (different concentrations of Ag nano film) on the bacteria inactivation while the data were analyzed at the level of α= 0.05. On the other hand, analysis of variance for the antibacterial action of the nanoparticles was carried out at different concentrations, by comparing the mean diameter of the inhibition zones. These measurements were also included measuring the frequency of the nano-particle size counted under the SEM.

Table 1. Estimated Marginal Means of $\log_{10}$ values of colony-forming units (n=9).

Ag-nano disc (ppm)	Time (h)	Mean±SE	95% confidence interval	
			Lower bound	Lower bound
Control	0	7.32±5.23	7.31	7.33
	24	7.51±4.91	7.52	7.52
	48	7.64±4.92	7.64	7.65
	72	7.74±5.26	7.75	7.75
1	0	7.32±5.23	7.31	7.33
	24	6.53±4.91	6.51	6.55
	48	7.66±4.92	7.66	7.66
	72	4.92±5.26	7.73	7.74
3	0	7.32±5.23	7.31	7.33
	24	5.49±4.91	5.14	5.68
	48	6.65±4.92	6.64	6.67
	72	7.74±5.26	7.74	7.74
5	0	7.32±5.23	7.31	7.33
	24	4.55±4.91	-5.14	5.32
	48	5.68±4.92	5.49	5.82
	72	7.76±5.26	7.76	7.77
7	0	7.32±5.23	7.31	7.33
	24	3.56±4.91	-5.23	5.25
	48	4.69±4.92	-5.09	5.35
	72	7.77±5.26	7.78	7.79

RESULTS

The results of preliminary test, multivariate statistical assess showed a strong and significant effects of independent variables, time (h) and concentration value of Ag nano disc (ppm) on dependent variable diameter of inhibition zone (mm) and counting the log $_{10}$ CFU of *S. aureus* (Pillai's Trace Value=2.90, Wilk's Lambda=0.00). The antibacterial effect of Ag-NPs as against *S. aureus* prepared on bacterial cultures and evaluation of their inhibitory diameters are shown in Figure 1.

According to the results of the antibacterial activity for the discs impregnated with 6, 7, 8 and 9 ppm of Ag-NPs, the mean diameter (mm) of the inhibition zones surrounding the discs was significantly different (*p*= .000, F=5971.57) from 2.41 to 6.19 mm with the bacteria *S. aureus* being sensitive to all Ag-NPs concentrations (Figure 1). The inhibited bacterial growth for 1 ppm of Ag-Nano disk (2.51±0.01 mm) was less than 3 ppm (3.21±0.02) and 5 ppm (4.27±0.01) as well as 7 (6.00±0.02 mm). Table 1 shows that the effect of interaction between the two variables (Time- nanosilver) on the measures of $\log_{10}$ CFU for *S. aureus.* Accordingly, the marginal means was significant for the 5 and 7 ppm at 24 and 48 h respectively, at which the estimated marginal mean values ($\log_{10}$) of CFU for the mentioned groups included zero at 95% confidence interval. However, the marginal mean value for the 7 ppm of Ag nanodisc (3.56±4.91) was obviously less than that of 5 ppm of Ag nanodisc (4.55±4.91) at 24 h. At 48 h, only its value for the 7 ppm of Ag nanodisc was significantly (*p*= .000) impressive and (*p*= .000) reached to 4.69±4.92 and increased to 7.77±5.26 at 72 h. As shown in Figure 3, the size of particles distributed with 15 kv and 5-10 kx magnifications was equal to 0.08 to 0.12 µm which infer on the size of 80-120 nm for the mean value of nano-particles in the discs.

