

Effect of Size and Surface Ligands of Silver (Ag) Nanoparticles on Waterborne Bacteria

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Abstract - Clean drinking water is a matter of concern for all countries, more so for many developing countries and consequently various methods of producing clean drinking water are being practiced for provision of such water to the consumers. At the same time an attempt is always made to adopt any new technique or technology to use it for commercially feasible production of such water. In recent years, with the advent of fast developing technologies, nanotechnology has also come into operation for economic and commercial production of clean drinking water. With a view to produce clean drinking water free from pathogens by the use of nanotechnology, we have investigated the antibacterial properties of silver (Ag) nanoparticles against waterborne bacteria in the public water supply sources in Islamabad, the capital of Pakistan. Water samples from over 50 water supply sources were collected and bacterial strains were identified by API20E method. Different silver nanoparticles were tested against 8 different bacterial isolates at concentrations varying from 26 to about 77 µg/mL. Nanoparticles of size ranging from 3-8 nm prepared by casein stabilization method showed the best activity against all isolated strains, *E. coli* being the most effectively inhibited with an average 14.9 mm zone of inhibition. *C. sakazakii* (12.6 mm) was most sensitive to citrate stabilized particles, followed by *E. coli* (12.3 mm), *K. pneumoniae* (12 mm) and *P. aeruginosa* (11.6 mm). These results provide a useful picture for the development of water filters incorporated with silver nanoparticles. We believe that this study is important for the public, particularly in the

developing countries and also for others working in the area of antibacterial effects of nanoparticles.

Keywords: Silver Nanoparticles, Waterborne Bacteria, Drinking Water, Antibacterial Effect, Surface Ligands

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1. Introduction

Among the many different types of metallic and metal oxide nanoparticle (NPs), Silver (Ag) NPs have proven to be the most effective against bacteria, viruses, and other eukaryotic microorganisms (Liu et al., 2009; Chamundeeswari et al., 2010; Huang et al., 2009; Sharma et al., 2008). Antibacterial activity of Ag is very well known from history. Ag NPs attack the respiratory chain and cell division that finally lead to cell death, while concomitantly releasing silver ions that enhance bactericidal activity (Klasen 2000).

Several investigators have assessed the biocidal efficacy of silver nanoparticles. (Baker et al., 2005; Panacek et al., 2006; Kim et al., 2007; Shrivastava et al., 2007; Li et al., 2010). Silver significantly reduces waterborne pathogens like *Pseudomonas aeruginosa* and *Aeromonas hydrophila* by

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inactivating them. It can also be used as a secondary disinfectant (Silvestry-Rodriguez et al., 2007). Kim et al. (2007) reported the effect of silver nanoparticles against yeast, *Escherichia coli*, and *Staphylococcus aureus*. They reported that Ag nanoparticles can be used as effective growth inhibitors against various microorganisms, which makes it capable of being applied in diverse medical devices and systems for antimicrobial control. Elechiguerra et al. (2005) studied the size dependent effect of silver nanoparticles on HIV-1 virus. They reported that silver nanoparticles inhibit the binding of virus to the host cells in vitro. Sondi and Salopek-Sondi (2004), studied the antibacterial effect of silver nanoparticles against *Escherichia coli*. Scanning and transmission electron microscopy (SEM and TEM) were used to study the biocidal action of this nano scale material. Their results confirmed that the treated *Escherichia coli* cells were damaged, showing formation of "pits" in the cell wall of the bacteria. They reported that these nontoxic nano materials, which can be prepared in a simple and cost-effective manner, may be suitable for the formulation of new types of bactericidal materials. In another study, the bactericidal effect of silver nanoparticles obtained by a novel electrochemical method on *Escherichia coli*, *Staphylococcus aureus*, *Aspergillus niger* and *Penicillium phoeniceum* cultures were studied. The silver nanoparticles show a pronounced antibacterial/antifungal effect (Khaydarov et al., 2009). Parameswari et al. (2010) compared the antimicrobial activity of silver nanoparticles and AgNO₃ against *E. coli* in terms of growth rate, time dependency and zone of inhibition. Ag nanoparticles created a zone of inhibition of 1.7 cm as compared to 0.8 cm by AgNO₃, thus proving Ag nanoparticles to be the efficient candidate for antimicrobial activity. Similarly, another study by Morones et al. (2005) on bactericidal effect of silver nanoparticles against gram negative bacteria showed that the effect is size dependent and the most effective range of nanoparticle sizes that provided direct interaction with the bacteria had a diameter of ~1–10 nm amongst the tested range of 1–100 nm.

