CHAPTER 5: ANTIBACTERIAL MECHANISM OF ACTION

5.1 INTRODUCTION

Development of novel antimicrobial agents is gaining tremendous importance due to emergence of resistant microbes and high cost of treatment. From common domestic sanitation to health care institutes, huge amount of antimicrobial agents in the form of antiseptics, disinfectants and antibiotics are used on a daily basis (Biswajoy et al., 2012).

Recent literature reports encouraging results about the bactericidal activity of AgNPs of either a simple or composite nature. Many researchers have been evaluating potential antibacterial effect of metals in their nanoparticle form. Metals including zinc, silver, and copper have been used as antibacterial agents for long time (Subbiahdoss et al. 2012). The advantage of using metals in their nanoparticle form is that these particles can be prepared to have very small diameter, and also have high surface area to volume ratio. It is thought that the high surface area to volume ratios and the resultant unique chemico-physical properties of the nanoparticles could contribute to their antimicrobial activities (Pal et al., 2007, Huh and Kwon, 2011 and Majed et al., 2014). Antimicrobial nature of nanoparticles makes it harder for bacteria to develop resistance against nanoparticles (Huang et al., 2008 and Nel et al., 2009).

Currently, the investigation of this phenomenon has gained more attention due to the increase of bacterial resistance to antibiotics, caused by their overuse. Bacteria are differentiated into two main groups, Gram-positive or Gram negative, based on a technique which detects the thick peptidoglycan cell wall characteristic of Gram-positive bacteria. Gram-negative bacteria are of particular biomedical, technological interest.
owing to their increasing antibiotic resistance and their utility in many biotechnological processes. *Escherichia coli*, found naturally in our digestive system and extensively used in biomedical research and industry. However, some strains may cause food poisoning, septicemia or meningitis while, in developing countries, it remains a major cause of infant mortality. In addition *E. coli* is one of the most frequent causes of many common bacterial infections including cholecystitis, bacteremia, cholangitis, urinary tract infection, traveler’s diarrhea, and other clinical infections such as neonatal meningitis and pneumonia (Ali et al., 2012 and Luke et al., 2013).

ROS are generated via respiratory chain or antioxidant enzymes dysfunction caused by thiol/Ag\(^+\) interactions. Similarly, bacteria treated with Ag\(^+\) released from a washing machine were found to have reduced viability; however *E. coli* was more susceptible than *S. aureus* (Woo et al., 2008). The mechanism of cell death was found to be a result of the cell membrane perturbation and separation from the cell wall leading to the release of intracellular contents (Woo et al., 2008).

Previous studies also showed that there are several mechanisms about the bactericidal effect of silver nanoparticles. AgNPs not only interact with the surface of the membrane, but can also penetrate into the bacterial cell membrane (Ivan and Salopek, 2004). In addition, silver nanoparticles can bind to the DNA inside the bacterial cells, preventing its replication or interaction with the bacterial ribosome (Lei et al., 2008). It has been discovered that silver nanoparticles can damage the structure of the bacterial cell membrane and reduce the activity of some membranous enzymes, which cause *E. coli* bacteria to die eventually (Wen-Ru et al., 2010).
Bacteria may be exposed to nanometer sized particles of sediment in their natural environment without adverse effects. However, the objective of the work is to study how AgNPs interact with bacteria using electron microscopy. The second approach is to study the effect of reactive oxygen species from the surface of AgNPs against well-characterized model system that is easily manipulated in the laboratory and has an international standard that can be made consistent between research groups. For this the model organism *Escherichia coli*, strain MTCC 46 was chosen.

In our investigation the antimicrobial mechanism of AgNPs against *E. coli* MTCC 46 by studying various parameters such as, bactericidal activity of AgNPs, bacterial growth kinetics against different concentration of AgNPs, interaction of bacteria with nanoparticles using electron microscopic studies, quantification of cellular uptake of AgNPs, release of ROS from the surface of AgNPs using ESR spectroscopy and ROS generation was also studied using DCFH-DA method and respiratory chain dehydrogenase activity was also reported.

5.1.1 *E. coli* as a Model Organism for Studying Bacterial-Nanoparticle Interactions

All bacteria have a cell membrane made up of phospholipid bilayers, which separates the internal contents of the cell from the external environment by creating a semipermeable barrier. Lipids are the bilayer composed of two layers of amphiphilic molecules bound by hydrophobic effect. The cell membrane regulates particles entering and exiting the cell, which are used or produced by the cell, during synthesis. Without this type of cell regulation, chemicals toxic to the cell could freely enter, causing cell dysfunction or death. Bacteria though have the increased protection of a peptidoglycan layer, an example being *Staphylococcus*. Other bacteria have a thin peptidoglycan layer,
and an outer membrane, an example being *E. coli*. Bacterial cells that have a thick peptidoglycan layer are classified as Gram positive and the bacteria with a thin peptidoglycan layer are Gram negative. Some bacterial cells can also gain extra protection from a thick slime capsule ([Dubey and Maheshwari, 2002](http://wikieducator.org/Bacterial_Structure) and [http://wikieducator.org/Bacterial_Structure](http://wikieducator.org/Bacterial_Structure)). Ultra structure of bacterial cell shown in (Fig. 5.1)

