The bactericidal potential of silver nanoparticles

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Nanotechnology is expected to open new avenues to fight and prevent disease using atomic scale tailoring of materials. Among the most promising nanomaterials with antibacterial properties are metallic nanoparticles, which exhibit increased chemical activity due to their large surface to volume ratios and crystallographic surface structure. In this work we conducted batch experiments to assess the efficiency of silver nanoparticles synthesized by citrate reduction method for their antimicrobial property. The antimicrobial activity of silver nanoparticles and $AgNO_3$ was compared in terms of *Escherichia coli* growth rate, zone of inhibition and time dependent antimicrobial activity. Silver nanoparticles showed 100 per cent growth reduction of *E. coli* when treated with 30 µg ml^{-1} concentrations, whereas the effect was much less at this concentration of $AgNO_3$. Zone of inhibition test was also done for identification of degree of inhibition by using different concentration of $AgNO_3$ and silver nanoparticles. It was found that, 10 µg ml^{-1} concentration was able to inhibit bacterial growth and created a zone of 0.8 cm by $AgNO_3$ and 1.7 cm by Ag nanoparticles. Thus Ag nanoparticles are found to be efficient candidate for antimicrobial activity than $AgNO_3$.

Key words: Antimicrobial potential, Silver nanoparticles, *Escherichia coli*.

INTRODUCTION

Over the past few decades inorganic nanoparticles, whose structures exhibit significantly novel and improved physical, chemical and biological properties, phenomena and functionality due to their nanoscale size, have elicited much interest. Nanophasic and nanostructured materials are attracting a great deal of attention because of their potential for achieving specific processes and selectivity, especially in biological and pharmaceutical applications (Pal *et al*., 2007).

Recent studies have demonstrated that specially formulated metal oxide nanoparticles have good antibacterial activity and antimicrobial formulations comprising nanoparticles could be effective bactericidal materials (Pal *et al*., 2007). Among inorganic antibacterial agents, silver has been employed most extensively since ancient times to fight infections and control spoilage. The antibacterial and antiviral actions of silver, silver ion, and silver compounds have been thoroughly investigated (Oloffs *et al.,* 1994). However, in minute concentrations, silver is nontoxic to human cells. The epidemiological history of silver has established its nontoxicity in normal use. Catalytic oxidation by metallic silver and reaction with dissolved monovalent silver ion probably contribute to its bactericidal effect (Oka *et al.,* 1994). Microbes are unlikely to develop resistance against silver, as they do against conventional and narrow-target antibiotics, because the metal attacks a broad range of targets in the organisms, which means that they would have to develop a host of mutations simultaneously to protect themselves. However, Ag^+ ions or salts have only limited usefulness as antimicrobial agents for several reasons as interfering effects of salts and the antimicrobial mechanism of continuous release of enough concentration of silver ion from the metal form. In contrast, these kinds of limitation can be overcome using silver nanoparticles.

MATERIALS AND METHODS

Preparation of silver nanoparticles

Silver nanoparticles were prepared by adopting the protocol of Pal *et al.,* 2007. Starch stabilized silver nanoparticles prepared by

adding silver nanoparticles to 0.2 per cent starch solution. The synthesized, bare and starch stabilized nanoparticles were characterized by UV-visible spectroscopy, Scanning Electron Microscope (SEM) and X- Ray Diffraction (XRD).

Organism preparation

Escherichia coli (MTCC-443) strain was grown overnight in Luria Bertani (LB) broth at 37°C. Bacterial cells were centrifuged at 6000 rpm for 15 min and washed cell pellets were resuspended in LB and optical density (OD) was adjusted to 0.1 corresponding to 10^8 CFU ml^{-1} at 600 nm.