DISCUSSION

An increasing trend of the inhibition zones diameter for the different Ag-nano concentrations from 1 to 7 ppm of Ag nano discs was seen. Based on the results given in Table 1, simultaneous interaction of the different concentrations of Ag nano discs (ppm) and time (h) were not significantly (*p*= .000) effective in reducing the marginal means of $\log_{10}$ values of CFU for groups,

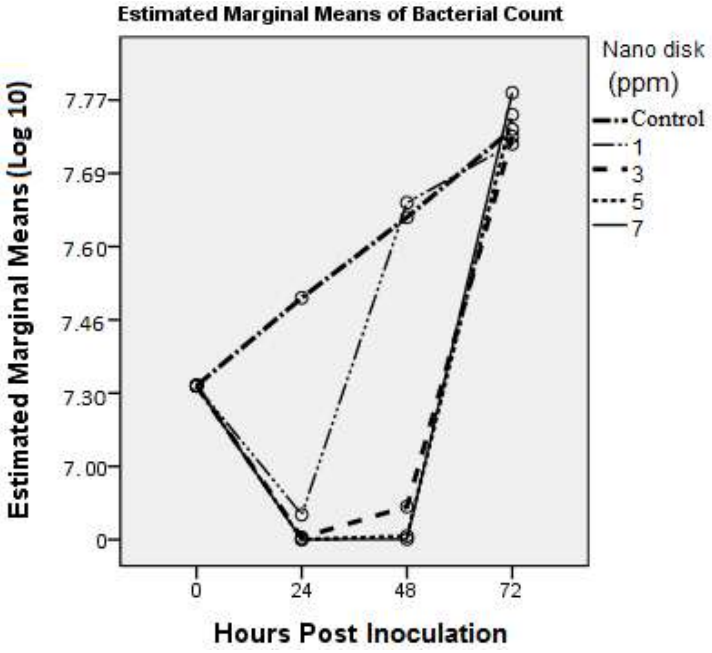

Figure 2. The effect of Ag-nano discs on estimated marginal means of log $_{10}$ CFU of *S. aureus.*

control, 1 and 3 ppm of Ag nano discs. An investigation (Rezaei and Kasra, 2013) representing a 3-log decrease from 6.0 at the beginning to 2.0 $\log_{10}$ CFU for about 16 h was constant till the end of the study (24 h), in exposing *S. aureus* with nanodisc of chitosan. This result is similar to our finding (marginal mean of $\log_{10}$ CFU) for 7 ppm of Ag nano disc for about 24 h but increased at 48 h in this study. Jung et al. (2008) showed reductions of more than 5 log10 CFU/ml of both *S. aureus* and *E. coli* bacteria that were confirmed after 90 min of treatment with the silver ion solution. Niakan et al. (2013) indicated that the zone of inhibition (mm) against *S. aureus,* with Ag-nanoparticles concentrations of 0.025 up to 0.0125 µg were 8.0 and 0.0 mm, respectively. Similar to our results, Shahverdi et al. (2007) confirmed the bactericidal property of Ag-NPs against *S. aureus* and on the other hand emphasized the presence of some antibiotics along with the Ag-NPs. Also, the results from a similar research (Kim et al., 2011) showed the minimum and more stable growth of *S. aureus* up to 8 h occurred with concentrations of 100 and 150 µg/ml of Ag-NPs. The results for Ag nano discs 5 and 7 ppm displayed significant (p= 0.000) decrease for the $\log_{10}$ CFU against *S. aureus* up to 24 and 48 h, respectively. Contrary to the present finding, Cho et al. (2005) indicated the antibacterial activity of Ag-NPs with 10 and 20 ppm is effective at 1 h after inoculation, being constant at the level of 3 logs CFU and reached 0.0 log CFU at the end of study (5 h). Accordingly, it showed that the stability property of Ag-NPs is remarkably dependent on the media culture PH while in this study and similar to Niakan et al. (2013), time was the important factor. In accordance with the result of Figure 2, the $\log_{10}$ CFU for 3, 5 and 7 ppm of Ag nano discs were effectively decreased at 24 h but statistically, the interaction between time and concentration of Ag-NPs was not significant for 1 ppm Ag-nano disc. Accordingly, the effectiveness of 5 and 7 ppm Ag-nano discs was remarkably decreased after 48 h, and only the interaction between time and concentration of Ag-NPs was significant for 7 ppm Ag-nano disc. The efficacy of the nano discs to decrease the log $_{10}$ marginal mean obviously decreased when it got to 72h. An investigation (Kim et al., 2007) was made on the effect of Ag-NPs on deactivating the yeast, *E. coli,* and *S. aureus*. Its results showed the inappropriate antibacterial activity for *S. aureus in contrary to* the yeast and *E. coli*, which is similar to our results that shows that the appropriate antibacterial activity for *S. aureus* was diminished after 24 h. This finding is also in agreement with the result of Guzman et al. (2012), which indicated Ag-NPs reasonable bactericidal activity against *E. coli*, *P. aeruginosa* and *S. aureus*. Also, Li et al. (2011) demonstrated that Ag-NPs passed the cell wall and interfered with the enzymatic formation. Subsequently, it entered the bacteria cell, condensed the DNA and prevented bacteria production.

