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Antimicrobial Effects of Silver Nanoparticles against Bacterial Cells Adhered to Stainless Steel Surfaces

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ABSTRACT

Given the increasing number of antibiotic-resistant bacteria and the need to synthesize new antimicrobials, silver has attracted interest in the scientific community because of its recognized antimicrobial activity. This study aimed to evaluate the antimicrobial effects of silver nanoparticles (NP) obtained by a new method and tested at concentrations of 6 μg/ml and 60 μg/ml against the species *Staphylococcus aureus, Listeria innocua, Salmonella* Choleraesuis, *Pseudomonas aeruginosa, Escherichia coli,* and *Bacillus cereus*. The ability of these nanoparticles to remove or kill vegetative cells adhered to stainless steel surfaces was also evaluated. We observed that the NP obtained with the new method, concentrated silver nanoparticles (CNP), and silver nanoparticles with added sodium chloride (NPNaCl) had high antimicrobial activities ($P < 0.05$). We also verified that the most effective condition for the removal of *P. aeruginosa* cells on stainless steel coupons (10 by 10 mm) was immersion of the surfaces in CNP. The CNP treatment produced a 5-log reduction of the microbial population after 30 to 60 min of immersion. The CNP treatment also performed better than water and sodium carbonate, a compound commonly applied in clean-in-place procedures in the food industry, in removing adherent *B. cereus* cells from stainless steel cylinders. Therefore, these results suggest that NP synthesized by a new procedure may be used as antimicrobials in the food industry, for example, for the sanitization of utensils that come into contact with foods.

The antibacterial properties of silver are well known, and it has been demonstrated that silver is nontoxic to human cells at low concentrations *(14).* In the past, silver was used in water containers and was also used to prevent the deterioration of foods and liquids. Silver ions were used to treat burns and as chemotherapeutic agents against pathogens such as *Staphylococcus aureus* and *Streptococcus pneumoniae*. The use of silver as an antimicrobial agent decreased after 1940, following the discovery of penicillin and its use in medicine. However, with the indiscriminate use of antibiotics, the selection of resistant microbial species, and the need to develop new antimicrobials, silver has again attracted attention from the scientific community *(2, 18).*

Nanoscale materials have recently emerged as new antimicrobials. The term ''nano'' is used to indicate a dimension of 10^{-9} m. These nanoparticles are clusters of atoms with sizes ranging from 1 to 100 nm *(15).* The novel medical use of nanoparticles as antimicrobial agents includes infection reduction and the prevention of bacterial colonization on the surfaces of prostheses, catheters, dental materials, and food processing surfaces, such as stainless steel *(8, 11).* Nanoparticles can also be used for microbial control in textiles and water treatment *(7).* The control of particle size is an important factor in the synthesis of nanoparticles; smaller particles exhibit greater antimicrobial effects *(17).*

The mechanisms of action of silver ions and silver nanoparticles (NP) on microorganisms are varied and are not well established. Some mechanisms have been proposed and accepted for silver ions. Extracellular binding or the precipitation of silver is believed to occur on microbial cell walls and membranes *(1).* Cell wall peptidoglycans contain negatively charged molecules that will likely interact electrostatically with silver ions. The silver ions can interact with cellular proteins, including key enzymes involved in ion transport, through sulfhydryl groups (–SH). This interaction can inactivate these proteins, blocking respiration and electron transfer, and subsequently inactivating the bacteria *(3, 9).*

Some researchers support the idea that NP exhibit efficient antimicrobial properties due to their large interfacial area, which allows better contact with the bacteria; the nanoparticles adhere to the cellular membrane and enter the bacterial cells. Nanoparticles and silver ions interact with sulfur-containing compounds found in bacterial membrane proteins and with phosphorus-containing compounds, such as DNA. Nanoparticles also release ions, further increasing their antimicrobial activity *(12, 19).*

The present study aimed to evaluate the antimicrobial effect of NP, obtained by reacting dodecyltrimethylammonium bromide (Dotab) and silver sulfadiazine (SAg), on *S.*

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aureus, Listeria innocua, Salmonella Choleraesuis, *Pseudomonas aeruginosa, Escherichia coli,* and *Bacillus cereus,* as well as to further evaluate the ability of these nanoparticles to remove or kill vegetative cells adhered to stainless steel surfaces.

