FORMATION OF HIGHLY BIOACTIVE SILVER NANOPARTICLES AND THEIR ANTIBACTERIAL ACTIVITY

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Summary

In the present study, we report the preparation of silver nanoparticles by chemical reduction of silver salt ($AgNO_3$) solution with enhanced anti-bacterial potency. As a reducer sodium citrate was used. UV-VIS spectrometry indicated formation of silver nanoparticles. The morphology and size of the silver nanoparticles was characterized by transmission electron microscopy (TEM) and X-ray diffraction patterns (XRD). The spherical shape particles formed are predominantly in the range of 50–150 nm. The antimicrobial activities of silver nanoparticles against Gram-negative *Pseudomonas aeruginosa, Citrobacter friundii* and *Pseudomonas stutzeri* and Gram-positive *Staphylococcus aureus* were investigated by disc susceptibility method and scanning electron microscopy. These silver nanoparticles exhibited greater bactericidal activity against *Citrobacter friundii, Staphylococcus aureus* and *Pseudomonas stutzeri* respectively compared with *Pseudomonas aeruginosa.*

Key words: Bactericidal activity, Silver nanoparticle, Reduction, Bacterial strains.

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Introduction

Nanotechnology is currently employed as a tool to explore the darkest avenues of medical sciences in several ways like imaging (1), sensing (2), targeted drug delivery (3) and gene delivery systems (4) and artificial implants (5). Hence, nanosized organic and inorganic particles are finding increasing attention in medical applications (6) due to their amenability to biological functionalization. Based on enhanced effectiveness, the new age drugs are been investigated for their antibacterial property (7-10). Silver nanoparticles can be synthesized using various methods: chemical, electrochemical, *ã*-radiation, photochemical, laser ablation (11-14) etc. The most popular preparation of silver nanoparticles is chemical reduction of silver salts by sodium citrate. Solution temperature, concentrations of the metal salt and reducing agent, reaction time influences particle size. Controlling size and shape of metal nanoparticles remains a challenge (15). The size-induced antibacterial properties of nanoparticles enable the development of new applications. Most of the bacteria have developed resistance to antibiotics. Thus there is a future need to develop a substitute for antibiotics (16). Silver nanoparticles are attractive as these are non-toxic to the human body at low concentration and have broad-spectrum antibacterial nature. Ag nanoparticles inhibit bacterial growth at a low concentration than antibiotics and have no side effects (17, 18).
In the present study, we employ the chemical reduction method to fabricate the silver nanoparticles with their antibacterial properties.

Material and Methods

Bacterial strains

Four bacterial strains, namely *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 35032)*, Citrobacter friundii* (ATCC 43864) and *Pseudomonas stutzeri* (ATCC 17588) were subjected to this analysis. They were procured from MTCC, Chandigarh. The other components like Mueller-Hinton medium, Silver nitrate ($AgNO_3$) and different antibiotics used in the study were supplied by Hi-Media Laboratories, Pvt. Mumbai, India. Analytical grade trisodium citrate ($C_6H_5O_7Na_3$) from Merck India Ltd., Mumbai was used for the synthesis of silver nanoparticles. For the preparation of the mixture solution deionized water was used.

Synthesis of silver nanoparticles

The silver nanoparticles were prepared by using chemical reduction method according to the description of Asta (19). All solutions of reacting materials were prepared in distilled water. In this experiment 50 ml of $1 \cdot 10^{-3}$ M $AgNO_3$ was heated to boiling. To this solution 5 ml of 1 % trisodium citrate was added drop by drop. Mechanism of reaction could be expressed as follows:

$$4Ag^{+} + C_6H_5O_7Na_3 + 2H_2O \longrightarrow 4Ag^{0} + C_6H_5O_7H_3 + 3Na^{+} + H^{+} + O_2$$

During the process solution was mixed vigorously. Solution was heated until color's change is evident (pale yellow). Color change indicates the formation of silver nanoparticles. Then it was removed from the heating element and stirred until cooled to room temperature. The pH of the nanoparticles thus formed was maintained at 7.4. The solutions of Ag nanoparticles were stored in glass vials under ambient conditions for future experiments.

Characterization of silver nanopartical

The bioreduction of Ag^+ in aqueous solution was monitored by periodic sampling of aliquots (0.2 ml) of the suspension, then diluting the samples with 2 ml deionized water and subsequently measuring UV–vis spectra of the resulting diluents. UV–vis spectroscopy analyses of silver nanoparticles produced were carried out as a function of bioreduction time at room temperature on UNICAM UV- 300 spectrophotometers at a resolution of 1 nm.

