

STUDY ON ANTIBACTERIAL POTENCY AND MECHANISM OF ACTION OF SILVER-NANO PARTICLES ON ESCHERICHIA COLI AS MODEL GRAM NEGATIVE BACTERIA

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Abstract

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Backgrounds: Emergence of bacterial resistance to several antibiotic are causing serious problem in health care. Even the broad-spectrum bactericidal effect of ionic form of silver sometimes found to be ineffective; few cases are reported to ascertain the incident of silver resistance. The purpose of the study was to examine the potency and mechanistic bactericidal actions of Silver Nano particles (AgNPs) on E. coli as model gram negative bacteria. **Methods:** Antibacterial activity of nano-sized silver particle was investigated against E. coli, Minimum Inhibitory Concentration (MIC) of AgNPs was determined following National Committee for Clinical Laboratory Standard (NCCLS) guideline, in Mueller- Hinton (MH) broth supplemented with different concentration of AgNPs. Growth pattern of bacteria at the MIC of AgNPs was studied and the mechanism of bactericidal action of AgNPs was observed by Transmission Electron Microscopy (TEM). **Results:** The antimicrobial activity AgNPs were investigated in liquid system and the results are showing that AgNPs were attached on the surface of the bacterial cell membrane; probably by the surface charge attraction followed by formation pits which destroy the integrity of the bacterial cell wall, resulting in death of the bacterial cells. **Conclusions:** AgNPs produce strong bactericidal action event at very low concentrations because of their high surface to volume ratio and remains effective for a longer duration. So it could be an effective therapy for open wounds if applied topically.

Introduction

Silver has been utilized by the mankind since the ancient time due to its potential antimicrobial effect. Nanotechnology, the science of engineering matter on an atomic and molecular scale utilizes the unique physiochemical properties of the particles at 1-100 nm size ranges for wide range of applications. However, healthcare and the drug delivery technologies are the one of the fast growing area of nanotechnology.

AgNPs, apart from its unique physiochemical properties; holds potential antibacterial effectiveness than its bulk form ^[1, 2]. Bacterial skin infections ranges from mild pyodermas to life threatening necrotizing infections, manifestation bacterial skin infection results from interaction of bacterial virulence proteins with immune status and underlying condition of the host ^[3].

In recent times incidence of bacterial resistance to antibiotics are being reported frequently due to their overuses [4], beside this some antimicrobial agents are extremely irritant and cytotoxic [5]. Silver ions and other form of silver based compounds are well known for their strong and broad spectrum biocidal effect on more than 16 species of bacteria [5].

Unlike conventional antibiotics, bacteria can not develop resistance easily against silver as it has multiple targeted biocidal actions [6], such as destruction of bacterial cell wall and destabilization of cell wall proteins, blocking of DNA replication, and inactivation of the vital enzymes of the bacterial respiratory systems [6-9]. However, AgNPs are relatively new as an antimicrobial agent [4] but it has drawn the interest of the modern research because of their high reactivity due to large surface to volume ratio. So that, AgNPs could produce strong antimicrobial effect even at a very low concentration as compared to other silver product. Hence, AgNPs has minimum chances of cellular toxicity (if any) to the host cells in comparison to other silver product.

In this work our objective was to find out the MIC of AgNPs as well as to study the interaction of bacterial cells with AgNPs by means of bacterial death Kinetics study and TEM analysis.

Materials and Methods

Preparation of AgNPs: AgNPs were prepared in our departmental laboratory by chemical reduction of silver nitrate [E (Merck) India Pvt. Ltd. Mumbai, India], in the presence of glucose [Himedia, Mumbai, India] and Di-ethyl amine [Loba Chemie Pvt. Ltd, Mumbai, India]; detailed process is not described here. The whole reaction was carried out in liquid system. Morphology of the AgNPs was analyzed by TEM and surface charge was tested by estimating Zeta potential.