MATERIALS AND METHODS

Synthesis of NP. NP were produced from a 0.0312 M aqueous solution of the surfactant Dotab (Sigma, São Paulo, Brazil) following a new method by Fernandes *(4).* This surfactant concentration corresponds to twice the critical micelle concentration of Dotab (0.0156 M). To this solution was added 0.028 M SAg (Sigma, São Paulo, Brazil). The dispersion was agitated sufficiently to ensure complete mixing and was centrifuged at 15,000 × *g* for 5 min in a Sigma 3K30 centrifuge (Sigma, Osterode am Harz, Germany) at room temperature (25°C). After centrifugation, the pellet was discarded, and the resulting yellow dispersion contained the NP. The nanoparticles were heated to partially evaporate water, thus generating concentrated silver nanoparticles (CNP). Additionally, sodium chloride (0.1 M; VETEC, Rio de Janeiro, Brazil) was added to silver nanoparticles (NPNaCl) to promote destabilization of the electrical double layer. The quantification of NP, CNP, and NPNaCl preparations was performed with an atomic absorption spectrophotometer SpectrAA-200 (Varian) employing flame atomization, at the chemistry department of the Federal University of Viçosa.

Determination of the hydrodynamic diameter of NP by light dynamic scattering. Light dynamic scattering of the nanoparticles was performed in the complex fluid laboratory of the Federal University of Viçosa. The samples were filtered using a 0.45-μm-diameter polyvinylidene fluoride filter. Measurements were obtained at room temperature (25°C) using an angle of 30° and a 150-mW solid-state laser. The correlation functions were calculated using a TurboCorr correlator (Brookhaven Instruments, Holtsville, NY).

TEM. Transmission electron microscopy (TEM) was carried out in the electronic microscopy center of the Federal University of Minas Gerais using a Tecnai G2-20 (FEI Co., Hillsboro, OR). Sample preparation consisted of adding a drop of dispersion onto a grid coated with amorphous carbon. After the sample was dried, the grid was observed by TEM.

Determination of antimicrobial effects by the agar diffusion test. In vitro evaluation of the antimicrobial activities of the various NP dispersions, Dotab (2× critical micelle concentration) and SAg was carried out using the agar diffusion method. Cultures of *E. coli* ATCC 11229, *S. aureus* ATCC 6538, *L. innocua* ATCC 33090, *Salmonella* Choleraesuis ATCC 6539, and *P. aeruginosa* ATCC 15442 were used. In addition to these bacteria, we evaluated the antimicrobial activity of the particles against *B. cereus* (RIBO 1 222-173-S4), a bacterium isolated from the stainless steel surface of a filling machine used for pasteurized milk *(1).* An inoculum of each microorganism was prepared by directly suspending colonies in a saline solution. The suspension was adjusted to match the turbidity of a 0.5 McFarland standard solution. The bacteria were used in their stationary phase. Mueller-Hinton agar (Sigma, São Paulo, Brazil) plates were inoculated with the microorganisms by swabbing them uniformly across the plate with sterile swabs. Small holes (0.6-cm diameter) were drilled into the surface of the inoculated agar, into which 30 μl of each antimicrobial was deposited. The plates were then incubated at an optimum temperature for each microorganism. Inhibition halo diameters were measured after 16 to 18 h. If the compound is effective against bacteria at a certain concentration, no colonies will grow where the concentration in the agar is greater than or equal to the effective concentration *(13).*

Immersion of stainless steel coupons with adherent bacteria into dispersions containing NP. Stainless steel coupons (10 by 10 by 0.05 mm) were cleaned, sterilized, and immersed in brain heart infusion broth (Merck, São Paulo, Brazil) containing an inoculum of *P. aeruginosa* (ATCC 15442); they were then incubated at 32°C for 18 to 24 h. After the incubation period, the coupons were removed, dried at 20 to 25°C, and immersed in Dotab surfactant (2× critical micelle concentration), CNP, or distilled water (control) for 30 or 60 min. Subsequently, the coupons from each treatment were transferred to 0.1 M sodium phosphate buffer for 1 min to remove planktonic cells and then again were immersed in sodium phosphate buffer and vortexed for 1 min to remove the sessile cells. Appropriate dilutions were prepared, and aliquots were plated onto plate count agar (Merck) and incubated at 32°C. The number of adherent cells was expressed as CFU per square centimeter. The initial number of cells adhering to the stainless steel coupons was determined using the same methodology described above, with the exception of the immersion in the antimicrobials.