X-ray diffraction (XRD) analysis of drop-coated films of silver nanoparticles in sample was prepared for the determination of the formation of silver nanoparticle by an X'Pert Pro x-ray diffractometer (X' Pert High Score Plus program) operated at a voltage of 40 kV and a current of 30 mA with Cu Kα radiation.

TEM samples of the aqueous suspension of silver nanoparticles were prepared by placing a drop of the suspension on carbon-coated copper grids and allowing water to evaporate. TEM observations were performed on an H-600 electron microscope (Hitachi, Japan) operated at an accelerating voltage of 120 kV. Size distribution of the resulting nanoparticles was estimated on the basis of TEM micrographs with the assistance of Sigma Scan Pro software.

Analysis of the antibacterial activity by silver nanoparticles

The effect of silver nanoparticles on gram-negative and gram-positive bacteria was investigated by culturing the organisms on Mueller-Hinton agar plates (10^6 colony forming units (CFU) of each strain per plate). Empty sterile discs having a diameter of 6 mm were impregnated with $1{\cdot}10^{-3}$ M silver nano solution at different concentrations (25 µl, 35 µl, 45 µl and 55 µl /disc, placed on inoculated surface of agar plate. Silver-free disc incubated under the same conditions were used as controls. These plates were incubating for 24 hour at 37°C and measured the zone of inhibition in millimeter (20). For SEM analysis bacterial cells treated with nanoparticles were collected by centrifugation of liquid samples at 10000 r/min for 10 min. The cell biomass were fixed on the aluminum stubs and coated with a thin layer of gold for SEM analysis (ZEISS EVO 40 EP).

Results and Discussion

The pale yellow colours observed in different solutions are symptomatic of the presence of silver nanoparticles in the solutions. UV-VIS absorption spectra have been proved to be quite sensitive to the formation of silver nanoparticles because silver nanoparticles exhibit an intense absorption peak due to the surface plamon (it describes the collective excitation of conduction electrons in a metal) excitation (21). Figure 1(a), shows the UV-VIS spectra of the silver nano in the range 300 – 700 nm. The absorption band in visible light region (350 – 500 nm, plasmon peak at 430 nm) is typical for silver nanoparticles. The plasmon peak and the full-width of half-maximum (fwhm) depend on the extent of colloid aggregation (22). Fig. 1(b) shows the plot of absorbance at 430 nm (λ_{max}) *vs.* time of reaction of trisodium citrate with Ag^+ ions and it can be inferred that the reduction of the silver ions occurs fairly rapidly. Since the varying intensity of the plasmon resonance depends on the cluster size.

Figure 2 show characteristic peaks (at 2θ = 30.8◦ and 64.4◦) of X-ray diffraction (XRD) patterns obtained for silver nanoparticles. A number of Bragg reflections corresponding to the (111) and (311) sets of lattice planes are observed which may be indexed based on the face-centred cubic structure of silver. The XRD pattern thus clearly shows that the silver nanoparticles formed by the reduction of Ag^+ ions by 1 % trisodium citrate are crystalline in nature.

A TEM micrograph and the particle size distribution are shown in Figures 3. The silver nanoparticles obtained by direct mixing of $AgNO_3$ and trisodium citrate solution. The spherical shape particles formed are predominantly. It is known that the shape of metal nanoparticles considerably changes their optical and electronic properties. The possibility of controlling the final size and size distribution of metal nanoparticles in the nanometer range is the aim of most modern nanochemistry. The particle size distribution was polydisperse with particle sizes ranging from 50 to 150 nm.

When a disk of repellent is placed in a Petri dish of Mueller-Hinton agar (10^6 colony forming units (CFU) of each strain per plate), bacteria will swim away from the repellent, creating a clear zone around the disk. Usually they sense repellents only at higher concentrations. Considering the growing interest in silver ions containing antimicrobial agents, we decided to test the antibacterial activity of silver nanoparticles against Gram-negative *Pseudomonas aeruginosa; Citrobacter friundii & Pseudomonas stutzeri* and Gram-positive *Staphylococcus aureus* were investigated. As the bacteria grew to form a confluent lawn, the extent of growth inhibition could be measured as the extent of the clear zone surrounding the disk shown in Figures 4. Out of these *Citrobacter friundii* showed maximum diameter of inhibition zone 10 mm, 14 mm, 16 mm, 18 mm respectively at 25, 35, 45 and 55 µl/disc concentration of silver nanoparticles solution, while *Pseudomonas aeruginosa* showed minimum diameter of inhibition zone 0 mm, 8 mm, 10 mm, 12 mm respectively at 25, 35, 45 and 55 µl/disc concentration of silver nanoparticles solution. Clear zone diameter of the bacterial inhibition zone was correlated to antibiotic activity of silver nanoparticles in Tables 1.