Culture of organisms: Field isolated strains of *E.coli* were used for this study. Organisms were harvested from an infected burn wound. Identification and culture of the organisms was done by the Department of Microbiology, in our Institution, following a standard clinical microbiology protocol.

Determination of MIC of AgNPs and Study of Bacterial growth curve: NCCLS guideline was followed to study the MIC and the dynamics of bacterial growth with different concentration and exposure time of AgNPs. Bacterial colonies from a fresh (18-24 h) culture plate were transferred to a sterile cation adjusted MH broth [Himedia, Mumbai, India] and matched with the 0.5 McFarland standard that is equivalent to the bacterial concentration of 1×10^8 to 2×10^8 CFU/ml, which was further diluted by following a standard protocol [10] to produce the final concentration of 5×10^5 CFU/ml of viable organisms in each micro-plate wells.

The final concentrations of the AgNPs in each well were adjusted as 300, 150, 75, 37.5, 18.75, 9.37, 4.68 and 2.34 ppm respectively. The last column of the micro-plate was set as control to which *E. coli* without AgNPs was added. Finally the plate was incubated at 37° C.

Dynamic of bacterial growth with the exposure of different concentrations of the AgNPs were observed in the same system by checking the OD₆₀₀ throughout the time series of 2, 4, 6, 8, 12, and 24 hours. MIC of AgNPs was recorded at the end of the experiment, in addition to this one loopfull of bacterial suspension from each well was taken and tricked on MacConkey agar plate to visualize the concentration (AgNPs) dependant growth inhibition of the *E. coli* cells.

Preparation of the *E. coli* for TEM analysis: *E. coli* cells with or without exposure of AgNPs were pelleted down by centrifugation. Cell pallet was washed for three times with PBS to remove the media, cellular derbies and unattached remaining AgNPs. A standard protocol was followed for cellular fixation, 2.5% glutaraldehyde solution was used as fixative. A drop of fixed bacterial suspension was dried on the TEM grid for the analysis.

Result

Determination of MIC of AgNPs: MIC of an antimicrobial agent gives quantitative estimate of susceptibility and is defined as the lowest concentration of antimicrobial agent required to inhibit the growth of the organisms. Here as the exposure of the AgNPs to *E. coli* increased gradually from 2.34 to 37.5 ppm, bacterial growth was found to be inversely proportional to the concentration of the AgNPs. No growth was seen at 37.5 ppm and further higher concentrations [Fig 1]. Hence, 37.5 ppm was recorded as the MIC of AgNPs with no visible growth of *E. coli* cells.

Analysis of the growth curve of treated *E. coli*: The purpose of doing bacterial death kinetics analysis was to evaluate the antimicrobial action and killing potencies of AgNPs at the predetermined concentration (MIC). Since,

inhibition of growth was assayed optically at OD₆₀₀ and as each examined micro-plate wells contained both the live and dead bacteria, positive OD reading were obvious for all wells, however, concentration (AgNPs) dependant growth inhibition was cross-checked by inoculating samples from each wells to separate MacConkey agar plates.

Growth curve of *E. coli* with the exposures of different concentrations of AgNPs was shown in [Fig 2], untreated control *E. coli* (A) reached the exponential phase rapidly but the prolonged Lag phase was noticed in case of *E. coli* treated with lower concentrations than the MIC of AgNPs (B-D). However, at MIC of AgNPs (E), growth of *E. coli* never reached the exponential phase. Moreover, the initial bacterial load which was set for the study was declined through the time series and no growth was observed at 6 hours up to 24 hours. This is an important phenomenon from the clinical point of view, for the treatment of open wounds as infection control remains the primary intention for such cases.

TEM analysis to study the bactericidal action of AgNPs on *E. coli*: Surface of the untreated *E. coli* cells were smooth [Fig 3a] while deposition and attachment of the AgNPs on the bacterial cell surface were seen in the TEM micrograph of treated *E. coli* cells. It is evident from the TEM analysis that the AgNPs were accumulated on the cell surface in a random fashion while some particles were found to be penetrated in to the bacterial cells that leads to formation of pits and gaps [Fig 3b & 3c] on the cell surface. Some of the treated cells showed leakage of intercellular substances as indicated by the red circle [Fig 3d], while some were found to be extremely fragmentary [Fig 3e] due to damage caused by AgNPs.