In Gram negative bacteria the peptidoglycan layer is relatively thin, but the cell also gains protection through an outer membrane. This membrane covered thin layer of peptidoglycan does not retain the purple stain in the gram staining process, so these bacteria are known as gram negative. Bacteria that have a deep cell wall made of peptidoglycan (carbohydrate polymers cross-linked by protein) will retain a purple colour when stained with crystal violet solution and are known as Gram-positive. Other bacteria have double cell walls with a thin inner wall of peptidoglycan and an outer wall of carbohydrates, proteins, and lipids. Such bacteria do not stain purple with crystal violet and are known as Gram-negative (Fig. 5.2).

Lipopolysaccharides (LPS) are large molecules consisting of a lipid and a polysaccharide joined by covalent bond. They are found in gram negative bacteria. They are contributing to the structural integrity of bacteria and protecting the membrane from certain kinds of chemical attacks. It also increases the negative charge of the cell membrane and help to stabilize the whole membrane structure. The cell structure comprises of three parts, Polysaccharide side chains, Core polysaccharide and Lipid A.
Fig. 5.1: Ultra structure of Gram positive and Gram negative bacteria
(Courtesy: http://wikieducator.org/Bacterial_Structure)

Fig. 5.2: Gram negative bacterial cell wall,
(Courtesy: http://wikieducator.org/Bacterial_Structure)
Ribosome consist of a small and large subunits made up of a complex proteins and RNAs. These are the sites of protein synthesis in the cell. The prokaryotic chromosome is a single circle of double stranded DNA, located in an irregularly shaped region called the nucleoid. Plasmids are extra chromosomal DNA capable of independent replication and can transfer between cells during recombination (http://wikieducator.org/Bacterial_Structure).

5.1.1 Bacterial-Nanoparticle Interactions

Cell membranes have evolved for the purpose of physically and chemically defining the boundary between an organism and the environment. Although some molecules may be sufficiently lipophilic to diffuse freely across lipid bi-layers, the exchange of hydrophobic and polar solutes may be stringently regulated by specialized transport proteins. Active transporters use energy from ATP hydrolysis or the proton motive force and are typically substrate specific, only moving solutes that satisfy specific steric and electrostatic binding parameters. Passive transporters are water filled protein channels that permit the specific or non-specific movement of small solutes down an electrochemical gradient.

The nanoparticle uptake by a bacterial cell considers the destructive nature of nanoparticles on bacterial membranes. This may enable nanoparticles to enter a cell by diffusion at a site of membrane damage. If this damage is not observed due to restrictions in the imaging technique then the nanoparticles would appear to be within an intact bacterium causing a false interpretation of an uptake process. For example, images from studies that depict AgNPs inside E. coli have been obtained using TEM (Steven et al., 2012). Therefore, while the cell wall may appear circumferential and intact in the
bacterial section, the integrity of the entire structure cannot be determined because only a thin section is viewed (Ivan and Salopek, 2004). *E. coli* cells showed cell wall damage, and in *S. aureus* cells, the cytoplasm membrane shrunk and became detached from the cell wall. Gram-positive organisms are more resistant towards silver ions due to extra protection offered by the peptidoglycan layer of the cell wall (Feng et al., 2000).

The bacterial-nanoparticle interactions, as observed in TEM images, leads to the hypothesis that dissolution from the attached nanoparticles yields a local concentration of Ag\(^+\) which can react directly with the bacterium at the bacterial-nanoparticle interface. Therefore, the bulk solution phase may contain a low concentration of the dissolved silver, but the anti-bacterial efficacy may be high. AgNPs cause considerable damage to the cell envelope through the activity of the silver ions on membrane proteins. Hence, might enter the cell by diffusion where the integrity of the cell wall is reduced. However, the cell envelope was circumferential in the TEM images and there was evidence of electron dense “pits”, representing sites of damage in the cell wall, which have been reported for silver nanoparticle treated *E. coli* (Ivan and Salopek, 2004).

The interaction between bacterial cells and AgNPs are due to electrostatic attraction between negatively charged cell membranes and positively charged nanoparticles, in a similar way as silver interacts with thiol groups of respiratory chain proteins and transport proteins, interfering with their proper function (Morones et al., 2005 and Catalina and Eric, 2010). AgNPs attached to bacterial cell membrane increases the permeability and disturb the respiration. Proteomic data shows the accumulation of envelope protein precursors in *E. coli* cells after exposure to AgNPs (Lok et al., 2006). Energy from ATP and proton motive force is required in order to newly synthesize
envelope proteins to be translocated to the membrane, therefore cytoplasmic accumulation of protein precursors suggests dissipation of proton motive force and depletion of intracellular levels of ATP (Lok et al., 2006).