Silver nanoparticles are highly active and show better biocidal results against Gram negative bacteria such as *E. coli* (Tiwari and Behari, 2009) as compared to Gram positive bacteria and the effects are dose dependent (Madigan and Martinko, 2005). The antimicrobial activity of Ag NPs is inversely dependent on size (Raimond et al., 2005; Sondi and Salopek-Sondi, 2004). Pal et al. (2007) reported that antibacterial activity of silver nanoparticles is also dependent upon the shape of the nanoparticles. There is another report showing the use of fullerenes and carbon nanotubes as antimicrobial agents (Kang et al., 2007; Badireddy et al., 2007). In this study, antibacterial activity of Ag nanoparticles (Ag NPs) was tested against different Gram positive and Gram negative waterborne bacterial strains which were isolated and identified from water samples taken from

different water supply sources of Islamabad working under Capital Development Authority (CDA).

2. Material and Methods

2.1. Synthesis of Ag Nanoparticle

Ag nanoparticles of three particles sizes (S1= 3-8nm, S2= 4-8nm and S3 = 5-9 nm) were prepared for the present investigation by following methods.

2.1.1. Synthesis of Casein (protein) Stabilized Silver Nanoparticles (S1)

In a typical experiment to synthesize casein (protein) stabilized silver nanoparticles, a given volume of a warm aqueous suspension of casein, set at a desired pH (7-8), was added quickly to a boiling aqueous solution of AgNO₃ (25 mL, 1 mM) under vigorous stirring. The color of the reaction mixture gradually changed from colorless to brown within 5 – 10 minutes. The reflux was continued for one hour to ensure complete reaction resulting in the formation of a brownish silver nanoparticles suspension. The nanoparticle suspension was then filter purified using centrifuge filters (Amicon® Ultra Centrifugal Filters by Millipore Corporation) having a molecular cut off value of 100kDa to remove excess casein and other impurities and then stored at room temperature for further analysis and use in subsequent experiments. Casein (molecular weight 75–100 kDa) used in this work was of Hammerstein grade purchased from MP Biomedicals. All solutions of casein were prepared in 50 mM Tris buffer and pH adjusted using 1 M NaOH/H₂SO₄. Ultrapure water with a resistivity of 18.2 MΩ. cm was used as the solvent in all preparations. The approximate size of these silver nanoparticles was found to be *ca.* 3-8 nm. FTIR spectroscopy confirmed that the hydrophobic part and amide functionality of casein is involved in the stabilization of silver nanoparticles (Ashraf et al., 2013).

2.1.2. Synthesis of Citrate Stabilized Silver Nanoparticles (S2)

This method is with slight modifications in the classical citrate reduction method by Turkevich et al. (1951) for nanoparticle synthesis. To an aqueous solution of 1mM AgNO₃, 1mM tri-sodium citrate solution was added and stirred for 2 hours. After 2 hours 5 mL NaBH₄ solution (0.8 mg/l mL) was added during stirring at room temperature. The reaction was allowed to complete for 5 hours. The particle solution was filter purified to remove access ligand and other impurities using centrifuge filters (Amicon® Ultra Centrifugal Filters by Millipore Corporation) having a molecular weight cut off value of 30 KDa, and then stored at room temperature for further analysis and use in subsequent experiments. The approximate size of these silver nanoparticles was found to be *ca.* 4-8 nm and pH was between 7 to 8.

2.1.3. Synthesis of a Polyhexamethylene biguanide (PHMB) Stabilized Silver Nanoparticles (S3)

In a typical experiment to prepare polymer (PHMB) functionalized silver nanoparticles, a given volume (15 mL, 20 $\mu\text{g/mL}$) of PHMB solution, was added to an aqueous solution of 1mM AgNO_3 under vigorous stirring. The reaction mixture was allowed to stir vigorously for 2 hours to let polymer form a complex with silver. Freshly prepared aqueous solution of sodium borohydride (5 mL, 0.8 mg/mL) was then added quickly to the polymer-Ag solution and the reaction was allowed to complete for 5 hours. The yellow/brownish suspension of silver nanoparticles was then filter purified to remove excess polymer and other impurities using centrifuge filters (Amicon® Ultra Centrifugal Filters by Millipore Corporation) having a molecular weight cut off value of 30 KDa, and then stored at room temperature for further analysis and use in subsequent experiments. PHMB (20% w/v) was purchased from Arch Chemicals (Norwalk, CT, USA). The approximate size of these silver nanoparticles was found to be ca. 5-9 nm. FTIR spectroscopy indicated the interaction of imine group of PHMB with nanoparticles confirming its functionalization with PHMB (Ashraf et al., 2012).