Minimum Inhibitory Concentration (MIC)

The bactericidal activity of silver nanoparticles was checked by determining the MIC. Minimal concentration of silver nanoparticles which inhibits the growth of *E. coli* is known as MIC. Bacterial cells were grown in LB medium and 500 µl of 24 h old bacterial culture (0.1 OD) was spread over LB agar plates, supplemented with 10, 20, 30, 40 and 50 µg ml^{-1} of silver nanoparticles. All plates were incubated at 37°C for 24 h. Antimicrobial test compound below the MIC cannot inhibit microbial growth.

Time dependent antibacterial activity

The silver nanoparticles were suspended in millipore water to conduct the time-dependent antibacterial study. *E. coli* cells were treated with 2.0 ml of each concentration (0, 10, 20, 30, 40 and 50 µg ml^{-1}) of silver nanoparticles as well as with varying time intervals for each concentration (0, 1, 3, 6 and 12 h). Before using the silver nanoparticles,the suspension was homogenized using ultrasonicator. Each treated bacterial culture was serially diluted till 10^6 dilution factor and 100 µl from each culture was homogeneously spread in LB agar plates. All plates were incubated at 37°C for 24 h and the number of colonies grown on agar plate was counted.

Growth pattern

Growth pattern of *E. coli* was studied with 0, 10, 20, 30, 40 and 50 µg ml^{-1} concentration of homogenized silver nanoparticles. *E. coli* cells were treated with varying concentrations of silver nanoparticles as mentioned above and inoculated in 250 ml of erlenmeyer flask. All the flasks were put on rotary shaker (180 rpm) at 37°C. Untreated culture flask was used as control. Optical density was measured after every hour (upto 16 h) using UV–Visible spectrophotometer at 600 nm.

Zone of inhibition

Zone of inhibition test was performed in LB agar plates supplemented with 0, 10, 20, 30, 40 and 50 µg ml^{-1} of silver nanoparticles. For this, 20 ml LB agar was poured in well rinsed, autoclaved petri plates, 1.0 ml of active bacterial culture was homogeneously spread in the agar plates and paper disc containing different concentration of Ag nanoparticles were placed in agar medium. The plates were incubated at 37°C for 24 h. The zone size was determined by measuring the diameter of the zone.

The antimicrobial activity of silver nanoparticles was compared with $AgNO_3$ for MIC, time-dependent antibacterial activity, growth pattern and zone of inhibition. The procedures followed for $AgNO_3$ were same as that of silver nanoparticles.

RESULTS

Synthesis and characterization of silver nanoparticles

The silver nanoparticles are prepared by citrate reduction method and characterized using XRD and SEM.

X- Ray Diffraction

Structural information of silver nanoparticles was obtained by oriented particulate monolayer X-ray diffraction (Plate.1). The XRD pattern clearly showed the crystalline nature of silver nanoparticles. It shows the XRD patterns of the nanoparticles lying flat with their basal planes parallel to the substrate. The remarkably intensive diffraction peak noticed at 2θ value of 37.879 from the {111} lattice plane of face-centered cubic silver unequivocally indicates that the particles are made of pure silver and that their basal plane, *i.e*., the top crystal plane, should be the {111} plane. It has been suggested that this plane may possess the lowest surface tension. This 2θ value reflection angle confirms the presence of silver nanoparticles.

Scanning Electron Microscope

Scanning Electron Microscope images of bare silver nanoparticles and starch stabilized nanoparticles are shown in plate.2. The images confirmed the sizes of the bare silver nanoparticles are 40 to 72 nm and starch stabilized particles between 53 to 91 nm. Though it appears, the size of such particles is in the range 40-90 nm. These larger particles are composed of van der Waals clusters of smaller entities. From geometry, it is clear that these individual particles are <10 nm in diameter, while the composite particles in lower resolution would appear to be higher particle size.