Discussion

In this study the bactericidal activity of AgNPs were investigated by growing *E. coli* cells in liquid MH medium which were supplemented with different concentration of AgNPs. Growth curve of *E. coli* that were treated with AgNPs, showing the evidence of delayed reproduction rate of bacterial cells and inhibition of growth could be achieved at or around the MIC of AgNPs.

In liquid medium lower concentration AgNPs (>MIC) could only delay the bacterial growth, that is prolongation of the Lag Phase. Because following interaction with bacterial cells, released intercellular substances of the damaged cells leads to the coagulation and removal of the AgNPs from the liquid system^[5]. Hence concentration of the AgNPs gradually decreases that allows the recurrence of bacterial cell growth [Fig 2].

However, inhibition bacterial growth depends on the relative concentration of the AgNPs as well as the CFU of bacteria used for the study. As reflected from the figure 1 complete inhibition of the growth was observed at MIC of the AgNPs. Since, the in-vivo bactericidal test involves high CFU of bacteria which are rarely found in real-life condition. So we could assume that these new generation AgNPs may potentially have excellent bactericidal effectiveness and can be proved to be good candidate for application such as formulation of bactericidal drugs.

Since long silver and its compounds are known to be broad spectrum bactericidal, still only a few rare strains of bacteria are reported to be silver resistance^[11-13]. Occurrence of resistance to an antimicrobial agent can be noticed either by 'intrinsic' or 'acquired' mechanisms. However, bacterial resistance to heavy metal ions can occur through energy dependant ion efflux systems^[11]. Though the bactericidal action of Metal Nano-particles (MNPs) are poorly understood, but as reported by Pan et.al^[14], MNPs could be absorbed on bacterial membrane first by surface charge attraction the destroy the membrane integrity by forming pits on the cell wall, which ultimately leads to the bacterial cell death^[14]. It is somewhat like mechanical damage caused externally to the bacterial cells, hence it can be assumed that bacterial cells are unable to develop resistance against MNPs.

E. coli was selected as a model for Gram negative bacteria, to study the bactericidal effect of AgNPs on the membrane structure and change in membrane permeability of bacterial cells. Membrane structure of native *E. coli* cell has already been studied extensively. The outer layer of the membrane is composed of tightly packed lipopolysaccharide (LPS) molecules that plays as an effective permeability barrier^[5] and imparts strongly negative charge to surface of Gram negative bacterial cells. Studies have shown that any alteration in LPS structures may leads to increase in permeability as compared that of native cells^[15]

However, interestingly, AgNPs used in this study are negatively charged [fig 4], still then as seen from the TEM photograph that the treated bacteria shows significant damage on the negatively charged cell membrane, the possible explanation could be, that the negative charge on the bacterial cell surface are not sufficient enough to cause repulsion of AgNP and similar effect were also reported^[16]. This result is in good agreement with as reported by

Sondi et.al^[5] and also to the similar effect as were reported with *E. coli* that were treated with metal oxide nanoparticles^[17]. Amro et.al have reported another study where they suggested that the formation of the irregular pits on the outer membrane may leads to progressive release of Lipopolysaccharide molecules (LPS) and membrane proteins^[15]. Here we may speculate, as it is evident from the TEM image, that the similar mechanisms involved in the membrane damage of *E. coli* cells while they were treated with AgNPs.

Moreover, It could be anticipated following several reports that AgNPs may release Ag⁺^[18, 19], which have traditional bactericidal effects that is inhibition of respiratory enzyme(s), generation of reactive oxygen species (ROS)^[20], additionally, sulfur-containing proteins in the membrane and phosphorus-containing elements like DNA are likely to be the preferential sites for Ag⁺ binding^[21-23] that consequently leads to the bacterial cell death.