Figure 5, SEM microphotographs showing effect of silver nanoparticles on bacterial cells. Bactericidal behavior of nanoparticles is attributed to the presence of electronic effects that are brought about as a result of changes in local electronic structures of the surfaces due to smaller sizes. These effects are considered to be contributing towards enhancement of reactivity of silver nanoparticles surfaces. Ionic silver strongly interacts with thiol groups of vital enzymes and inactivates them. It has been suggested that DNA loses its replication ability once the bacterium are treated with silver ions (18). Two dimensional electrophoresis and proteins identification analysis of antibacterial action of silver nanoparticles have disclosed accumulation of envelope proteins precursors. Silver nanoparticles destabilize plasma membrane potential and depletion of levels of intracellular adenosine triphosphate (ATP) by targeting bacterial membrane resulting in bacterial cell death (16).

Outer membrane of bacrerial cells is predominantly constructed from tightly packed lipopolysaccharide (LPS) molecules, which provides an effective permeability barrier overall charge of bacterial cells at biological pH values is negative because of excess number of carboxylic groups, which upon dissociation makes the cell surface negative. The opposite charges of bacteria and nanoparticles are attributed to their adhesion and bioactivity due to electrostatic forces. Nanoparticles have larger surface area available for interactions, which enhances bactericidal effect than the large sized particles; hence they impart cytotoxicity to the microorganisms. Silver has a greater tendency to react with sulfur or phosphorus-containing soft bases. Thus, sulfur-containing proteins in the membrane and phosphorus-containing elements like DNA are likely to be the preferential sites for silver nanoparticle binding (23). Figure 5 (A) shows the zero loss SEM images of the cell structures and figure 5 (B) showing clumped and damaged cell membrane treated with silver nanoparticle. It has been suggested (24) that disruption of membrane morphology may cause a significant

increase in permeability, leading to uncontrolled transport through the plasma membrane and finally, cell death.

Conclusion

Silver nanoparticles were proved to have excellent antibacterial ability against Gram-negative *Pseudomonas aeruginosa, Citrobactor*, *Pseudomonas stutzeri* and Gram-positive *Staphylococcus aureus.* Studied by disc susceptibility method and scanning electronic microscopy.

Table 1 Antimicrobial Effect of Silver Nanoparticle Solutions against gram +ve and gram –ve bacteria

Bacterial strain	Concentration of Silvernano solution* (µl/disc)	Zone of inhibition (in mm)
Pseudomonas aeruginosa	25	8 mm
	35	10 mm
	45	12 mm
	55	14 mm
Citrobacter friundii	25	10 mm
	35	14 mm
	45	16 mm
	55	18 mm
Staphylococcus aureus	25	8 mm
	35	10 mm
	45	12 mm
	55	14 mm
Pseudomonas stutzeri	25	Nil
	35	8 mm
	45	10 mm
	55	12 mm

***** 1 mM aqueous solution of Silver nanoparticle

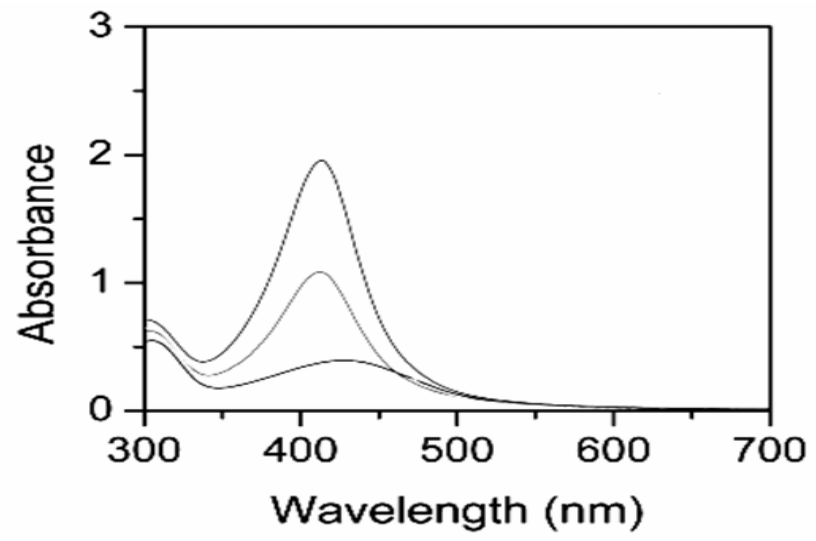

Figure 1 (a). UV–visible absorption spectra during various stages of reduction of silver ions to silver nanoparticles.