2.2. Characterization of Silver Nanoparticles

The silver nanoparticles were characterized using UV-visible spectroscopy in the range of 300 to 800 nm. All the three preparations have a clear surface plasmon resonance band. The particles produced by borohydride reduction in the presence of sodium citrate seem relatively more uniform as indicated by the narrow absorption band.

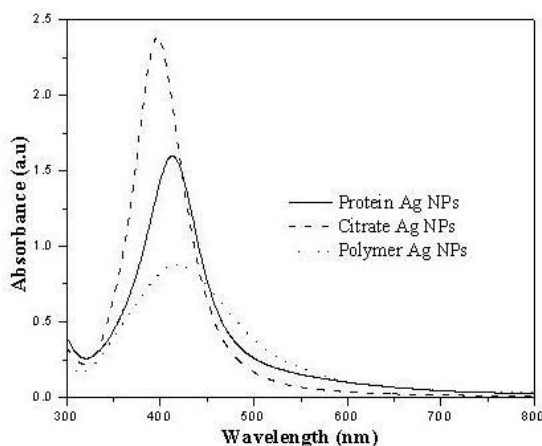


Fig.1. UV-visible spectra of silver (Ag) nanoparticles produced using different methods.

Three samples of different particle sizes (S1= 3-8 nm, S2= 4-8 nm and S3 = 5-9 nm) used in this study with three

different concentrations 60, 77 and 26 $\mu\text{g/mL}$ of Ag nanoparticles in the solutions respectively.

2.3. Bacterial Strains

Bacterial strains were isolated from water supply of different sectors of Islamabad and identified by API 20E method. These include *Enterobacter cloacae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Cronobacter sakazakii*, *Pantoea agglomerans*, *Serratia marcescens*, *Aeromonas hydrophila* and *Klebsiella pneumoniae*.

2.4. Bioassay

Antibacterial activity of Ag nanoparticles was tested in triplicate by using agar well diffusion method (Perez et al., 1990). 1.5×10^8 Colony Forming Units (CFU/ml) of each microorganism was inoculated on Muller Hinton (MH) agar. Working volume 120 μL of different Ag nanoparticle sample was used with 100 μL of distilled water as control. Diameter of zones of inhibition was noted after 24 hrs of incubation at 37 $^{\circ}\text{C}$.

3. Results

Antibacterial activity of Ag nanoparticles (Ag NPs) was tested against different Gram negative waterborne bacterial strains which were isolated from water supply of Islamabad working under Capital Development Authority (CDA). Casein stabilized (S1) and Citrate stabilized (S2) silver nanoparticles showed activity against various water borne bacterial strains. To find out the effect of different surface ligands of Ag NPs on bacteria, the concentration difference was eliminated by normalizing the results to the particle concentration. Casein stabilized particles (S1) were more effective than citrate stabilized particles (S2) against all the tested bacterial strains as shown in Figure 2.

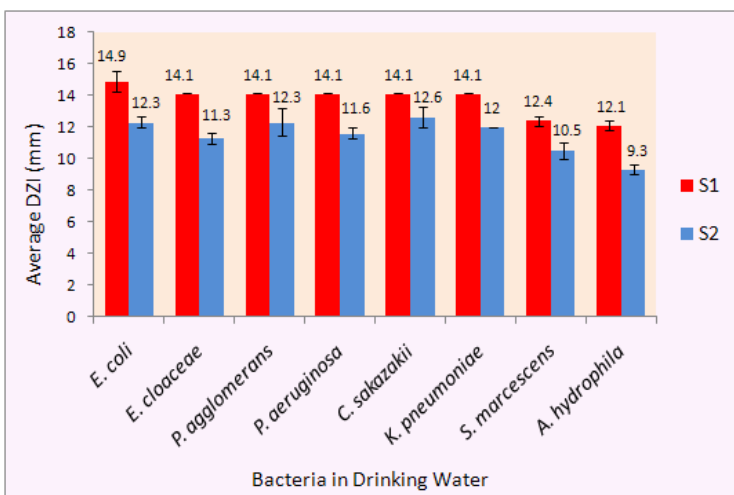


Fig. 2. Diameter of Zones of Inhibition (DZI) in millimeter (mm) of different Ag NPs against drinking water bacteria.

