# Antibacterial Characterization of Silver Nanoparticles against *E. Coli* ATCC-15224

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Silver nanoparticles of mean size 16 nm were synthesized by inert gas condensation (IGC) method. Crystalline structure, morphology and nanoparticles size estimation were conducted by X-ray diffraction (XRD) and transmission electron microscopy (TEM). Antibacterial activity of these silver nanoparticles as a function of particles concentration against gram-negative bacterium *Escherichia coli* (*E. coli*) was carried out in liquid as well as solid growth media. Scanning electron microscopy (SEM) and TEM studies showed that silver nanoparticles after interaction with *E.coli* have adhered to and penetrated into the bacterial cells. Antibacterial properties of silver nanoparticles are attributed to their total surface area, as a larger surface to volume ratio of nanoparticles provides more efficient means for enhanced antibacterial activity.

**KEY WORDS: Silver; Nanoparticles; Antibacterial; Electron microscopy**

## 1. Introduction

Nanomaterials display unique, superior and indispensable properties and have attracted much attention for their distinct characteristics that are unavailable in conventional macroscopic materials. Their uniqueness arises specifically from higher surface to volume ratio and increased percentage of atoms at the grain boundaries. They represent an important class of materials in the development of novel devices that can be used in various physical, biological, biomedical and pharmaceutical applications[1–4].

Synthesis of nanosized drug particles with tailored physical and chemical properties is of great interest in the development of new pharmaceutical products[5]. Emergence of new resistant bacterial strains to current antibiotics has become a serious public health issue, which raised the need to develop new bactericidal materials[6]. However, the phenomenon of enhanced biological activity and certain material changes resulting from nanoparticles is not yet understood fairly. Investigations have shown encouraging results about the activity of different drugs and antimicrobial formulation in the form of nanoparticles. Activity of nanoemulsions of oil droplets against bacteria and spores has been analyzed[7].

Silver is a nontoxic, safe inorganic antibacterial agent used for centuries and is capable of killing about 650 type of diseases causing microorganisms[8]. Silver has been described as being 'oligodynamic' because of its ability to exert a bactericidal effect at minute concentrations[9]. It has a significant potential for a wide range of biological applications such as antifungal agent, antibacterial agents for antibiotic resistant bacteria, preventing infections, healing wounds and anti-inflammatory[10]. Silver ions ($Ag^+$) and its compounds are highly toxic to microorganisms exhibiting strong biocidal effects on many species of bacteria but have a low toxicity towards animal cells. Therefore, silver ions, being antibacterial component, are employed in formulation of dental resin composites, bone cement, ion exchange fibers and coatings for medical devices[11,12].

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[13]. 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[14].

Compounds of silver such as silver nitrate and silver sulfadiazine are used to prevent bacterial growth in drinking water, sterilization and burn care. It is economical to consolidate silver in polymers, composites, fabrics and catheters for antibacterial functionality[15–18]. The present study was conducted to synthesize silver nanoparticles by inert gas condensation (IGC) process and characterization of their crystalline structure, morphology, estimation of mean size and distribution by XRD and TEM techniques. Antibacterial activity of these silver nanoparticles as a function of particles concentration has been demonstrated for inhibiting the growth and killing of *E. coli* ATCC-15224 strain in liquid as well as solid growth

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media. The ultimate objective was to study the interaction between bacteria and silver nanoparticles.

## 2. Experimental

### 2.1 Synthesis of silver nanoparticles

IGC method was used to synthesize silver nanoparticles by metal evaporation in inert atmosphere. Pieces of 5 mm thick bulk silver wire (Sigma Aldrich, 99.9% purity) were placed in the Mo boat. Process vacuum chamber was cleaned by achieving a base pressure of the order of $1.33\times10^{-4}$ Pa. Argon gas (99.99% purity) was introduced into the chamber through a moisture trap at a pressure of 13.33 Pa. Silver was evaporated by supplying electric current to the boat. There was a disc shutter over the boat, which was opened when supersaturation conditions were achieved. The particles formed in the gas phase were allowed to deposit at the surface of a stainless steel flat surface cooled by flowing liquid nitrogen. Synthesized nanoparticles were collected by brushing them off the stainless steel plate with a teflon scrapper. The collected silver nanoparticles were characterized by XRD and TEM for estimation of crystalline structure, mean size and morphology. For TEM analysis, dilute suspensions of nanoparticles in pure acetone were prepared by ultrasonication. Drops of the suspension were put on carbon coated copper grids and dried. TEM analyses were done on a JEOL-1010 microscope.