Antimicrobial activity

Minimum Inhibitory Concentration (MIC)

The MIC of $AgNO_3$ and silver nanoparticles were compared in *Escherichia coli.* The growth of the *E. coli* cells are inhibited at a concentration of 10 µg ml^{-1} of silver nanoparticles. This is the minimum concentration of the nanoparticles which inhibit the growth of the *E. coli* cells, *i.e* MIC of Ag nanoparticles. Both Ag nanoparticles and $AgNO_3$ inhibited the growth of *E. coli* cells at the same concentration but the rate of inhibition appears to be slow with increasing concentration of $AgNO_3$ compared to

Plate1. XRD pattern of synthesized silver nanoparticles

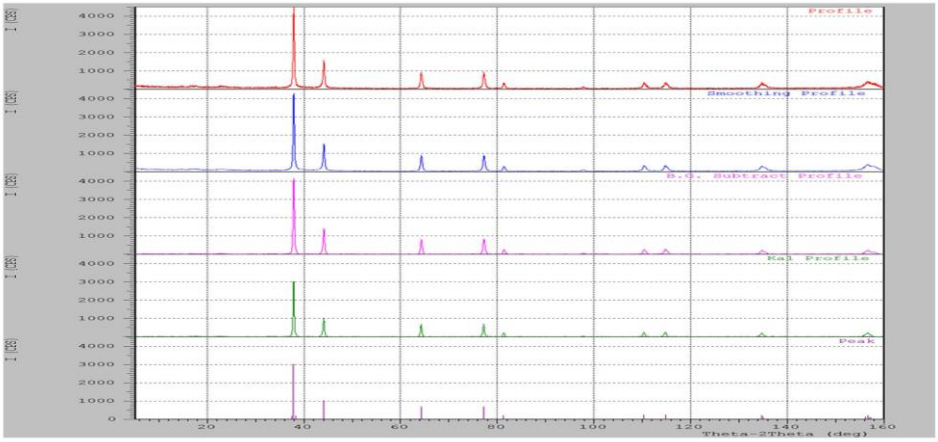
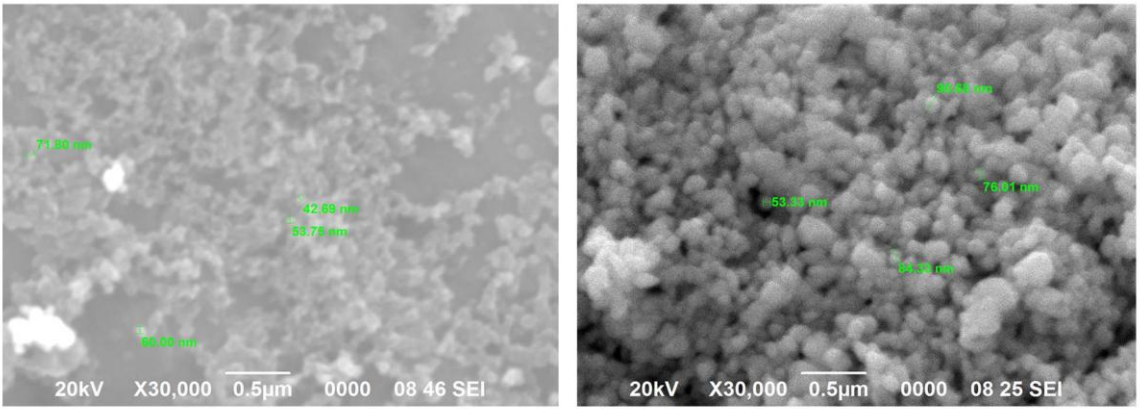


Plate 2. SEM images of synthesized silver nanoparticles



a) Silver nanoparticles **b) Starch stabilized silver nanoparticles**

silver nanoparticles. It suggested that the MIC of $AgNO_3$ is higher than silver nanoparticles.