Structural changes in the cell membrane of bacteria increases cell permeability, leading to an uncontrolled transport through the cytoplasmic membrane, and ultimately cell death occurs. It has also been proposed that the mode of antibacterial activity of AgNPs is related to the formation of free radicals and subsequent free radical-induced membrane damage (Jun et al., 2007).

The efficacy of silver ions to strongly interact with thiol groups of vital enzymes and phosphorus-containing bases and on the presence of AgNPs inside the bacterial cells (Morones et al., 2005), it is likely that further damage could be caused by interactions with compounds such as DNA. This interaction may prevent cell division and DNA replication from occurring, and also ultimately lead to cell death. Other studies have suggested that AgNPs may modulate the phosphotyrosine profile of putative bacterial peptides that could affect cellular signaling and, therefore, inhibit the growth of bacteria (Siddhartha et al., 2007).

In the antibacterial mechanism of AgNPs the AgNPs can release Ag\(^{+}\) ions, which have been well documented to cause toxicity in bacteria. One such study revealed that Ag\(^{+}\) release was dependent on both proton and dissolved oxygen content, and that release was pH and temperature dependent (Liu and Hurt, 2010). They also discussed that AgNPs will not persist as particles but will undergo dissolution when in biological conditions.
5.1.6 Reactive Oxygen Species

Oxidative stress depicts the existence of products as free radicals and reactive oxygen species (ROS), which are formed under normal physiological conditions but become deleterious when not being eliminated. In fact, oxidative stress results from an imbalance between the generation of reactive oxygen species and endogenous antioxidant systems (Neelofar and Sharma 2013).

ROS are major sources of primary catalysts that initiate oxidation in vivo and in vitro and create oxidative stress which results in numerous diseases and disorders (Halliwell, 1994 and Rackova et al., 2007) such as cancer (Kinnula and Crapo., 2004), cardiovascular disease (Singh and Jialal., 2006), neural disorders (Sas et al., 2007), Alzheimer's disease (Smith et al., 2000) mild cognitive impairment (Guidi et al., 2006) Parkinson's disease (Bolton et al., 2000), alcohol induced liver disease (Arteel 2003), ulcerative colitis (Ramakrishna et. al., 1997), ageing (Hyun et al., 2006), atherosclerosis (Upston et al., 2003). As well as, microbial resistance to antibiotics is also increasingly becoming a concern to public health. Currently used antibiotic agents are failing to bring an end to many microbial infections due to super resistant strains. For this reason the search is ongoing for new antimicrobial agents, either by the design and synthesis of new agents or through the search of natural sources for as yet undiscovered antimicrobial agents.

5.1.7 Free Radicals and Oxidative Stress:

The production of oxygen free radicals is a natural consequence of aerobic metabolism, with these molecules being constantly generated in the body by normal metabolic processes (Schipper, 1998). Most atoms and molecules remain reasonably
stable when placed in contact with living cells. However, free radicals are group of particles that are considered to be less benign. Free radicals are unstable, highly reactive molecules characterized by the presence of unpaired electrons in their outermost shells (Halliwell, 1995).

The generation of oxygen free radicals is an important contributing factor in several chronic human diseases, including atherosclerosis and related vascular diseases, mutagenesis and cancer, neurodegeneration, immunologic disorders, and even the ageing process (Fig. 5.3).

Free radical is a chemical compound which contains an unpaired electron spinning on the peripheral layer around the nucleus. Any free radical involving oxygen can be referred to as ROS (Reactive Oxygen Species) or ROS is the family of free radicals generated from oxygen. Molecular oxygen is required by living organisms and biological systems to survive whereas, any free radical involving oxygen (ROS) causes damage to other molecules by extracting electrons from them in order to attain stability. ROS are ions, atoms or molecules that have the ability to oxidize reduced molecules. ROS are various forms of activated oxygen, including free radicals such as superoxide anion radicals (O$_2^-$) and hydroxyl radicals (‘OH), as well as non-free radicals (H$_2$O$_2$) and singlet oxygen (Halliwell, 1995). Oxygen derived free radicals such as superoxide anions; hydroxyl radicals and hydrogen peroxide are cytotoxic and give rise to tissue injuries (Jainu and Shyamala, 2005).

Excessive amount of ROS is harmful because they initiate bimolecular oxidation which leads to cell death and creates oxidative stress.
Fig. 5.3: Generation of oxygen free radicals (Courtesy: Beyer et al., 1998)
In addition, oxidative stress causes inadvertent enzyme activation and oxidative damage to cellular system (Wiseman and Halliwell, 1996). In the body, free radicals are derived from two sources: endogenous sources, e.g. nutrient metabolism, ageing process etc and exogenous sources e.g. tobacco smoke, ionizing radiation, air pollution, organic solvents and pesticides, etc. (Buyukokuroglu et al., 2001).