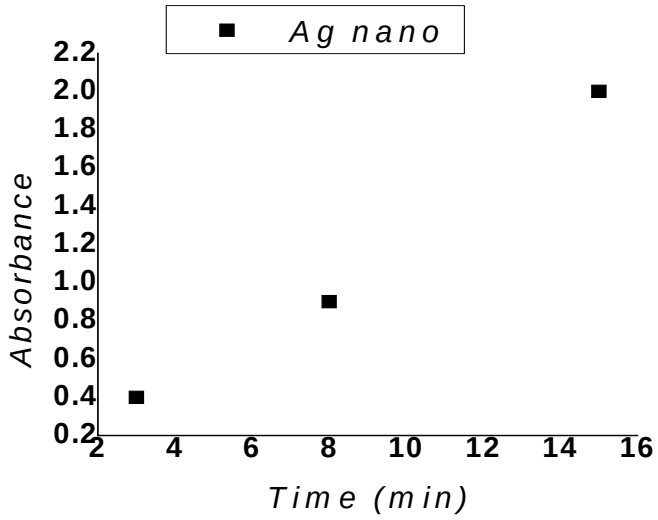

Figure 1 (b). Plot of maximum absorbance vs time of reaction for the silver nanoparticles

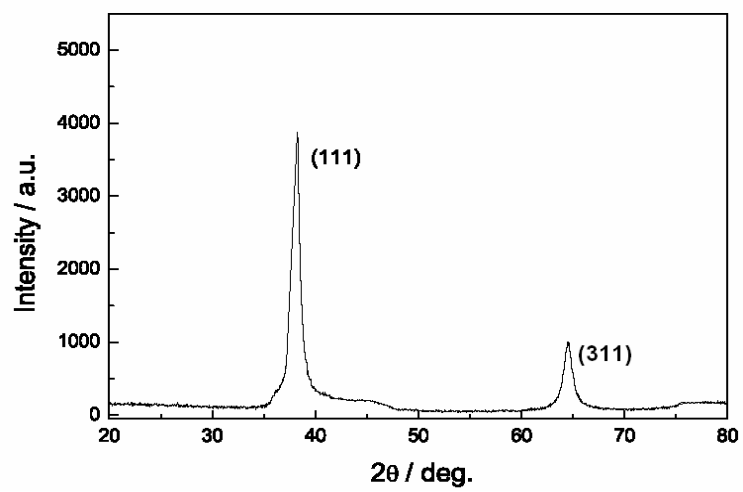

Figure 2. XRD patterns recorded from drop-coated films on glass substrate of silver nanoparticles.

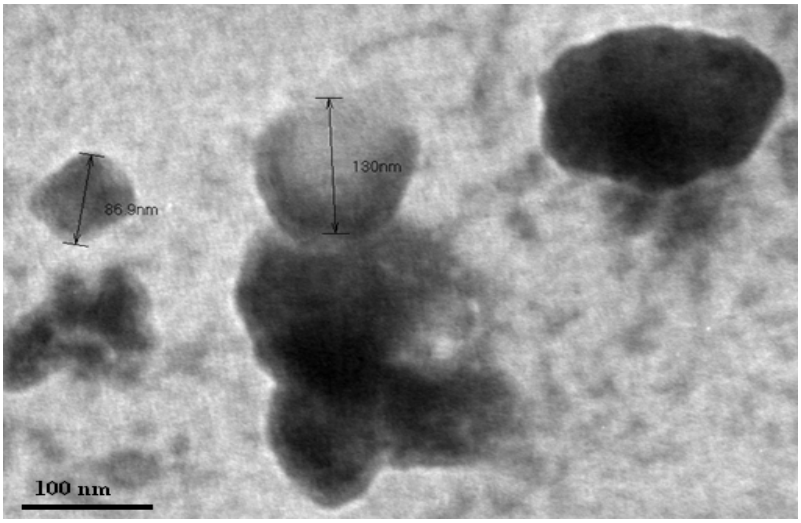

Figure 3. Transmission electron micrographs of silver nanoparticles. Scale bars: 100 nm.