Nevertheless, AgNPs are neither an alternative nor a competitor to an antibiotic; moreover studies from corners of the world, suggesting that synergistic effect of AgNPs with antibiotics would prevent the emergence of resistance and may empower the antimicrobial properties of an antibiotic^[24].

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Figure legend

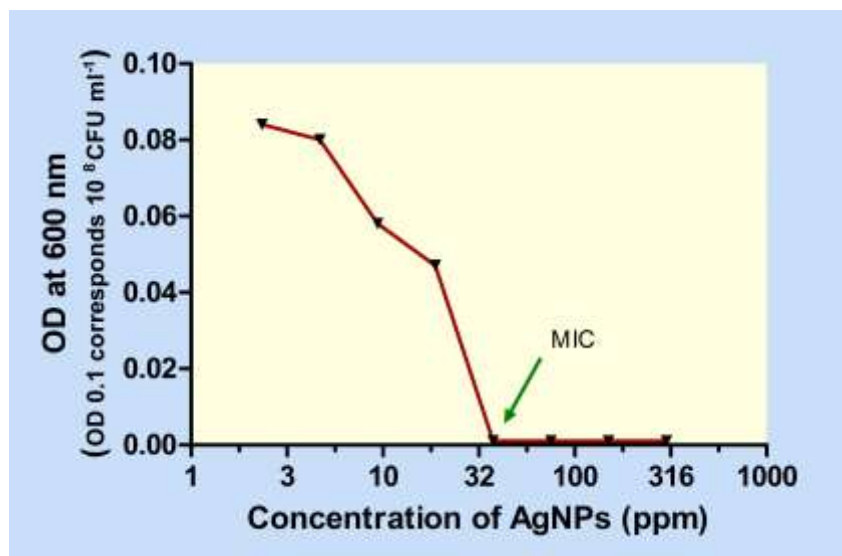


Figure 1: Determination of MIC of AgNPs on *E. coli*.