5.2 MATERIALS AND METHODS

5.2.1 Bactericidal Activity of AgNPs (MBC)

Agar dilution method was used to study the bactericidal activity of AgNPs on *E.coli*. Nutrient Agar (NA) supplemented with various concentration of AgNPs (0, 2.5, 5, 10 and 15 µg/ml) and each plate was inoculated with $10^7$ CFU/ml of *E.coli* by spread plating. Plates inoculated with *E.coli* but without AgNPs were used as control. Number of surviving bacteria in agar plates was counted after 24 hours of incubation at 37°C.

5.2.2 Bacterial Growth Kinetics against Different Concentration of AgNPs (MIC)

To study the bacterial growth curve, *E.coli* culture was inoculated with fresh colonies and incubated for 12 hour overnight at 37°C in NB. Bacterial growth curves were determined by measuring the optical density (O.D) at 600 nm using spectrophotometer. At this juncture the OD obtained was 1.0. To different concentration (0-15 µg/ml) of AgNPs the aforesaid *E. coli* cultures were added and the turbidity was measured at different time intervals (0, 5, 10, 15, 20 and 25 hrs).
5.2.3 Effect of AgNPs on Respiratory Chain Dehydrogenase

The dehydrogenase activity was measured as per iodonitrotetrazolium chloride method (INT) (Wen-Ru et al., 2010 and Kim et al., 2011). Under physiological condition, colorless INT is reduced by bacterial respiratory chain dehydrogenases to a purple water insoluble iodonitrotetrazolium formazan (INF). Thus the dehydrogenase activity can be determined by the change of the spectrophotometric value of INF.

Different volumes of NB medium, AgNPs and E.coli cells were added into 10 ml cultures separately resulting in final concentration of 10 µg/ml AgNPs and 10⁷ CFU/ml E.coli. Experiment conducted in the absence of AgNPs was used as control. Cultivations were performed at 37°C with shaking at 150 rpm. One ml of the culture was sampled out separately and centrifuged at 12000 rpm, then the supernatants were discarded and the bacteria washed with phosphate buffered saline (PBS) twice and 0.9 ml of PBS was added to suspend bacteria. INT solution (0.1 ml of 0.5%) was added, the culture was incubated at 37°C in dark for 2h, and then 50 µl of formaldehyde was added to terminate the reaction. The culture was centrifuged to collect the bacteria and 250 µl solutions of acetone and ethanol 1:1 in volume were used to distill the INF twice. The dehydrogenase activity was then calculated according to the maximum spectrophotometric absorbance of INF at 490 nm by spectrophotometer.

5.2.4 Interaction of AgNPs with Bacterial cells using TEM

The interaction of AgNPs with E.coli was assessed using TEM. Broth containing E.coli exposed to AgNPs was subjected to TEM at a time intervals of 1, 5, 8 and 12 hrs respectively. The samples which were subjected to TEM studies were also sent for ESR
studies to detect the free radical generation from the surface of silver. ESR is an analytical method to detect the free radical generated from the surface of silver. It is based on absorption of microwave radiation by an unpaired electron when it is exposed to a strong magnetic field. AgNPs that contain free radicals therefore be detected by ESR. Free radical generation from the surface of Ag\(^+\) was recorded using ESR spectrophotometer.

5.2.5 Release of ROS from the Surface of AgNPs

The generation of ROS from the surface of AgNPs was measured using 2’, 7’-Dichlorofluorescein diacetate (DCFH-DA) (Hengyi et al., 2012). DCFH-DA is one of the most widely used technique for directly measuring the redox state of a cell. DCFH-DA, a cell permeable, nonfluorescent precursor of DCF can be used as an intracellular probe for oxidative stress. It has many advantages over other techniques developed as it is very easy to use, extremely sensitive to changes in the redox state of a cell, inexpensive, and can be used to follow changes in ROS over time.

10\(^7\) CFU/mL of cells were treated with 10\(\mu\)g/mL of AgNPs and incubated at 37°C for 5 h then centrifuged at 4°C for 15min at 600 rpm and the obtained supernatant was treated with 100 \(\mu\)M DCFH-DA for 1h. The ROS formed was measured using Fluorescence spectrometry.

5.2.6 Antioxidant activity of AgNPs against E. coli

To check the inhibition of \textit{E. coli} targeted through the ROS produced by AgNPs from \textit{A.niger}, we conducted a separate experiment using Ascorbic acid as an antioxidant which acts as a scavenger. NA plates supplemented with AgNPs (10 \(\mu\)g/ml) were also
incorporated with 10 mM Ascorbic acid as a scavenger. Thus the plates were inoculated with fresh 12 hrs cultures of *E.coli* and the surviving rate was counted after 24 hrs of incubation (Jun et al., 2007).