Removal of vegetative *B. cereus* cells adhered to stainless steel cylinders. Individual 1-ml suspensions of *B. cereus* (RIBO 1 222-173-S4) (containing approximately 1.0×10^8 CFU/ml) were added to individual 100-ml samples of ultrahigh-temperature sterilized milk. These samples were placed in American Iron and Steel Institute 304 stainless steel proof tubes (AcelorMittal Acesita, Timóteo, Minas Gerais, Brazil) to simulate the process of bacterial removal by clean-in-place procedures. The proof tubes were subjected to rotational motion and then were incubated at 32°C for 24 h to promote the adhesion of the microorganisms to the stainless steel. Following adhesion, the inoculums contained in the tubes were discarded, and the tubes were filled with one of the following treatments: distilled water at 25°C, Dotab (0.0312 M), sodium carbonate (40 mg/liter), or CNP (60 mg/liter). This process aimed to evaluate the efficacy of the various treatments in removing or killing the adhered cells. After filling with the treatment solutions, the tubes were again rotated. The number of cells that adhered to the surfaces of tubes and survived the removal procedures was determined by rinsing with Ringer's solution and using a stainless steel rod to aid in the removal of the cells. After a final rinse with Ringer's solution, appropriate dilutions were performed, inoculated onto plate count agar (Merck), and incubated at 32°C for 24 h. The results were expressed in CFU per square centimeter *(16).*

Statistical analysis. All experiments were conducted in a completely randomized design with three repetitions conducted in triplicate. The data were analyzed by analysis of variance at a probability of 5%; when significant differences were observed, Tukey's test was used. The analyses were performed using SAS software version 9.1 (SAS Institute Inc., Cary, NC).

RESULTS

Nanoparticle diameters. In our study, NP, CNP, and NPNaCl synthesized by the method of Fernandes *(4)* had average diameters of 2.8, 3.7, and 4.4 nm, respectively.

TEM. We observed that the addition of NaCl promoted better dispersion of the nanoparticles. The TEM images in

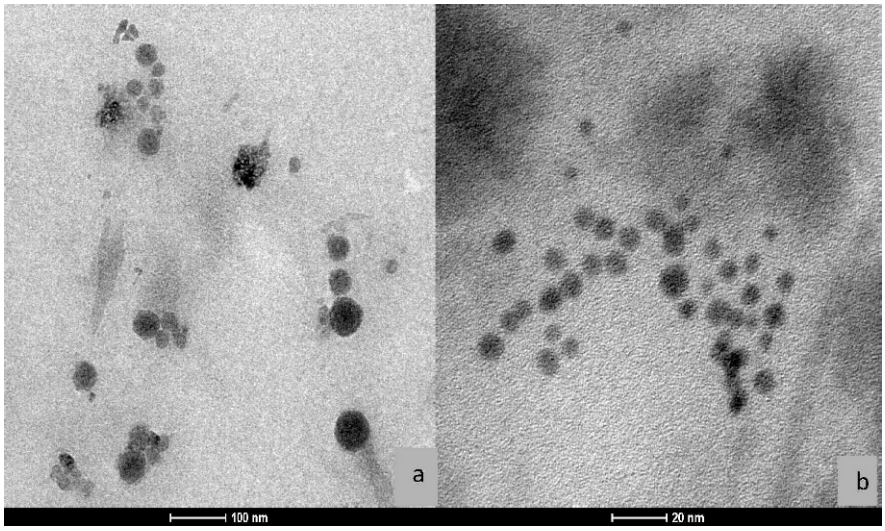

FIGURE 1. *Silver nanoparticles (a) and silver nanoparticles with added NaCl (b) taken with transmission electron microscopy.*

Figure 1 show the dispersion promoted by salt and indicate that the nanoparticles are spherical.

Agar diffusion test. We observed that NP, CNP, and NPNaCl produced following the new methodology proposed by Fernandes *(4)* exhibited high antimicrobial activity. Based on the size of the inhibition halos, there was no difference ($P > 0.05$) between the inhibition induced by the NP and NPNaCl treatments, whereas the CNP treatment was the most effective (Table 1). In the case of *L. innocua,* the antimicrobial action was the same for the NP, CNP, and NPNaCl treatments. For *B. cereus,* there was no difference between the NP and CNP treatments, whereas the inhibition caused by NPNaCl was lower. We note that there was no significant difference ($P > 0.05$) in bacterial inhibition by the antimicrobials Dotab and SAg for the bacteria *P. aeruginosa*, *E. coli, L. innocua, Salmonella* Choleraesuis, and *B. cereus*. Smaller inhibition halos were obtained for Dotab and SAg treatments than for the others (NP, CNP, and NPNaCl).

