



Biofilm Removal and Antimicrobial Activities of Agar Hydrogel Containing Colloid Nano-Silver against *Staphylococcus aureus* and *Salmonella typhimurium*

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ABSTRACT

Background: Antibacterial and biofilm removal effects of agar hydrogel incorporating silver nanoparticles (SNP) at various concentrations were studied against *Staphylococcus aureus* and *Salmonella typhimurium* *in vitro*.

Methods: The minimum inhibitory concentrations (MIC) of SNP was determined by agar dilution method. Then, hydrogels were prepared by mixing of 0.5% w/v agar and SNP (1/2 MIC, MIC, and 2 MIC) and their inhibitory efficacies against planktonic and biofilm forms of bacteria were measured using agar spot and microtiter test, respectively.

Results: The MIC value was 125 µg/ mL for both bacteria. All SNP hydrogels represented antibacterial activity against *Staphylococcus aureus* and *S. typhimurium* on agar culture, which was significant compared to control group (silver sulfadiazine cream). The developed biofilm of *S. aureus* and *S. typhimurium* were strongly (85% reduction) and moderately affected (60% reduction) by SNP hydrogels during 15 min contact time, respectively. A dose-dependent biofilm reduction was not demonstrated when different SNP concentrations were tested. Moreover, the results from this study confirmed the moderate sanitizing ability of SNP loaded hydrogel against planktonic forms of both bacteria, which SNP (2MIC) hydrogel decreased only 2.3 log₁₀ CFU/ mL in a primary population of *S. typhimurium* during 15 min exposure time.

Conclusion: We recommended SNP incorporated agar hydrogel as an effective biofilm removal sanitizer.

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Introduction

Hydrogels are a three-dimensional network formed from cross-linked hydrophilic polymers. Each hydrogel has its own characteristics, which should be considered when using them as a carrier of functional compounds. Polymer selection for hydrogel preparation depends on 1) the amount of water to be absorbed by the hydrogel and 2) the method of polymer chains connection within the gel network (1, 2). For hydrogel preparation, a variety of natural and synthetic polymer compounds may be used separately or in combination with each other (3). Anionic polymers (carrageenan and pectin), cationic (chitosan and poly-L-lysine), amphoteric (collagen and gelatin) and neutral (dextran and agar) are among the most important compounds used for the preparation of hydrogel (4, 5). The ability of hydrogels to control microorganisms may be improved by incorporating antimicrobial compounds. Many antimicrobials have been reported to improve antimicrobial effectiveness of gels (6-9).

Silver nanoparticles (SNPs) are widely known as highly toxic to 16 major species of bacteria due to their multiple mechanisms of action. The small size and high surface to the volume ratio of SNPs, allowing them to contact with microorganisms, and represent antimicrobial activity (6, 10). In this case, SNPs attack cellular membranes and interrupt membrane normal performance. Metal nanoparticles also interacting with sulfur-containing proteins and damage phosphorus-containing compounds like DNA (11).

Currently, research dealing with the use of gel in sanitation schedule are on the increase. Due to heavy microbial contamination, food plants can be recognized as the target industry to use gel-sanitation technology for cleaning and disinfection. The technology offers many advantages over traditional sanitation methods, including, easy and safe operation, less use of detergents or sanitizers and the high speed and performance. Contrary to the foam sanitation which needs the physical addition of air into sanitizing solution, gel formation resulting from

internal interactions that occur by adding water into a gel-producing material. This polymeric network could be used as a place to incorporate sanitizer or detergent (12).

The aim of this study was to assess the antibacterial and biofilm removal properties of agar hydrogel prepared by incorporation of different concentrations of SNPs. We also investigated the sanitizing activity of SNP hydrogels against *Staphylococcus aureus* and *Salmonella typhimurium*.

Materials and Methods

Materials

Ready-to-use nanoparticles solution (4000 µg/mL with the size of 35 nm) was purchased from Pars Nano Nasb Co (Tehran, Iran). The stock solution was sterilized by filtration through 0.22 µm filters. Luria-Bertani (LB) broth and agar, Mueller Hinton agar, peptone water, phosphate buffered saline (PBS) and Agar-agar were obtained from Sigma Chemical Co (St. Louis, MO., USA) while all chemicals were acquired from Merck (Darmstadt, Germany). *Staphylococcus aureus* ATCC 25923 and *Salmonella typhimurium* ATCC 14028 were supplied by the Department of Food Hygiene and Quality Control, Urmia University, Urmia, Iran.