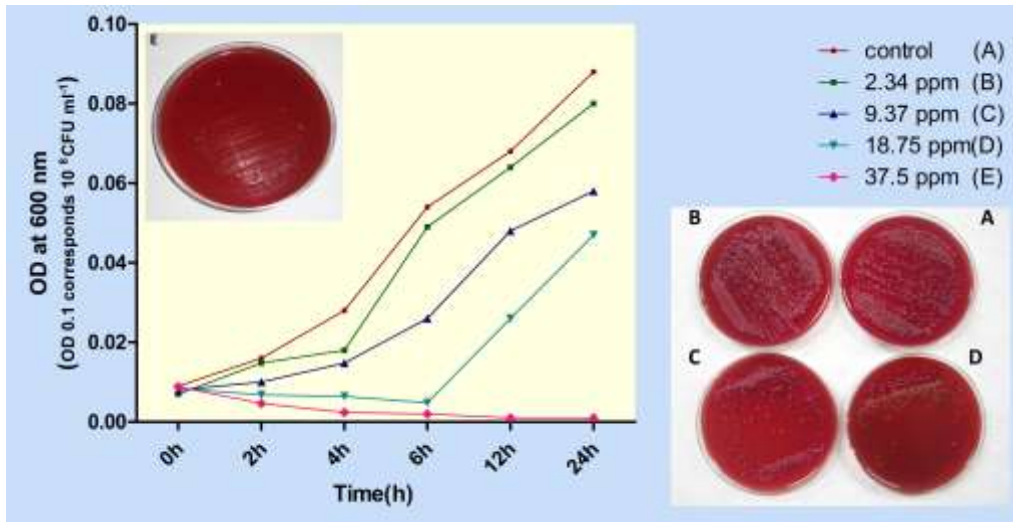
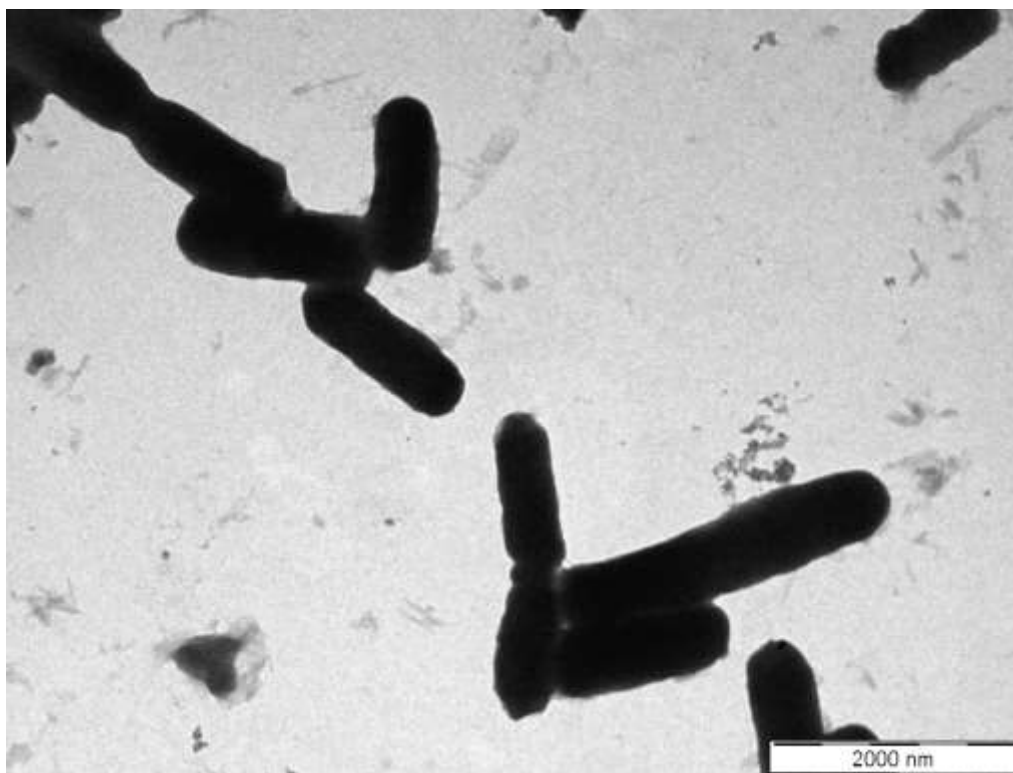