Removal or death of adhered bacterial cells in stainless steel coupons by NP. We also observed that the CNP treatment was the most effective in removing or killing adhered *P. aeruginosa* cells ($P < 0.05$) from stainless steel coupons after 30 or 60 min of contact time (Table 2).

Removal or death of adhered bacterial cells in stainless steel cylinders. *B. cereus* was adhered to stainless steel cylinders, and antimicrobial agents were tested for their ability to remove or kill adherent cells (Table 3). The CNP treatment exhibited the best performance ($P < 0.05$).

DISCUSSION

Nanoparticle size. The antimicrobial effect of nanoparticles is influenced by the size of the particle; smaller particles have a larger effect due to increased interfacial area, which provides better contact with the bacteria.

Microscopy electron transmission. The average size of the nanoparticles determined by TEM produced with or without addition of NaCl was 10 nm. The size obtained by TEM was different from that observed by dynamic light scattering because preparing samples for microscope viewing involves drying samples on the grid, which promotes aggregation of nanoparticles.

Agar diffusion test. The antimicrobial activity of the nanoparticles obtained by the new synthesis may be related to the size of NP (average diameter, 2.8 to 4.4 nm). The greater interfacial area of smaller nanoparticles allows greater interaction with the bacteria, thus facilitating contact and antimicrobial action *(17)*. The higher surface area–to–volume ratio of these nanoparticles means that a greater number of silver atoms are in contact with the solution on a per-unit weight basis. Moreover, smaller-sized nanoparticles

TABLE 1. *Mean diameters of inhibition halos of vegetative cells exposed to the action of the different antimicrobial agents dodecyltrimethylammonium bromide (Dotab), silver sulfadiazine (SAg), silver nanoparticles (NP), concentrated silver nanoparticles (CNP), and silver nanoparticles with added NaCl (NPNaCl)*[a]

Bacteria	Dotab	SAg	NP	CNP	NPNaCl
Staphylococcus aureus	3.60 ± 0.1 C	1.17 ± 0.06 D	4.33 ± 0.15 B	4.63 ± 0.05 A	4.27 ± 0.15 B
Pseudomonas aeruginosa	1.60 ± 0.08 C	1.27 ± 0.06 C	3.86 ± 0.3 B	4.57 ± 0.22 A	4.13 ± 0.26 B
Bacillus cereus	1.60 ± 0.26 C	1.76 ± 0.15 C	4.00 ± 0.1 A	4.73 ± 0.57 A	2.87 ± 0.51 B
Listeria innocua	1.92 ± 0.20 B	1.95 ± 0.35 B	4.36 ± 0.23 A	4.40 ± 0.19 A	4.00 ± 0.10 A
Escherichia coli	1.90 ± 0.24 C	1.84 ± 0.11 C	3.13 ± 0.45 B	4.26 ± 0.25 A	2.84 ± 0.36 B
Salmonella Choleraesuis	2.12 ± 0.17 C	2.20 ± 0.3 C	3.07 ± 0.05 B	4.03 ± 0.15 A	3.32 ± 0.12 B

[a] Values are in centimeters. Means followed by the same letter in the same row do not differ by Tukey's test ($P < 0.05$).

TABLE 2. *Number of* Pseudomonas aeruginosa *adhering to stainless steel coupons after immersion in different antimicrobials*[a]

Treatment	30-min immersion (log CFU/cm^2)	Log reduction	60-min immersion (log CFU/cm^2)	Log reduction
Adhered cells (before immersion)	6.06 A	—	6.64 A	—
Water (control)	5.81 B	0.25	6.24 B	0.40
Dotab	4.20 C	1.86	3.54 C	3.10
CNP	<1.00 D	>5.06	<1.00 D	>5.64

[a] Means followed by the same letter in the same column do not differ by Tukey's test ($P < 0.05$).

release greater amounts of silver ions and therefore have higher antimicrobial efficacies *(6)*.

Given that the size of the nanoparticles was similar for all three of these treatments (NP, CNP, and NPNaCl), the concentration of nanoparticles in the CNP suspension likely contributed to its greater effect because the concentration in the CNP treatment was 60 μg/ml, but the concentrations in the NP and NPNaCl treatments were 10 times lower (each 6 μg/ml). Notably, the antimicrobial effect of NPNaCl is only due to the nanoparticles present because the concentration of NaCl in the treatment (0.586%) is unlikely to be sufficient to promote inhibition of these microorganisms.