Antibacterial activity of S1 and S2 varied from 9.3 to 14.9 mm for different bacterial strains. Previously different studies were carried out on antibacterial activity of Ag NPs (Savithramma et al., 2011; Ruparelia et al., 2008) but due to different particles size, method of preparation and initial bacterial concentration used, direct comparison is difficult. S2 showed 12.3 mm inhibition zone against *E. coli* and 12.6 mm against *C. sakazakii*. Theivasanthi and Alagar (2011) also studied the effect of silver nanoparticles (24 nm particle size) against *E. coli* and reported 12 mm zones of inhibition. Furthermore, S2 showed 12 mm and 11.6 mm inhibition zones against *K. pneumoniae* and *P. aeruginosa* respectively. Prasad et al. (2011) reported a 6 mm zone of inhibition against *K. pneumoniae* when they used silver nanoparticles of 59 nm produced phylogenically. Another antibacterial study of silver nanoparticles produced from three different medicinal plants showed 12, 7 and 12 mm zones of inhibition against *Klebsiella* and 9, 6, 15 mm inhibition zones against *Pseudomonas* (Savithramma et al., 2011). The variation in results is most likely due to different particle sizes and methods used for synthesis of silver nanoparticles. S1 particles showed better activity than S2 against all the tested strains of bacteria.

E. coli was most effectively inhibited with 14.9 mm zone of inhibition and *A. hydrophila* was observed to be the most resistant against silver nanoparticles among the eight Gram negative bacteria tested. S3 showed no activity. Our results have shown good relevance with the previous literature although direct comparison is difficult due to differences of parameters regarding the use of initial bacterial count and amount of Ag NPs tested.

4. Conclusion

We evaluated the effect of surface stabilizers of silver nanoparticles between the range of 3-10 nm on the waterborne bacterial strains. Citrate stabilized silver nanoparticles and Casein stabilized silver nanoparticles showed antibacterial activity against all the tested strains. The biocidal action of citrate stabilized particles against multidrug resistant bacteria is known (Borah et al., 2012). *C. sakazakii* (12.6 mm) was most sensitive to citrate stabilized particles, followed by *E. coli* (12.3 mm), *K. pneumoniae* (12 mm) and *P. aeruginosa* (11.6 mm). Casein stabilized particles (Sample 1) have shown marked antibacterial effect as compared to citrate stabilized particles. Therefore, it can be concluded that casein is a better surface ligand in terms of antibacterial effect as compared to citrate, *E. coli* being the most effectively inhibited with 14.9 mm zone of inhibition.

Enterobacter cloacae, *Pantoea agglomerans* and *Cronobacter sakazakii* were tested for the first time to our knowledge. A detailed study is, however, required for the production of silver nanoparticles with better surface stabilizers for improved antibacterial activity. Further, the effect of the shape of the Ag nanoparticles against the

bacterial strains used is under investigation. Polymer stabilized silver nanoparticles (S3) showed no activity against any strain tested. This might be due to low concentration (26 µg/mL) of silver nanoparticles in the sample. Other similar studies reported the antibacterial activity of silver nanoparticles. Bankura et al. (2012) studied the antimicrobial activity of dextran stabilized silver nanoparticles in aqueous medium and they reported the significant reduction of bacteria, *Bacillus subtilis*, *Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Another study by Ashraf et al. (2012) reported the antibacterial activity of Polyhexamethylene biguanide (PHMB) functionalized silver nanoparticles against *E. coli*.

Further investigations are under process and we plan to study Ag nanoparticle incorporated clay filters which are cheap and commercially affordable for low income group people for the provision of clean drinking water, particularly in rural areas. We believe that this study is important for the public, particularly in the developing countries and also for others working in the area of antibacterial effects of nanoparticles in drinking water.

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