Time dependent antibacterial activity

The number of *E. coli* colonies decreased for the increased concentration of $AgNO_3$ as well as silver nanoparticles. The duration of treatment markedly affected the *E. coli* population. When treatment duration increased from 1 to 12 h, 10 µg ml^{-1} concentrations was sufficient to inhibit 96 and 60 per cent bacterial growth by silver nanoparticles and $AgNO_3$ respectively. On the other hand 50 µg ml^{-1} of silver nanoparticles cause 100 per cent growth inhibition but at the same concentration of $AgNO_3$ inhibit only 80 per cent of growth during initial phase of treatment (Plate.3). In case of 12 h treatment using silver nanoparticles, 100 per cent growth inhibition recorded from 30 to 50 µg ml^{-1}. But $AgNO_3$ showed inferior performance, even in the 50 µg ml^{-1} concentration, more colonies grew on the plate.

Growth pattern

The bacterial growth kinetics was monitored in 100 ml LB medium (initial bacterial concentration, 10^7 CFU ml^{-1}) supplemented with different amounts of $AgNO_3$ and silver nanoparticles. Figure 1 shows that at all amount, $AgNO_3$ and nanoparticles caused a growth delay of *E. coli*, increasing concentration of $AgNO_3$ and silver nanoparticles decreased the growth of *E. coli*, and even in the lower concentration at which growth stopped altogether was observed in silver nanoparticles than $AgNO_3$.

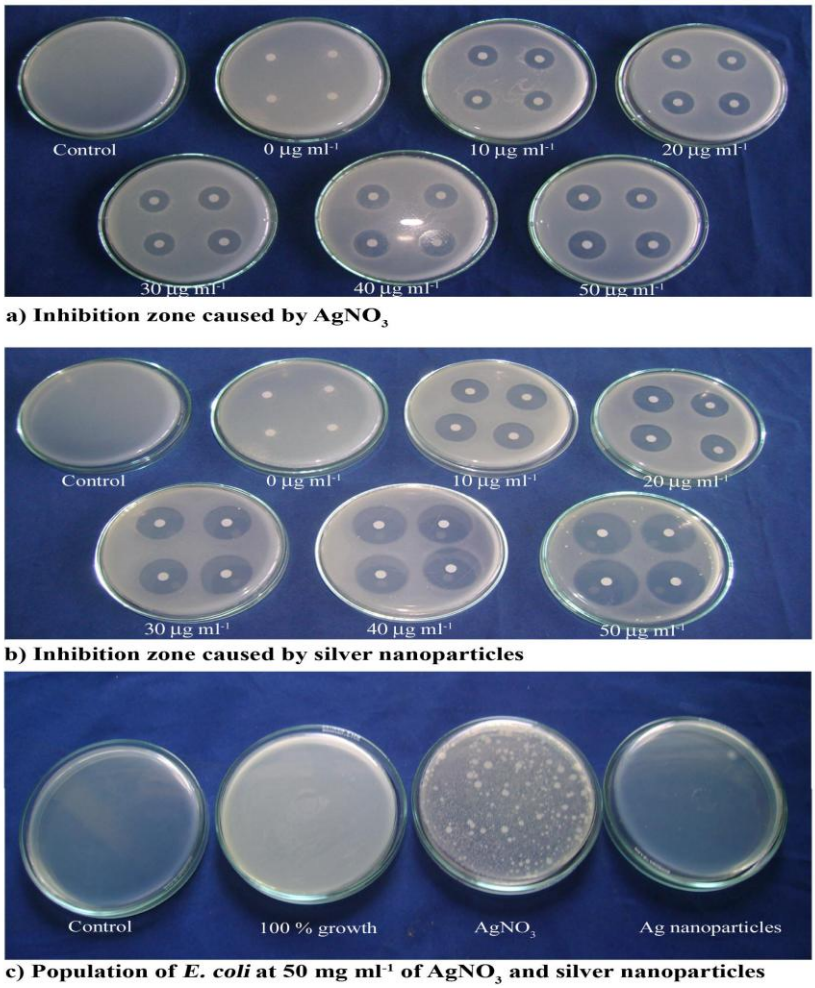


Plate 3. Comparison of E.coli growth inhibition by AgNO3 and silver nanoparticles

The growth rate of bacteria increased steadily with the increase in time at all concentrations, the growth was slightly affected in 10 µg ml^{-1} of $AgNO_3$ but greater reduction was observed under same concentration of silver nanoparticles. The silver nanoparticles caused 100 per cent growth reduction when treated with 30 to 50 µg ml^{-1} concentrations, whereas the effect was much less at this concentration of $AgNO_3$.