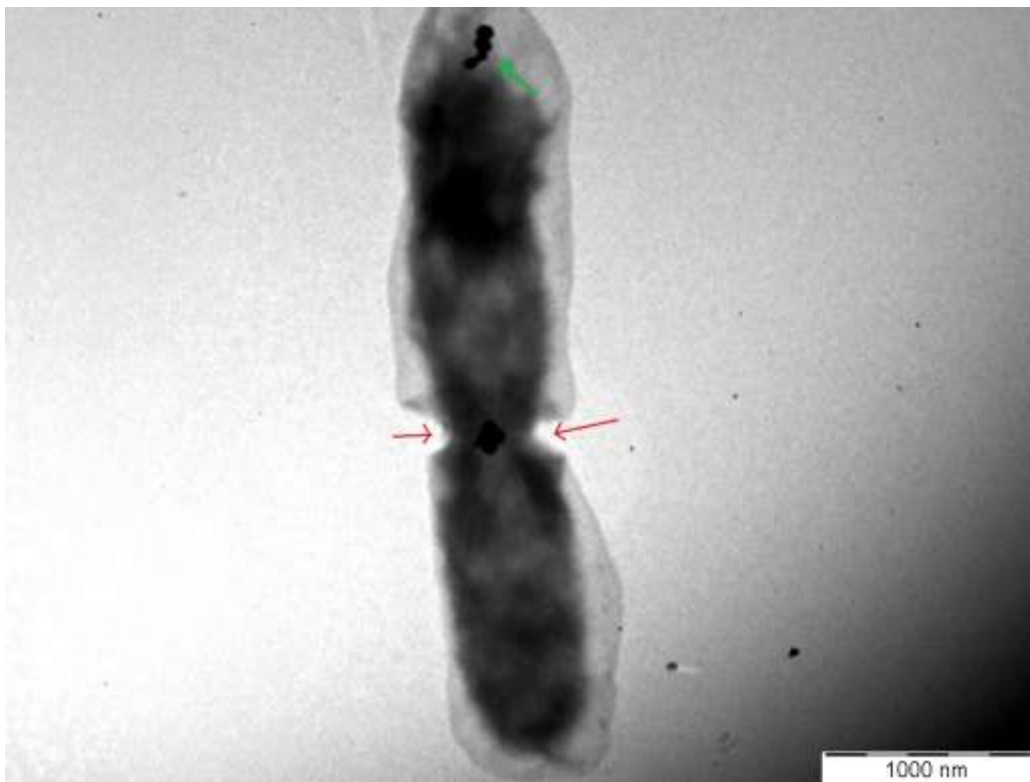
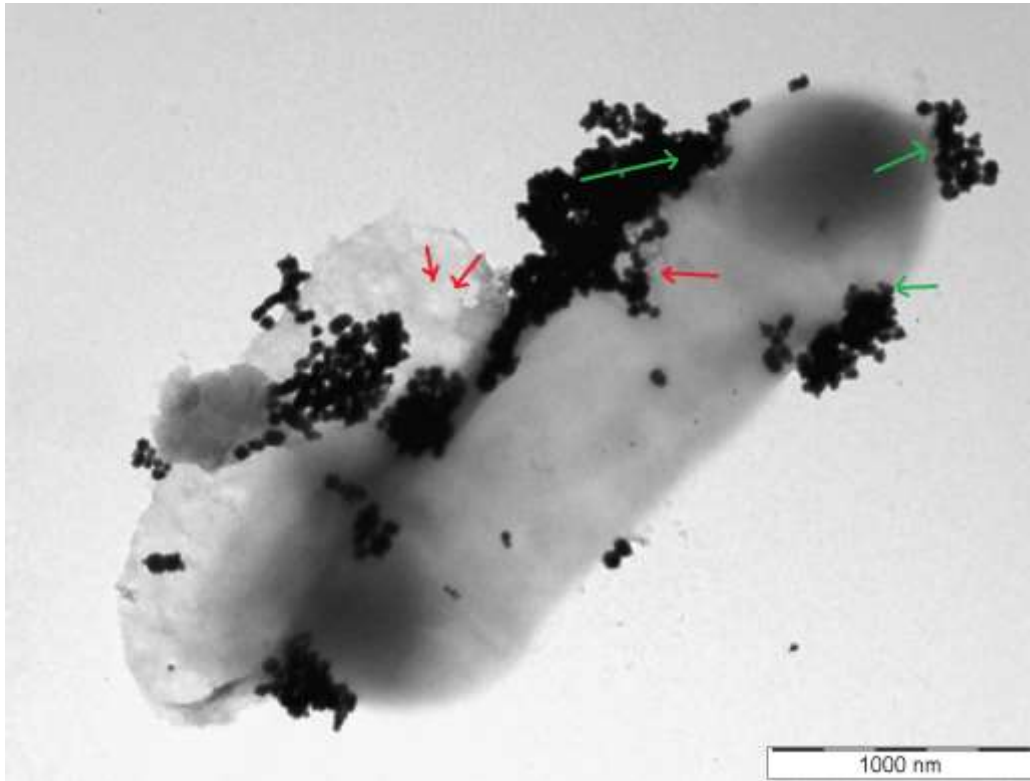