5.2.7 **Quantification of cellular uptake of AgNPs**

The nanoparticles uptake by the cells was quantified using inductively coupled plasma-optical emission spectroscopy (ICP-OES) (Asharani et al., 2009). Broth containing *E.coli* exposed to AgNPs was subjected to TEM at a time intervals of 1, 5, 8 and 12 hrs respectively, after 12 hrs of bacteria-AgNPs interaction the lysate was taken for ICP-OES analysis to calculate the rate of uptake of AgNPs by bacterial cells, and the results were discussed in light of the cellular uptake of AgNPs and also in context of the similar work done by other workers.

5.3 **RESULTS AND DISCUSSION**

5.3.1 **Bactericidal Activities of AgNPs (MBC)**

The bactericidal activity was performed against Gram negative *E.coli*, on NA plates containing different concentrations of AgNPs such as 0, 2.5, 5, 10 and 15µg/ml (Fig. 5.4). Number of bacterial colonies grew on NA plate as a function, the concentration of AgNPs when the overnight *E.coli* culture was applied to the plates. The presence of AgNPs at a concentration of 10 and 15 µg/ml inhibited bacterial growth by 100%. There was luxurious growth at 0µg/ml i.e. without AgNPs while at 2.5µg/ml of AgNPs there was substantial growth, may be that the concentration of 2.5µg/ml of AgNPs is not sufficient to kill *E.coli*, while at 5µg/ml there was decreased growth due to enhanced AgNPs concentration.
Fig. 5.4: Bactericidal activities of AgNPs on *E.coli*
Our results indicate that 10µg/ml of AgNPs are sufficient to inhibit 10^7 CFU/ml of *E.coli*. For confirmation we went for 15µg/ml concentration of AgNPs against *E.coli*, here also there was complete inhibition, indicates that a concentration of 10µg/ml of AgNPs is sufficient to completely kill 10^7 CFU/ml of *E.coli*.

Wen- Ru et al., (2010) also reported that 10µg/ml of AgNPs are sufficient to completely inhibit 10^7 CFU/ml of *E.coli*, indicating the MIC of AgNPs to *E.coli* was 10µg/ml, though the AgNPs were not biologically synthesized, but purchased commercially, yet our results correlate with them. Ivan and Salopek (2004) reported that a concentration of 20 µg/ml cm^-3 completely prevented bacterial growth if 10^4 CFU of *E.coli* were used. While Kim et al., (2011) reported a MIC of 100µg/ml of AgNPs to cause complete death of *S. aureus* and *E. coli*. Comparing our results with the above said authors, AgNPs produced by our isolate *A.niger* are more effective in completely inhibiting *E.coli* at the minimum dose of 10µg/ml.

Siddhartha et al., (2007) revealed that LB agar plates incorporated with increasing concentrations of silver nanoparticles and inoculated with 10^6 CFU of different bacterial strains, the inhibition increased to 90% in plates with 10 µg ml^-1 nanoparticles, whereas the concentration of 25 µg ml^-1 of AgNPs ensured complete inhibition of bacterial growth. In the case of ampicillin-resistant *E. coli* and multi-drug resistant strains of *S. typhi*, 70–75% inhibition in growth was observed in plates supplemented with 10 µg ml^-1 of nanoparticles, whereas 25 µg ml^-1 or higher concentration of nanoparticles elicited 100% inhibition in growth of bacteria. In a study by Ali et al., (2012) silver nanoparticles with spherical shape exhibited the most significant antibacterial activity among the four shapes of silver nanostructures against, *E. coli* and *Bacillus subtilis* bacteria. Cubic, wiry,
and triangular structures showed the most antibacterial activities against *E. coli* and *Bacillus subtilis* against spherical PVP coated AgNPs.

**5.3.2 Bacterial Growth Kinetics against Different Concentration of AgNPs (MIC)**

The growth curve of *E.coli* treated with AgNPs was determined by using NB supplemented with 0, 2.5, 5, 10 and 15µg/ml of AgNPs (Fig. 5.5). The concentration of 2.5 and 5 µg/ml showed the growth of *E.coli* but comparatively less than the growth curve shown without AgNPs (0 µg/ml). It is very interesting to observe that the incorporation of 10 and 15µg/ml AgNPs to NB showed complete inhibition of *E.coli* which is evident from graph.

The dynamics of bacterial growth was monitored in liquid LB broth supplemented with $10^7$ *E. coli* cells with 10, 50, and $100\ \mu g\ cm^{-3}$ of AgNPs at all these concentrations caused a growth delay of *E. coli*. Yan et al., (2012) also reported that a concentration of 10µg/mL of AgNPs showed strong antibacterial activity against *E. coli*.