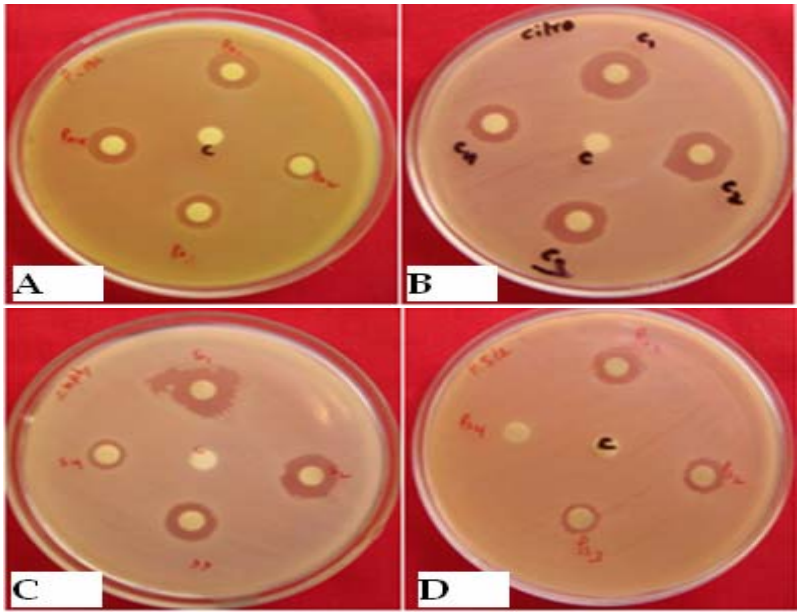

Figure 4. Antibacterial activity of silver nanopartical against **(A)** *Pseudomonas aeruginosa* **(B)** C*itrobactor* **(C)** *Staphylococcus aureus* and **(D)** *Pseudomonas stutzeri* with $1 \cdot 10^{-3}$ M nanosilver solution at different concentrations 25 µl, 35 µl, 45 µl, and 55 µl /disc and in center 'c' is control.

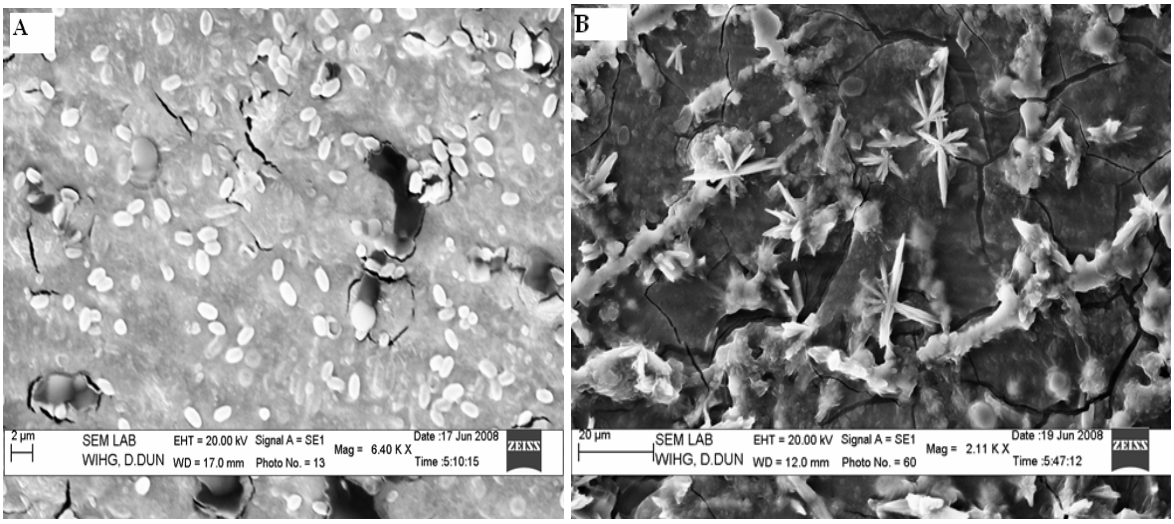

Figure 5. SEM microphotographs showing effect of silver nanoparticles on bacterial cells. **(A)** Bacterial cells before treatment at 6.40 KX magnification **(B)** Bacterial cells after treatment with silver nanoparticles at 2.11 KX magnifications.

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