Preparation of bacterial suspension

Ten microliters of frozen stocks (at -20 °C) of *S. aureus* and *S. typhimurium* were transferred to 10 mL of LB broth and incubated at 37±1 °C for 24 h. The CFU/mL of the bacterial suspensions was adjusted via spectrophotometer (Amersham Pharmacia Biotech Inc., Buckinghamshire, UK) at 600 nm and confirmed by plating and counting on LB after 24 h at 37±1 °C.

Antimicrobial testing of SNP

Agar dilution method was selected to determine the minimal inhibitory concentration (MIC) of

SNP against *S. aureus* and *S. typhimurium*. Firstly, serial two-fold dilution of the SNP was dispensed in cooled (48 ± 1 °C) molten agar to achieve final concentrations ranging from 7.8 to 200 µg/ mL. Tubes were mixed well before pouring into Petri dishes and then the agar allowed to dry. Two microliters of bacterial suspension (10^5 CFU/mL per drop) was dropped on the surface of the agar. The inoculum was allowed to be absorbed before incubation at 37 ± 1 °C for 24 h. The MIC was reported as the lowest SNP concentration at which there is no visible growth of the bacteria.

Hydrogel preparation

Agar hydrogel was prepared according to Incoronato et al. (2011) (13) with modification. Hydrogel developed by adding 5 grams agar into one liter distilled water. The final solution was autoclaved at 121 °C for 15 min. SNP at different concentrations was poured in molten agar at 50 °C achieving final hydrogels with $\frac{1}{2}$ MIC, MIC, and 2 MIC of SNP. Control hydrogels were developed identically but without the addition of SNP.

Antimicrobial testing of hydrogels

Antimicrobial activity of hydrogels was performed by spot test. Mueller Hinton agar plates were swabbed with a bacterial suspension (106 CFU/ mL) and then, 10 µL of hydrogels containing 0, $\frac{1}{2}$ MIC, MIC and 2 MIC concentrations of SNP was placed on inoculated agar. After incubation at 37 ± 1 °C for 24 h, the zone surrounding the droplets was measured. Silver sulfadiazine cream was used as control (14). The same steps were performed with $\frac{1}{2}$ MIC, MIC and 2 MIC concentrations of SNP to investigate antibacterial activity of SNP in alone.

Biofilm removal properties of hydrogels

Biofilm properties of SNP hydrogels were examined by Microtiter plate (MTP)-based systems using according to the procedure

described by Mahdavi et al. (2007) (15) Reduction percent of biofilms was according to this formula:

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

C= OD540nm of control wells, B= OD540nm of negative controls and T= OD540nm of treated wells.

The same steps were performed with $\frac{1}{2}$ MIC, MIC and 2 MIC concentrations of SNP to investigate the biofilm removal activity of SNP in alone.

Sanitizing properties of hydrogels

Sanitizing effects of SNP- agar hydrogels were investigated according to Phongphakdee & Nitisisinprasant (2015), (16) with some adaptations. Bacterial cultures (1×10^7 CFU/ mL) (0.5 mL) were added to tubes containing 4.5 mL of either agar hydrogel or hydrogel with SNP at a final concentration of $\frac{1}{2}$ MIC, MIC, and 2 MIC. Tubes without hydrogel were used as a control. All tubes were kept at 23 ± 2 °C for 15 min and then samples from each tube were taken, serially diluted in 0.1 % peptone water, and plated on LB agar. All plates were then incubated for 24 h at 37 °C, and CFU were counted. The same steps were performed with $\frac{1}{2}$ MIC, MIC and 2 MIC concentrations of SNP to investigate the sanitizing activity of SNP in alone.

Statistical analysis

Each experiment was replicated three times and data were expressed as means \pm S.E. Data were analyzed by analysis of variance (ANOVA, P<0.05) with Newmann Keuls posttest using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA (www.graphpad.com).

Results

Antimicrobial activity of SNP

In the current study, and in terms of applicability and relevance, the agar dilution method was preferred to broth dilution for the rapid antimicrobial susceptibility. The MIC of SNP against both *S. aureus* and *S. typhimurium* was 125 $\mu\text{g mL}^{-1}$, which suggest that Gram-positive is as susceptible to SNP as Gram negative bacteria.

Antimicrobial properties of hydrogels

Images of agar hydrogel and SNP hydrogels dispersed on the surface of stainless steel are given in Figure 1. SNP hydrogels were yellowish in color and translucent with no signs of grittiness. Antimicrobial effectiveness of hydrogels was observed in terms of the zone of inhibition conducted according to the spot method. The zone of inhibition increased with the increasing SNP concentration in a hydrogel with no statistic significant trend (Table 1) (Figure 2).