Figure 2: Growth curves of *E. coli* treated with different concentrations (ppm) of AgNPs.





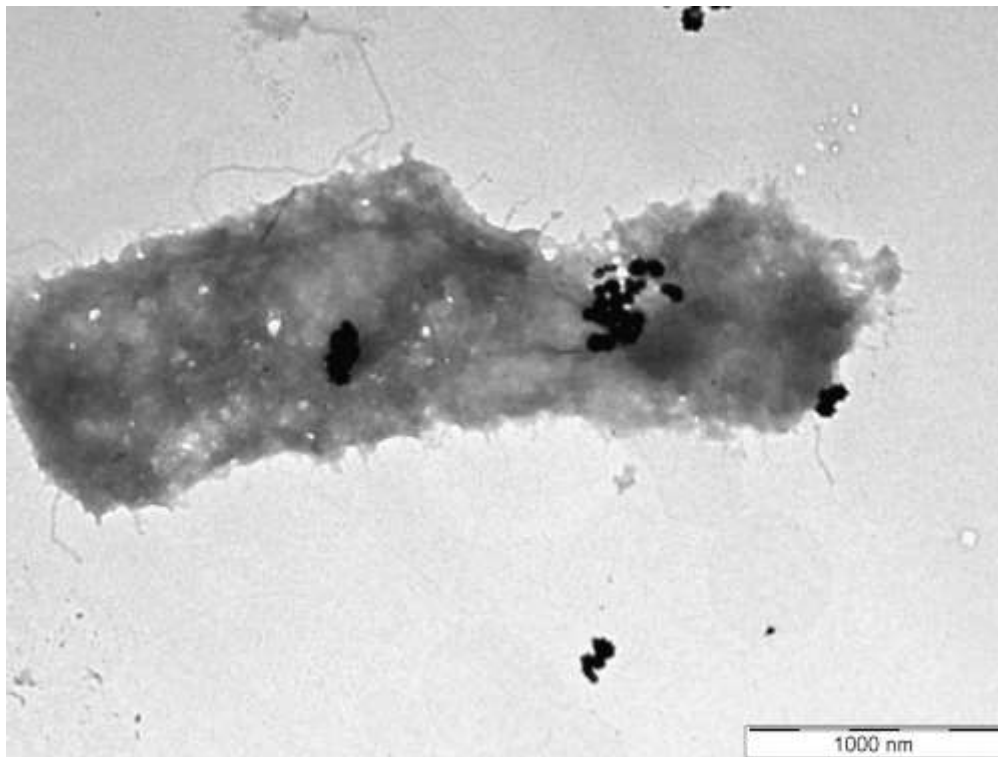
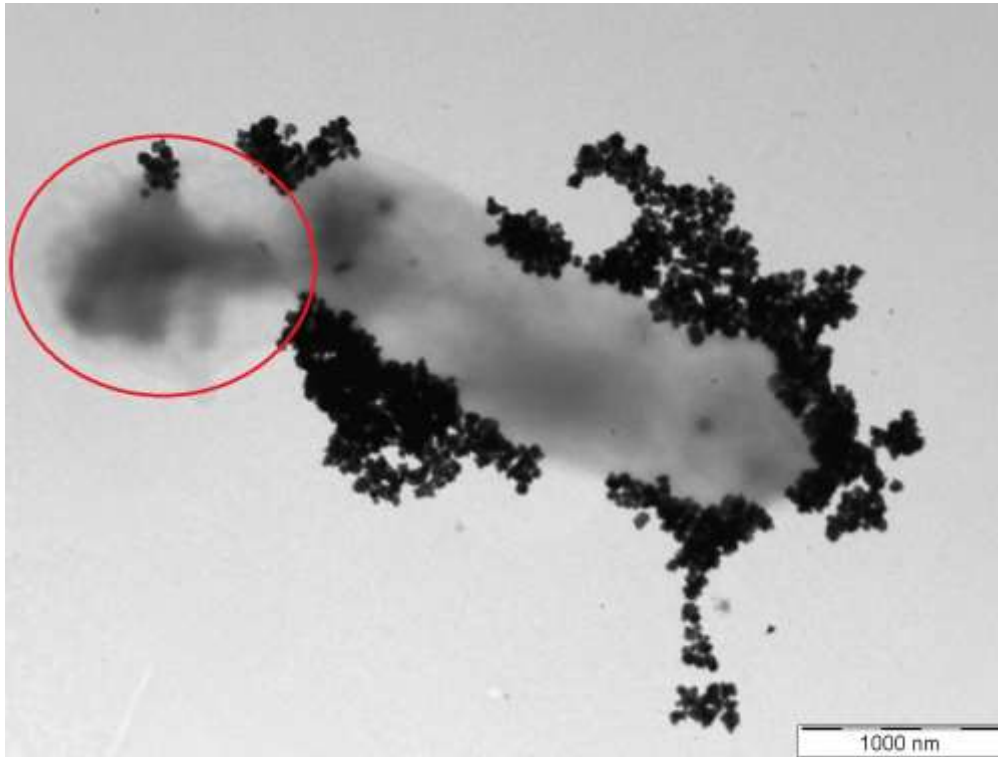