### 2.2 Antibacterial test

Bactericidal effect of silver nanoparticles was studied against Gram-negative bacteria. These nanoparticles were dispersed in autoclaved deionized water by ultrasonication (Yamato-934, Japan). Aqueous dispersions of silver nanoparticles of desired concentrations were made. An axenic culture of *E. coli* ATCC-15224 was grown in liquid nutrient broth medium CM-01 (Oxoide, England) (containing (g/l): Lab lemco powder 1 g, NaCl 5 g, peptone 5 g and yeast extract 2 g). For this experimental investigation, freshly grown bacterial inoculum ($10^4$ cells/ml) of *E. coli* was incubated in the presence of a range of silver nanoparticles loadings of 0, 20, 40, 60, 80 and 100 $\mu$g/ml added in each flask to observe the bacterial cell growth at 37°C and 150 r/min. Total solution volume in each flask was kept 50 ml. In liquid medium, the growth of *E. coli* was indexed by measuring optical density (OD) at $\lambda_{\max}$ 600 nm against abiotic control using UV-Vis spectrophotometer (Agilent-9453, USA) after every 2 h up to 24 h. Samples were collected for colony formation units (CFU) measurements onto the solid medium. Samples treated with nanoparticles were spread on nutrient agar plates and after incubation at 37°C for 24 h, and the numbers of CFU were counted. Bacterial cells treated with nanoparticles were collected by centrifugation (Beckman-Coulter Microfuge-18, USA) 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 (LEO-440 *i*). Bacterial cells incubated along with nanoparticles in liquid growth medium were collected on the carbon coated copper grids for TEM analysis. All experiments were performed under sterile conditions and in triplicate.

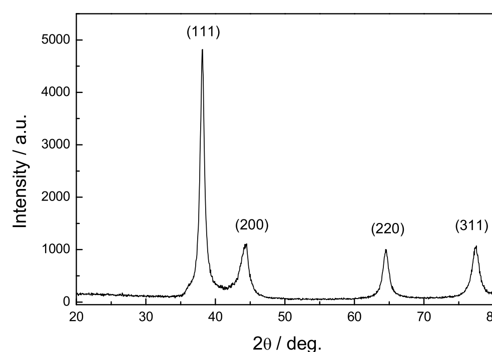


**Fig.1** XRD pattern of silver nanoparticles synthesized by inert gas condensation process

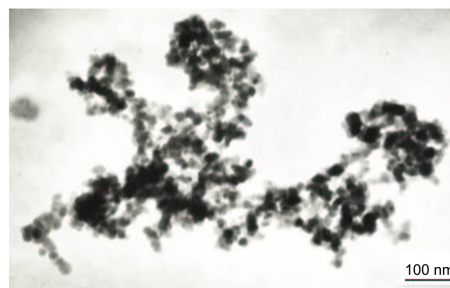


**Fig.2** TEM micrograph of silver nanoparticles synthesized by inert gas condensation process

## 3. Results and Discussion

XRD pattern of silver nanoparticles produced by IGC method is shown in Fig.1. All diffraction peaks correspond to the characteristic face centered cubic (FCC) silver lines. XRD patterns were analyzed to determine peak intensity, position and width. Full-width at half-maximum (FWHM) data was used with the Scherrer′s formula to determine mean particle size. Scherrer′s equation is given by

$$d = \frac{0.9\lambda}{\beta\cos\theta}$$

where $d$ is the mean diameter of the nanoparticles, $\lambda$ is wavelength of X-ray radiation source, $\beta$ is the angular FWHM of the XRD peak at the diffraction angle $\theta$[19]. The mean size of nanoparticles estimated by XRD is 14 nm.

TEM micrograph of silver nanoparticles is shown in Fig.2, which demonstrates agglomerated clusters of spherical shape and narrow particle size distribution. Nanoparticles formed in gas phase synthesis processes follow lognormal distribution function (LNDF)[20–23]. The mean particle size estimated by TEM is 16 nm, which compares well with the size estimated from XRD data. The mean size of these silver nanoparticles is comparable to the particles that have been synthesized by sol-gel technique in our previous work[24].

Silver nanoparticles were dispersed in autoclaved deionized water by ultrasonication so that soft aggl-

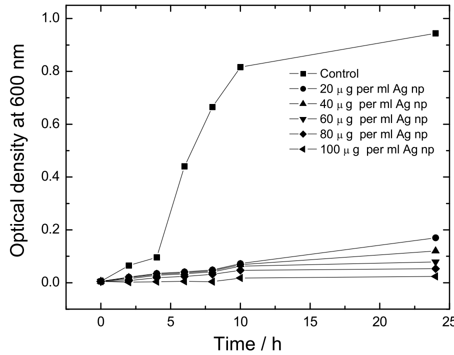


**Fig.3** Optical density as a function of time in the solution studies

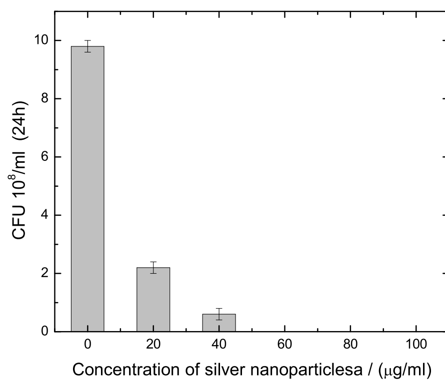


**Fig.4** Antibacterial characterization by CFU as a function of silver nanoparticles concentration on nutrient agar plates after 24 h incubation time