According to criteria proposed by Villanueva et al. (2016) (7) the inhibition zone of all hydrogels prepared in this study stand as good, display the sufficient release of SNP to the media. The property of “good” applied for hydrogels if the inhibitory zone is observed both around and under the spotted gel.

Biofilm removal properties of hydrogels

The results showed that the SNP loaded hydrogels had a more effect on biofilm than SNP under similar conditions. It is clear from Figure 3 that increase of the concentration of SNP, for one-day-old biofilm of both bacteria, resulted in no increase in biofilm activity. In the case of *S. typhimurium*, by increasing the concentration of SNP, the biofilm removal properties of SNP were significantly decreased ($P < 0.05$). This could be shown by a decrease from 52 % to 40 % when the concentration was increased from $\frac{1}{2}$ MIC to 2 MIC. However, in contrast to the biofilm removal

activity of SNP, by increasing the concentration of SNP in hydrogel the biofilm removal effect did not decrease.

Sanitizing properties of hydrogels

Figure 4 show the susceptibility of *S. aureus* and *S. typhimurium* to different concentrations of SNP and SNP-hydrogels during 15 min contact time. Statistically significant difference between control and treated groups were shown. In contrast to solid medium, increasing concentrations reduced the antimicrobial effects of SNP on both bacteria in a liquid medium. At $\frac{1}{2}$ MIC concentration, SNP exhibited up to 1.5 and 1.1 log reductions in 15 min on *S. aureus* and *S. typhimurium* population, respectively, while the 2 MIC concentration did not change the viable population of both bacteria after 15 min.

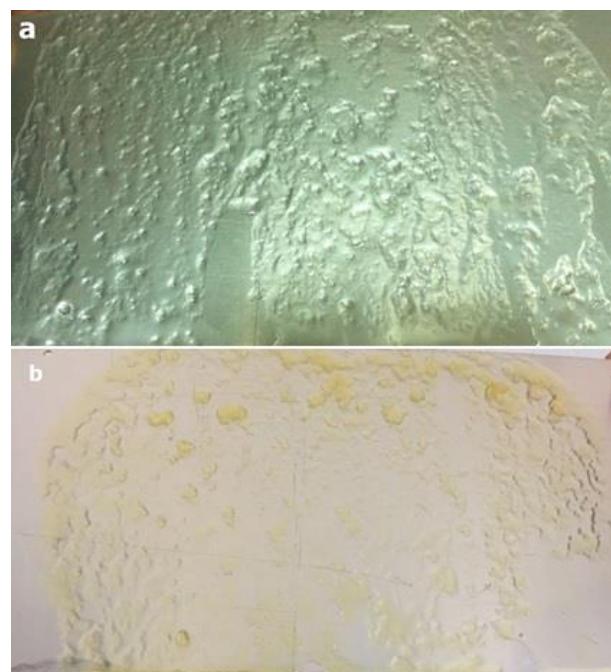


Figure 1. Lateral view of the agar hydrogel (a) and hydrogel containing silver nanoparticles (b) spread on the surface of stainless steel.

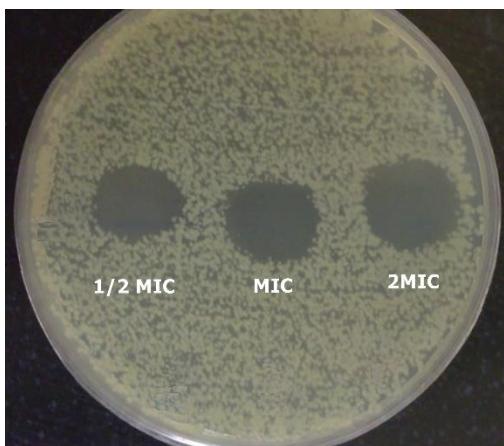


Figure 2. Zone of inhibition of different concentration of SNP loaded agar hydrogel according to spot test. MIC: minimum inhibitory concentrations.