Increasing the food life span using nano covers can be done by decreasing the size of materials lesser than 100 nanometer and that their properties follow the quantum

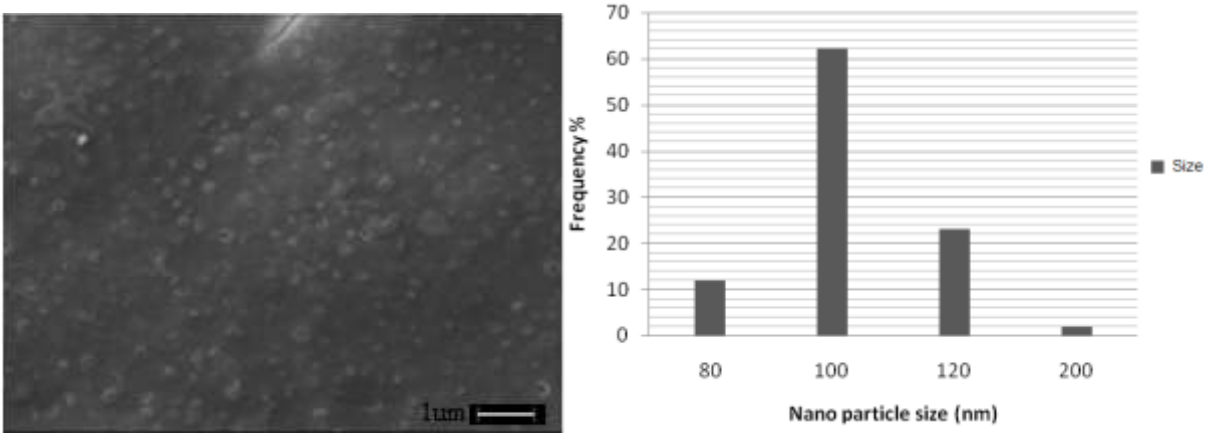

Figure 3. Left) Scanning electron microscope (SEM) of Ag-NPs, EHT=15.0, WD=10 mm, Mag. =10.0 kx; Particle size histogram of the silver particles is shown in the right hand picture.

physics rules, similar to the finding of Birla et al. (2009). These findings are in accordance with the results of Shahbazzadeh et al. (2011) who concluded that nanosilver has growth inhibiting on cancer cells two times more compared to natural and normal cells in the cases of Mesenchymal stem cells, natural fibroblast (HF2) and osteoblast (G292).

It is therefore concluded, the probability that the best concentration of Ag- nanodisc can inhibit the growth of *S. aureus* is 7 ppm at the size of 80-120 nm up to 24 h after inoculation and the present study results suggest that the efficacy of the Ag-NPs is decreased after the aforementioned time.

Conflict of interest

The authors have not declared any conflict of interest.

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