NaCl was added to the synthesized NP to destabilize the electrical double layer of the micelle and to observe the influence of this break in the shielding on synthesis of nanoparticles because destabilization of the electrical double layer favors access of the sulfadiazine to the micelle. We noted that the salt does not influence the size of nanoparticles, but it does influence the dispersion of nanoparticles.

Martínez-Castañón et al. *(10)* determined the MIC of synthesized NP against *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 and observed that nanoparticles that had a diameter of 7 nm showed the greatest antimicrobial effect. According to the authors, these nanoparticles, due to their smaller size, can easily reach the core content of the bacterium and they present a larger surface area, providing greater contact with the bacterium.

In our research, a nanoparticle concentration of only 6 μg/ml was able to cause inhibition halos for the species tested here. In another study *(8),* NP had a wider spectrum of inhibition against *E. coli, Bacillus subtilis, S. aureus,* and *P. aeruginosa* when the concentration of NP in the suspension was 2,000 μg/ml.

Morones et al. *(12)* studied the effect of adding nanoparticle powder to a matrix of carbon. A range of concentrations of NP (0, 25, 50, 75, and 100 μg/ml) was evaluated for antimicrobial activity against *E. coli, Vibrio cholera,* and *P. aeruginosa*. The results showed that the concentration of nanoparticles that prevented cellular growth differed for each bacterial species, and *P. aeruginosa* and *V. cholerae* were more resistant than *E. coli*. At concentrations of 75 and 100 μg/ml, there was no significant growth for any of the tested bacteria.

From the results presented in this study, it is clear that the classification of bacteria as gram positive or gram negative did not influence inhibition trends. *L. innocua* was the most sensitive of the bacteria studied because the halos were similar regardless of the types of nanoparticles used. In the case of *L. innocua,* a concentration of only 6 μg/ml of NP and NPNaCl was sufficient to produce the same inhibition as 60 μg/ml of CNP.

Dotab is a surfactant that has known antimicrobial effects. However, its efficacy was lower than that of NP obtained by the new synthesis procedure. We propose that the bacterial inhibition promoted by these nanoparticles can be attributed partly to the effect of Dotab and partly to the antibacterial effect of the NP. Therefore, there is a positive interactive effect between Dotab and NP.

In our study, we did not observe relevant antimicrobial effects of SAg. Reports in the literature indicate that ionic silver (Ag^+) inhibits microorganisms well. One possible cause may be that it is difficult to produce homogenous dispersions of this component, which may have resulted in impaired diffusion of silver ions into the agar, thus reducing the antimicrobial activity.

Removal or death of adhered bacterial cells in stainless steel coupons by NP. A 3-log reduction is required for a sanitizing solution to be considered effective at removing or killing adhered cells. Immersing the coupons in the CNP treatment for either 30 or 60 min produced 5-log reductions of adhered cells of *P. aeruginosa,* an opportunistic pathogen that is able to produce biofilms on devices used in the medical field, indicating that these nanoparticles exhibit high antimicrobial efficacies. Thus, NP may be used in the food industry for their antimicrobial effect and their ability to reach bacterial colonization sites, which are often in cracks in stainless steel surfaces.

Removal or death of adhered bacterial cells in stainless steel cylinders. Simulated-use tests are often used to analyze a situation that may actually occur in the food industry. The observed effect of CNP meets the recommendations of the American Public Health Association, which advocates a limit of 2 log CFU/cm^2 of mesophilic bacteria on stainless steel surfaces that come in contact with food if those surfaces are to be considered hygienic or sanitary.

TABLE 3. *Number of* Bacillus cereus *adhered to the stainless steel surface of a pipe used for processing milk*[a]

Treatment	Log CFU/cm^2
Water	3.14 A
Sodium carbonate (control)	2.04 B
CNP	0.80 C

[a] Means followed by the same letter do not differ by Tukey's test ($P < 0.05$).

Studies with stainless steel surfaces coated with zeolites containing 2.5% silver ions and 14% zinc ions demonstrated significant reductions (3 log) in vegetative cells of *B. subtilis, B. anthracis,* and *B. cereus* within 24 h. The spores of *B. cereus* were completely resistant under the same conditions *(5, 17).*

Based on these results, we propose that NP obtained by the new synthesis methods described here may be used as antimicrobials in the food industry. For example, they may be used for the sanitization of utensils that come into contact with foods to reduce bacterial adhesion and thereby increase the safety of foods offered to consumers.

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