Hengyi et al., (2012) reported the exposure of the bacteria to AgNPs at a concentration of 5 mg/L inhibited bacterial growth by 50%. AgNPs with a concentration of 12.5 mg/L almost totally inhibited bacterial growth but AgNPs used in their studies were polymer coated and suggested the antibacterial activity due to positive charge on silver ions ($Ag^+$) and perhaps with AgNPs that resulted in an electrostatic attraction with the negatively charged bacterial cell membrane resulting in cell damage.

Sujoy and Enrico, (2010) reported that the dynamics of bacterial growth was monitored in liquid LB broth supplemented with $10^7$ *E.coli* cells with 10, 50 and $100\ \mu g\ cm^{-3}$ of AgNPs. At all these concentrations a growth delay of *E.coli* was observed.
Fig. 5.5: Effect of AgNPs on *E.coli*
Siddhartha et al., (2007) reported that a concentration of 25µg/ml AgNPs showed strong inhibition against bacteria at a noticeable time of 8h, in LB broth.

Kornphimol et al., (2010) reported 10 g/L silver suspension was sufficient for preparing antibacterial fabrics. At this treatment concentration, the fabrics induced a 99.83% and 99.93% reduction of proliferation competency in S. aureus and E. coli, respectively. This study provides the unprecedented data on detecting silver released from antibacterial fabric products using artificial sweat as a model to represent the human skin environment.

5.3.3 Effect of AgNPs on Respiratory Chain Dehydrogenase

To determine oxidative stress induced damage of respiratory system of cells, the respiratory chain dehydrogenase activity was measured. The dehydrogenase activity was measured as per iodonitrotetrazolium chloride method (INT) (Wen-Ru et al., 2010 and Kim et al., 2011). The dehydrogenase activity can be determined by the change of the spectrophotometric value of INF. Under physiological conditions, colorless INT is reduced by the bacterial respiratory chain dehydrogenase to a dark red water insoluble iodonitrotetrazolium formazan (INF), while that of E.coli treated with 10 µg/ml AgNPs remains colourless. Thus, the dehydrogenase activity was determined efficiently using spectrophotometric value of INF.

The effect of AgNPs on respiratory chain dehydrogenase of E.coli is represented here. Activity of respiratory chain dehydrogenase in cells treated with AgNPs inhibited the enzymatic activity thus there is no change in color, while that of cells devoid of AgNPs turns the colorless INT to dark red colour indicates the enzymatic activity of E.coli (Fig. 5.6, a and b).
Fig. 5.6: (a) Effect of Ag-NPs on respiratory chain lactate dehydrogenase of *E. coli*.

Fig. 5.6: (b) Effect of AgNPs which inhibits dehydrogenase activity of *E. coli*.
Our results present that the ROS formed by AgNPs inhibit dehydrogenase, an important enzyme in cellular respiration. AgNPs targets sulfur containing proteins and also thiol groups of respiratory chain proteins and transport proteins, interfering with their proper function. Kim et al., (2011) reported the effect of AgNPs on dehydrogenase activity of E.coli and S. aureus and discussed that dehydrogenase activity of E.coli cells increased considerably with time, while that of cells treated with 100 µg/ml AgNPs decreased slightly when INF studies were done. They also reported that the AgNPs cause inhibition of bacterial growth.

Kyung et al., (2005) reported that the E.coli completely inhibited when 100ppm of AgNPs were used suggesting that the Ag ions in AgNPs solution or dissolved Ag ions might affect the inhibition of intracellular enzyme activity. Holt and Brad (2005) also reported that Ag\(^+\) inhibited respiration of E. coli by determining change of oxygen dissolved in culture resolution. Kim et al., (2008) reported that Ag\(^+\) interact with thiol (–SH) group of cysteine by replacing the hydrogen atom to form –S–A, thus hindering the enzymatic function of affected protein to inhibit growth of E.coli. Our results are correlating with the reports discussed by Kyung et al., (2005), Holt and Brad (2005) and Kim et al., (2011).

5.3.4 Interaction of AgNPs with Bacterial cell using TEM

Gram negative, E.coli was selected as a model to study the effect of ROS released from the surface of AgNPs on the permeability and the membrane structure of E.coli cells. The interaction of AgNPs with bacterium was analyzed using TEM micrographs. E.coli samples were incubated with AgNPs for 12 hours and were analyzed employing TEM. Figure 5.7 (a) shows normal E.coli cells with its well integrated cell wall.
the *E.coli* cells were made to interact with AgNPs for 1 hr, it can be seen from Figure 5.7 (b) that the AgNPs were trying to adhere to the surface of *E.coli* cells. After 5 hrs interaction a closure look at the bacterial cell membrane reveals that AgNPs anchored onto the cell surface of *E.coli* and many pits and gaps appeared in the micrograph and their membrane was fragmented as presented in Figure 5.7 (c).