Zone of inhibition

Zone of inhibition test was done for identification of degree of inhibition by different concentration of $AgNO_3$ and silver nanoparticles. It was found that 10 µg ml^{-1} concentration was able to inhibit bacterial growth and create a zone of 0.8 cm by $AgNO_3$ and 1.7 cm by Ag nanoparticles (Plate.3). The increasing concentration of $AgNO_3$ and Ag nanoparticle showed a consistent increase in the zone size and reached the maximum of 1.2 and 2.5 cm diameter in $AgNO_3$ and silver nanoparticles, respectively at 50 µg ml^{-1} concentration.

DISCUSSION

The size of metallic nanoparticles ensures that a significantly large surface area of the particles is in contact with the bacterial cells. Such a large contact surface is expected to enhance the extent of bacterial elimination. The synthesis and characterisation of nanoscaled materials in terms of novel physico-chemical properties is of great interest in the formulation of bactericidal materials. Although growth on agar plates is a more ready means of distinguishing antimicrobial properties of silver nanoparticles, in this study liquid growth experiments showed similar results. But a previous study (Sondi and Salopek, 2004) pointed out a distinct difference between these two methods. In this study, complete inhibition of bacterial growth was

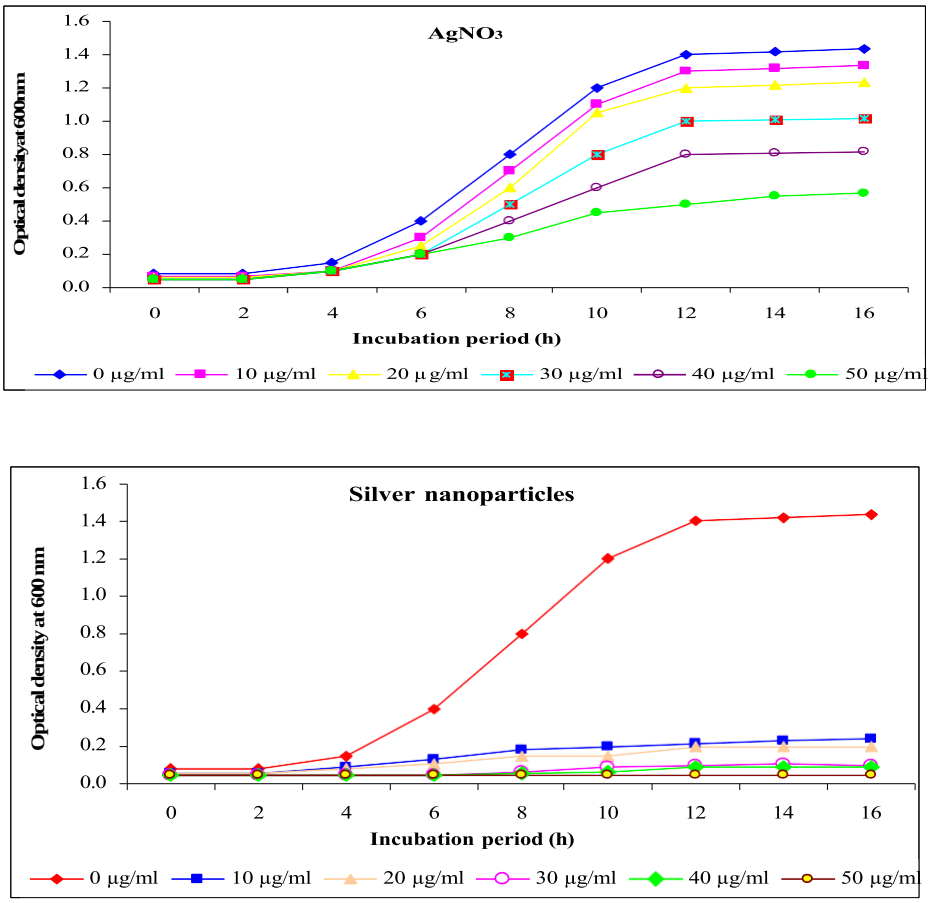


Figure 1.Growth pattern of *E.coli* in different concentrations of $AgNO_3$ and silver nanoparticles