omerates were broken down and resulted into smaller nanoparticles. Aqueous dispersions of these nanoparticles at desired concentrations were made. Number of bacterial cells ($10^4$ cells/ml) added in each flask containing 50 ml of solution were closer to the real life situations. Shaking provided bacteria aeration and homogeneity. Control flasks containing all the initial reaction components except the silver nanoparticles showed no antibacterial activity.

Silver nanoparticles were added in the solution at the beginning of bacterial cell growth. Optical densities as a function of time measured periodically up to 24 h of the control and solutions containing different concentrations of silver nanoparticles are shown in Fig.3. Bacterial cells grow by a process called binary fission in which one cell doubles in size and splits into halves to produce two identical daughter cells. Bacterial cell growth enhances the turbidity of the liquid nutrient medium and as a result the absorption increases. It has been observed that optical density of the growth medium decreased in comparison to the control with increasing concentration of silver nanoparticles. This has been attributed to the reduced growth of bacterial cells. Silver nanoparticles at concentrations 60 $\mu$g/ml and higher were found effective bactericides, and there was virtually no bacterial growth as optical absorption was insignificant.

Figure 4 shows the number of bacterial colonies grown on nutrient agar plates as a function of concentration of silver nanoparticles. The bacterial cell colonies on agar-plates were detected by viable cell counts. Viable cell counts are the counted number of colonies that are developed after a sample has been diluted and spread over the surface of a nutrient medium solidified with agar and contained in a petri dish. The number of CFU reduced significantly with increasing the concentration of silver nanoparticles. There was virtually no CFU observed in the samples containing silver nanoparticles loading 60 $\mu$g/ml and higher. The bacterial growth inhibition trend observed from CFU data has matched well with the results of optical density.

Silver nanoparticles in the membrane of the bacteria as well as in its interior were observed by electron microscopy. Figure 5(a), shows the SEM micrograph of bacterial biomass treated with silver nanoparticles. It has been observed that silver particles adhered to the bacterial cell wall surface. The presence of elemental silver on the bacterial cells was supported by EDX analysis shown in Fig.5(b). TEM micrograph shows silver nanoparticles not only adhered at the surface of cell membrane, but also penetrated inside the bacterial cells. Figure 6(a) shows that silver nanoparticles were bound to the cell wall of bacteria. Figure 6(b) reveals that nanoparticles have penetrated inside the bacterial cells. Electron microscopy determined the distribution and location of the silver nanoparticles, as well as the morphology of the bacteria after treatment with silver nanoparticles. Bacterium have different membrane structures on the basis of which these are classified as Gram negative or Gram positive. The structural difference lies in the organization of peptidoglycan, which is the key component of membrane structure. Gram-negative bacterium exhibit a thin layer of peptidoglycan (about 2–3 nm) between the cytoplasmic membrane and the outer cell wall. Outer membrane of *E. coli* cells is predominantly constructed from tightly packed lipopolysaccharide (LPS) molecules, which provides an effective permeability barrier[5]. The 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. It is logical to state that binding of nanoparticles to the bacteria depends on the surface area available for interaction. Nanoparticles have larger surface area available for interactions, which enhances bactericidal effect than the large sized particles; hence they impart cytotoxicity to the microorganisms[18]. The mechanism by which the nanoparticles are able to penetrate the bacteria is not understood completely, but studies suggest that when *E. coli* was treated with silver, changes took place in its membrane morphology that produced a significant increase in its permeability affecting proper transport through the plasma membrane, leaving the bacterial cells incapable of properly regulating transport through the plasma membrane, resulting into cell death. It is observed that silver nanoparticles have penetrated inside the bacteria and have caused damage by interacting with phosphorus and sulfur containing compounds such as DNA[13]. Silver tends to have a high affinity to react with such compounds.