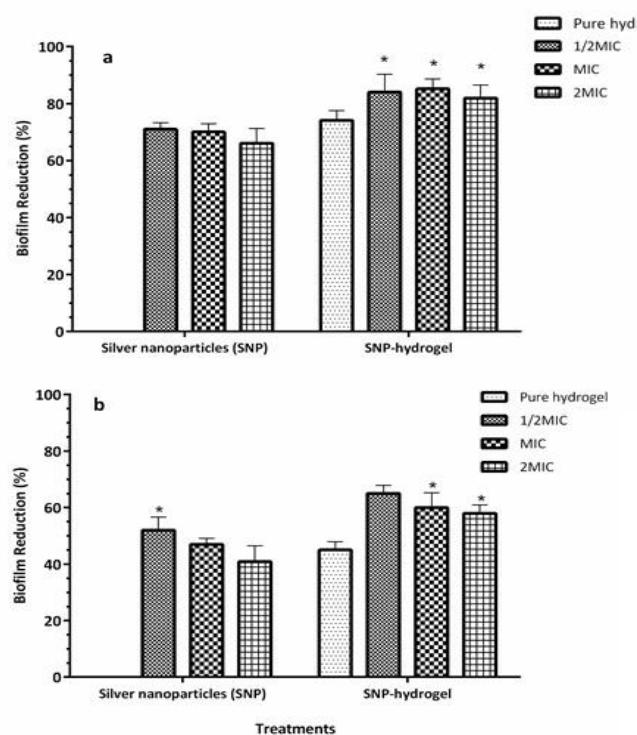
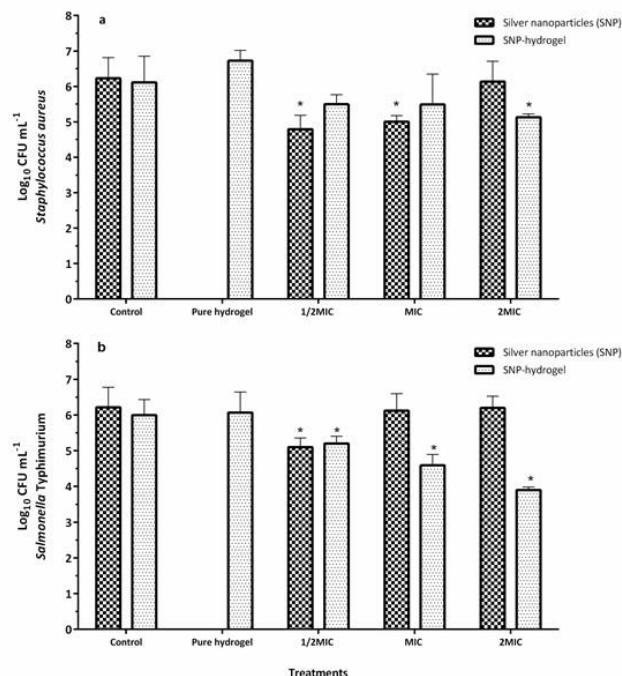


Figure 3. The effect of SNP and SNP loaded agar hydrogel on reducing one-day old biofilm of *Staphylococcus aureus* (a) and *S. typhimurium* (b) developed on polystyrene surface with 15 min contact time.* indicates a significant difference ($P < 0.05$) among SNP and SNP loaded agar hydrogel treatment at each concentration. MIC: minimum inhibitory concentrations.

Figure 4. The effect of SNP and SNP loaded agar hydrogel on the survival of *S. aureus* (a) and *S. typhimurium* (b) during 15 min exposure time .* indicates a significant difference ($P < 0.05$) among each treatment with a control. MIC: minimum inhibitory concentrations.

Discussion

SNP are proposed as a well-known and very interesting antimicrobial compounds with high effectiveness on a broad range of microbes at low doses used. The results of antimicrobial activity of SNP (Table 1) are in agreement with Zarei et al. (2014) (17) who reported the MIC values of similar SNP against *Listeria monocytogenes*, *S. typhimurium*, *Escherichia coli* O157:H7 and *Vibrio parahaemolyticus* in the range of 3.12- 6.25 $\mu\text{g}/\text{mL}$ according to microdilution method in Tryptic soy broth. Moreover, SNPs manifest distinguished antibacterial mechanisms on microorganisms by adsorption of SNP onto the cell and formation of ‘pits’ which resulting in the destruction of bacterial cell integrity. The production of free radicals may also physically damage the cell membrane and cause the killing of a bacterial population (10, 18). It has been revealed

in several reports that antibacterial efficacy of SNP varied between different prepared SNPs. Given the fact that factors such as the type of bacteria, the media of inoculation, type and the size of nanoparticle and the method of preparation must be considered when compare the antibacterial activity of different nanoparticles (19-22).