Figure 3 a-d: (a) Transmission Electron Micrograph of Control *E. coli* cells, (b, c) treated *E. coli* cells showing deposition of AgNPs on cell membranes (green arrow) leading to formation of pits (red arrow), (d) release of intracellular components through the pits, (e) complete damage of cell membrane.

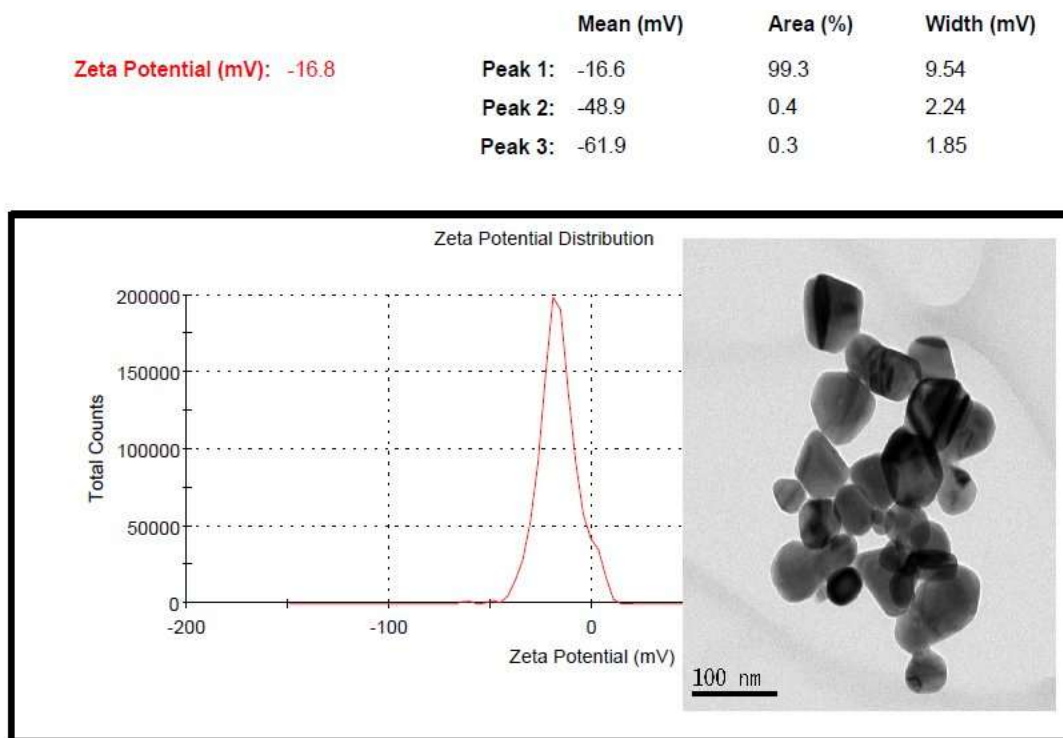


Figure 4: Transmission Electron Micrograph and zeta potentials (surface charge) of AgNP.