Figure 5.7 (d) shows that AgNPs are trying to get inside the bacterial cell through the ruptured cell membrane. After 8 hours, the AgNPs surround the bacterial cell surface and almost cover the sides of the cell and nanoparticles are visible in the cytoplasm also.

In addition electron dense particles or precipitates were also observed around the damaged bacterial cell Figure 5.7 (e). The interaction can either completely disintegrates the cell or may cause cell lyses after 12 hours Figure 5.7 (f) shows AgNPs eventually leading to the bacterial cell death.

Another proposed mechanism of *E.coli* membrane damage by AgNPs relates to metal depletion i.e. the formation of pits in the outer membrane and change in membrane permeability by the progressive release of Lipopolysaccharide (LPS) molecules and membrane proteins. A bacterial membrane with this morphology exhibits a significant increase in permeability, leaving the bacterial cells incapable of properly regulating transport through the plasma membrane and, finally, causing cell death. It is well known that the outer membrane of *E. coli* cells is predominantly constructed from tightly packed LPS molecules, which provide an effective permeability barrier (Sujoy and Enrico (2010). It has been also proposed that the sites of interaction for AgNPs and membrane cells might be due to sulfur containing proteins in a similar way as silver interacts with thiol groups of respiratory chain proteins and transport proteins interfering with their
Fig. 5.7: TEM micrograph of *E.coli* loaded with AgNPs. (a) Normal *E.coli* cell with its well integrated cell wall. (b) Anchoring of AgNPs on cell wall of *E.coli*. (c) Ruptured cell membrane and entry of AgNPs into the cytoplasm. (d) Complete damage of cell wall and cell membrane. (e) Clear AgNPs within the *E.coli* cell. (f) Complete destruction of *E.coli* cell and overloading of AgNPs.
proper function (Feng et al., 2000, Yoshinobu et al., 2003, Mikihiro et al., 2005, Morones et al., 2005 and Woo et al., 2008).

Wen-Ru et al., (2010) also reported the sites of interaction for AgNPs and membrane cells might be due to sulfur containing proteins in a similar way as silver interacts with thiol groups of respiratory chain proteins and transport proteins, interfering with their proper function.

Ivan and Salopek, (2004) reported TEM images with small clusters and individual silver nanoparticles attached to the E. coli double envelope, some of the nanoparticles appeared within the periphery of the cell, on the left and right of the image, but proximal to the cell wall, and some of the AgNPs appeared to be integral with the cell envelope and cytosol. Silver nanoparticles are also located distal from the cell. However, most of the detached nanoparticles were scattered in the same orientation with respect to the nearest bacterium, thus may have been moved by the sectioning knife.

The bacterial growth inhibition by formation of free radicals from the surface of AgNPs was also observed by employing ESR spectroscopy (Fig. 5.8). Excess generation of reactive oxygen species can attack membrane lipids and then lead to a breakdown of membrane function. Reactive Oxygen Species are natural byproducts of the metabolism of respiring organisms (Aruna and Arunachalam, 2010 and Feng et al., 2000). Induction of ROS synthesis leads to the formation of highly reactive radicals that destroy the cells.

Excess generation of reactive oxygen species can attack membrane lipids and then lead to a breakdown of membrane function. Certain transition metals might disrupt the cellular donor ligands that coordinate Fe. Mounting evidence suggests that the primary targets for various metals are the solvent exposed [4Fe-4S] clusters of proteins.
Fig. 5.8: ESR spectrum of AgNPs recorded at room temperature, m1 (332.715) and m2 (341.418) indicate the control peak of Mn and the peak (mT: 337.475) indicates the release of free radical from AgNPs. Instrument Setting: JEOL-JES-TE 200 spectrophotometer micropower, 4mW, MOD, 100 khz and the time const 0.03 sec
The direct or indirect destruction of [4Fe-4S] clusters by metals could result in the release of additional Fenton-active Fe into the cytoplasm resulting in increased ROS formation. The ability to induce Fe release from these proteins, as well as from other Fe-containing proteins, might account for observations that some Fenton inactive metals (such as Ag, Hg, and Ga) generate ROS and that cells require or upregulate ROS-detoxification enzymes to withstand toxic doses of these nanoparticles (Joseph et al., 2013).

5.3.5 Release of ROS from the Surface of AgNPs

The ROS production from the surface of AgNPs was measured using DCFH-DA method. DCFDA is a popular fluorescence-based probe for reactive oxygen species (ROS) detection in vitro. After 5 h of incubation, ROS formed in the sample was detected at 523nm of emission wavelength using fluorescence spectroscopy (Fig. 5.9). Intracellular esterases cleave DCFH-DA at the two ester bonds producing a relatively polar and cell membrane impermeable product, H2DCF. This nonfluorescent molecule accumulates intracellularly and subsequent oxidation yields the highly fluorescent product DCF.