An increase in activity of SNP hydrogels may be due to the migration of SNP from the agar matrix and interact with lipid layer of membrane (23). The zone of inhibition in *S. aureus* was similar to *S. Typhimurium* (Table 1). This is in contrast with the results of Zakaria et al. (2016) (8) which reported the susceptibility of Gram-negative strains into gel formulations containing cefotaxime than Gram-positive. In addition, the efficacy of SNP hydrogels against *S. aureus* and *S. typhimurium* was significantly higher than the well-known standard (silver sulfadiazine cream) ($P < 0.05$), particularly at a high concentration. The similarity of inhibition zones of SNP with SNP loaded hydrogel unveiled the fact that incorporation of antimicrobial compounds into hydrogels does not decrease its effectiveness against both Gram-positive and Gram-negative strains, this entirely associated with spreadability characteristic of hydrogel (8). Additionally, the neat hydrogel showed no antibacterial activity against tested bacteria. According to Alshehri (2016) (24) the AgNPs-hydrogel prepared from carboxymethyl cellulose, polyvinyl alcohol, followed by the incorporation of AgNPs by microwave radiation revealed higher antibacterial activity against *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *S. aureus* and *Proteus mirabilis* compared to the neat hydrogel (24). The nature of the base gel, antimicrobial agents, and the environment display a substantial role in the release of nanoparticles from the gel into medium (25).

Observed variation in biofilm removal activity of SNP or SNP loaded hydrogels on tested strains (Figure 3) may be related to the surface characteristics of Gram positive and Gram negative bacteria. There could be several reasons for decreasing antimicrobial effectiveness by

increasing SNP concentration. SNP aggregation which occurs at a high concentration of SNP reduces the contact surface of nanoparticles with bacteria and thus diminish its biological activity. SNP reveal agglomeration after adding into the media. When SNP is suspended in nutritious media such as LB, rather than the pure solution, the aggregation was very noticeable. A work performed by Zhou et al. (2015) (26) support our results and verified that SNP aggregation depends on the media and antimicrobial concentration. Perez-Diaz et al. (2016) (6) studied the biofilm removal activity of SNP loaded chitosan gel on oxacillin-resistant *P. aeruginosa* and *S. aureus*. In this research, gels had a greater effect on MRSA, by 6 log reduction at the concentration of 100 ppm of SNP and 3.3 log reduction of *P. aeruginosa* at a concentration of 1000 ppm.

Due to antibacterial property, SNP is widely used in medicine as a disinfectant and antibacterial agents. It is believed that this novel material can be used as a new technology for the production of new sanitizing agents. Zakaria et al. (2016) (8) studied the cefotaxime gel formulations on bacterial pathogens survival. In this research, the antibacterial activity of cefotaxime loaded gels were worthwhile, 4 to 5-log reduction against *P. aeruginosa* and *E. coli* after 24 h incubation. Observed variation in cell reduction (Figure 4) may be related mainly to exposure time and also to the type and concentration of gel base, type, and concentration of loaded antimicrobial agents and the type of bacteria strains.

In contrast to SNP, the sanitizing activity of SNP- hydrogels were enhanced with increasing SNP concentration from 1/2 MIC to 2 MIC (Figure 4), reveals this fact that incorporation of SNP into hydrogel improve the antimicrobial activity by eliminating agglomeration of SNP. Higher reduction (2.3 log₁₀ CFU/ mL) was obtained on *S. typhimurium* by using agar hydrogel containing SNP at 2 MIC concentration.

Table 1. Diameters of inhibition zones (mm) of silver nanoparticle (SNP) and hydrogels against *S. aureus* and *S. typhimurium*.

Bacteria	Silver nanoparticle			SNP-hydrogels			Control		
	½ MIC	MIC	2MIC	½ MIC	MIC	2MIC	Silver sulfadiazine cream	Chloramphenicol 30 µg	Ampicillin 10 µg
<i>S. aureus</i>	1.00±0.09	1.20±0.03	1.50±0.1	0.86±0.05	1 ±0.00	1.13±0.05	0.8±0.00	28.40±0.5	23.75±0.7
<i>S. Typhimurium</i>	1.20±0.07	1.20±0.1	1.30±0.1	1 ±0.1	1.10±0.1*	1.13±0.05	0.8±0.00	10.25±0.24*	18.74±0.27*

* indicates a significant difference ($P < 0.05$) among different treatments in a column.

Conclusion

To our knowledge, this is the first report of the antimicrobial and biofilm removal activity of agar hydrogel incorporated with SNP. The results of the current study revealed that antimicrobial property of SNP loaded hydrogel is mainly affected by the nature of the medium (broth or agar), the concentration of nanoparticles and somewhat by the type of bacteria. In general terms, the SNP hydrogel activity has proved more effective than SNP in alone in the liquid and solid medium. On the other hand, the decrease in antimicrobial effects of SNP with increasing concentration, by loading SNP into hydrogel nanoparticles. SNP hydrogel showed also displayed biofilm removal activity against both tested bacteria in an SNP concentration-dependent manner.

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Conflict of interest

Authors declare no conflict of interest in this study.

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