observed on agar plates supplemented with silver nanoparticles. The extent of inhibition depends on the concentration of the silver nanoparticles as well as on the initial bacterial population. On the other hand, silver nanoparticles in liquid medium, even at lower concentrations showed *E. coli* growth delay and the growth was resumed rapidly with a decrease in the concentration of nanoparticles. This was supported by Sondi and Salopek (2004) who, reported that the interaction of these particles with intracellular substances from lysed cells caused their coagulation and the particles were thrown out of the liquid system.

The number of *E. coli* colonies decreased as increased concentration of silver nitrate ($AgNO_3$) and silver nanoparticles. The duration of treatment markedly affected the *E. coli* population. When treatment duration increased from 1 to 12 h, 10 µg ml^{-1} concentration was sufficient to inhibit 96 and 60 per cent bacterial population by silver nanoparticles and $AgNO_3$, respectively. The corresponding results are expressed as log (1+x) = f (c), i.e., log (1 +x) as a function of concentration of silver nanoparticles (c), where x is the number of CFU grown on agar plates. The decrease in number of viable cells with increasing amounts of $AgNO_3$ and silver nanoparticles can be fitted with a first-order exponential decay curve with a linear regression coefficient (R^2) ranging from 0.73 to 0.97 and 0.78 to 0.98, respectively.

The growth rate of bacteria increased steadily with the increase in time at all concentrations and the growth was slightly affected at 10 µg ml^{-1} of $AgNO_3$ but greater reduction was observed under same concentration of silver nanoparticles. The silver nanoparticles at 30 µg ml^{-1} concentration showed 100 per cent growth reduction, whereas in $AgNO_3$ much less effect was observed. Zone of inhibition test was also done for assessing the degree of inhibition by different concentration of $AgNO_3$ and silver nanoparticles. It was found that 10 µg ml^{-1} concentration inhibited bacterial growth and created a zone of 0.8 cm by $AgNO_3$ and 1.7 cm by silver nanoparticles. When the concentration of $AgNO_3$ and silver nanoparticles increased, the inhibition zone also increased. It was observed that silver nanoparticles are more toxic to *E. coli* than $AgNO_3$. The similar results were reported by Sharma *et al.* (2009), they proposed that the silver nanoparticles might attach to the surface of the cell

membrane, disturbing permeability and respiration functions of the cell. It is also possible that silver nanoparticles not only interact with the surface of membrane, but also penetrate inside the bacteria (Sharma *et al.*, 2009).

The mechanism of inhibitory action of silver ions on microorganism shows that upon Ag^+ treatment, DNA loses its replication ability and expression of ribosomal subunit proteins, as well as other cellular proteins and enzymes essential to ATP production, becomes inactivated (Yamanaka *et al.*, 2005). It has also been hypothesized that Ag^+ primarily affects the function of membrane bound enzymes, in the respiratory chain. However, the mechanism of bactericidal actions of silver nanoparticles is still not well understood. The positive charge on Ag^+ is an important factor for its antibacterial nature, through electrostatic interaction between the negatively charged cell membrane of the microorganisms and positively charged nanoparticles. It is proposed that the electrostatic force might be an additional cause for the interaction of the nanoparticles with the bacteria (Tiwari *et al*., 2008). In a previous report (Pal *et al*., 2007) on the bactericidal activity of silver nanoparticles, it was shown that the interaction between silver nanoparticles and constituents of the bacterial membrane caused structural changes and damage to membranes, finally leading to cell death.

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