The redox state of the sample can be monitored by detecting the increase in fluorescence. The result confirms the generation of free radicals and from the surface of AgNPs, which becomes toxic to bacterial cells leading to death. Our result corroborates with Kim et al., (2011) who reported the oxidative stress can cause damage to bacterial cell membrane, protein structure, and intracellular system against S. aureus and E. coli using DCFDA method.
5.3.6 Antioxidant activity against *E. coli*

To determine the involvement of ROS in the antibacterial activity of AgNPs, we used Ascorbic acid as scavenger. This antioxidant was used to scavenge the ROS produced by the AgNPs. Protective activity of antioxidant against bactericidal activity of AgNPs was observed. In control plate, the bacterial colonies were clearly seen without antioxidants and AgNPs, but in the plate supplemented with AgNPs (10µg/ml), no bacterial growth was observed revealing that AgNPs completely inhibited bacterial growth due to ROS formation. Surprisingly the bacterial colonies were observed in the plate supplemented with both AgNPs and antioxidant, which clearly indicates that the Ascorbic acid used as an antioxidant serves as a scavenger hindering the ROS release from AgNPs. It is determined that the antioxidant prevents the formation of a silver oxide layer on the AgNPs surface and consequently formation of the Ag⁺ reservoir (Fig. 5.10).

Bacterial cells exposed to AgNPs suffer morphological changes such as cytoplasm shrinkage, detachment of cell wall membrane, DNA condensation and localization in an electron-light region in the centre of the cell and cell membrane degradation allowing the leakage of intracellular contents (Ivan and Salopek, 2004, Feng et al., 2000 and Meiwan et al., 2011). Physiological changes occur together with the morphological changes, bacterial cells enter an active but non culturable state in which physiological levels can be measured but cells are not able to grow and replicate (Yoshinobu et al., 2003 and Wang et al., 2012).

Fig. 5.9: Formation of ROS in *E.coli*, using fluorescence spectroscopy

Fig. 5.10: Antioxidant activities of silver nanoparticles on *E.coli*
5.3.7 Quantization of cellular uptake of AgNPs

Attempts to identify the uptake routes of AgNPs by bacterial cells leads to the conclusion that AgNPs were taken up through formation of pits and the reactive oxygen species from the surface of AgNPs damaged the bacterial cells interacted with intracellular contents of cells and finally cell death occurs. After 12 h interaction the cells showed the detectable level of AgNPs with concentration of 0.57ppm.

Zong et al., (2012) reported AgNPs themselves do not significantly exert direct particle-specific toxicity on bacteria. AgNPs could be engineered with different particle formations (e.g., surface coatings) to release Ag$^+$ at desired rate and location. Furthermore, AgNPs may serve as a vehicle to deliver Ag$^+$ more effectively to the bacterial cytoplasm and membrane (Fig. 5.11), whose proton motive force would decrease the local pH (as low as pH 3.0) and enhance Ag$^+$ release.

Amber et al., (2011) using freshly prepared AgNP-ZM (zeolite membrane), reported that the release of Ag$^+$ into the broth can be as high as 20 ppm after 48 hours. Since the zeolite membranes were extensively ion-exchanged with Na$^+$ prior to these experiments, any Ag$^+$ in solution would have to occur by AgNP oxidation and release. Slow release of Ag$^+$ from the AgNP-ZM is also supported by the observation that bacteria sequestered in the plates were not killed as quickly as those incubated with supernatants conditioned for three hours.

Siddhartha et al., (2007) reported different stages of interaction between Gram-negative bacteria and AgNPs. The cumulative effect of these factors would lead to retardation in bacterial growth but not complete inhibition.
Fig. 5.11: Schematic of AgNPs, Ag\(^+\) and cell interactions (Courtesy: Zong et al., 2012)
Interaction with nanoparticles resulted in perforations in the cell wall, contributing to the antibacterial effects of the nanoparticles as demonstrated by TEM. Inferences from the reculture experiments were consistent with the entry of nanoparticles inside bacterial cells and/or strong association with bacterial cellular components. Once inside the cell, nanoparticles would interfere with the bacterial growth signaling pathway by modulating tyrosine phosphorylation of putative peptide substrates critical for cell viability and division.

Elvio et al., (2011) reported cysteine capped AgNPs were stable under physiological conditions and antibacterial tests gave MICs values of 180 and 15 μg/mL for S. aureus and E. coli, respectively. Dialysis experiments demonstrated that Ag\(^+\) released from the capped colloid is less than MIC values under physiological conditions. Hence, the antibacterial activity of dispersed cysteine capped Ag NPs can be ascribed to the direct action of metallic silver NPs, rather than to the bulk release of Ag\(^+\).

Silver nanoparticles are a highly effective vector for delivery of silver ions to E. coli because they may interact directly with the bacterial surface. This nanoparticle-specific silver ion delivery and associated anti-bacterial action is a novel hypothesis, which has gross implications for the design and development of silver nanoparticle based antimicrobials.