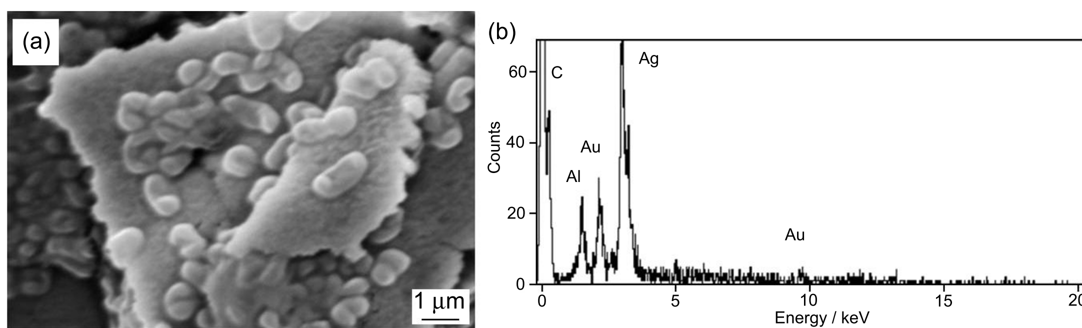


**Fig.5** (a) SEM micrograph of *E. coli* treated with silver nanoparticles, (b) EDX analysis

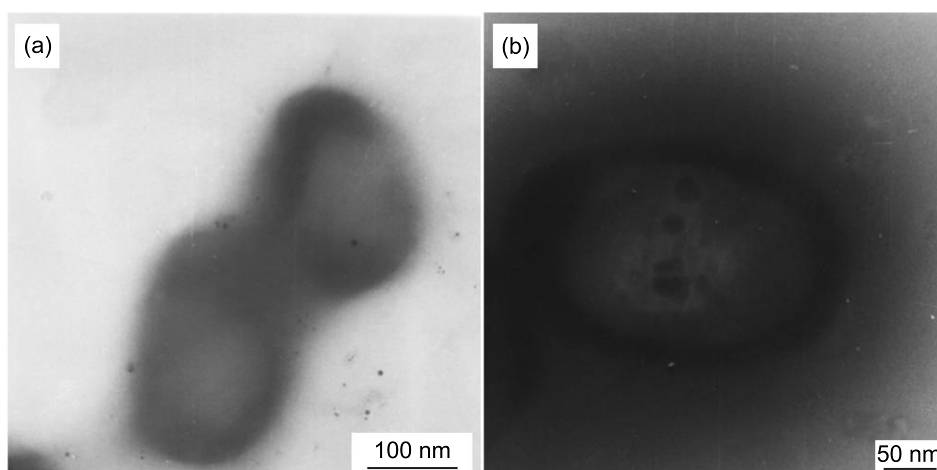


**Fig.6** TEM micrograph: (a) interaction of silver nanoparticles with *E. coli*, (b) penetration of silver nanoparticles in *E. coli*

In our study, it is believed that DNA may have lost its replication ability and cellular proteins become inactive after treatment with silver nanoparticles. Another reason would be the release of silver ions from nanoparticles, which will have an additional contribution to the bactericidal efficacy of silver nanoparticles. Heavy metals are toxic and react with proteins, therefore they bind protein molecules; as a result cellular metabolism is inhibited causing death of microorganism[4]. High activity of silver nanoparticles is attributed to species difference as they dissolve to release $Ag^0$ and $Ag^+$ clusters, whereas other silver sources such as silver nitrate and silver sulfadiazine release $Ag^+$ only[10]. It is believed that silver nanoparticles after penetration into the bacteria have inactivated their enzymes, generating hydrogen peroxide and caused bacterial cell death[25].

Experimental observations of this study have explained significantly the antibacterial behavior of silver nanoparticles. It is observed in Fig.5(a), that bacterial cells show critical changes and damage in the membrane structure. When *E. coli* was treated with highly reactive metal oxide nanoparticles, an inhibitory effect took place[7]. Silver nanoparticles after adherence to the surface of the cell membrane disturbed its respiration as $Ag^+$ interact with enzymes of the respiratory chains of bacteria[26]. It is observed in our study that a degradation of the membrane structure of *E. coli* took place. Metal depletion causes formation of irregular-shaped pits in the outer membrane of bacteria which is caused by progressive release of LPS molecules and membrane proteins[27]. In addition, it is believed that silver binds to functional groups of proteins, resulting in protein denaturation[28]. As observed in Fig.3, lag phase of bacterial growth has a delayed trend at lower concentrations of silver nanoparticles loadings as compared with biotic control. Complete bacterial inhibition depends upon the concentrations of silver nanoparticles and on the number of bacterial cells. It reflects that silver nanoparticles have an excellent biocidal effect and potential in reducing bacterial growth for practical applications.

## 4. Conclusion

Silver nanoparticles of mean size 16 nm were synthesized by inert gas condensation process using argon gas. Antibacterial characterization has been demonstrated against *E. coli* ATCC-15224 on both liquid as well as solid growth media. Silver nanoparticles with concentrations as low as 60 $\mu$g/ml have demonstrated a complete cytoxicity to the *E. coli* bacterial strain. Silver nanoparticles adhered to the cell wall of bacteria and penetrated through the cell membrane. This resulted into inhibition of bacterial cell growth and multiplication.

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