Abstract

Antibacterial agents are compound(s) which interfere with the bacterial growth either by inhibiting their reproduction or by killing them. The widespread and sometimes needless use of antibacterial agents (antibiotics and drugs) leads to development of resistance in the bacterial species. Antimicrobial resistance shown by a particular microbial species to multiple antimicrobial drugs is termed as multidrug resistance (MDR). It is well known that due to their unique outer membrane, which is present outside the peptidoglycan cell wall, Gram-negative bacteria are less vulnerable to attack by antibiotics. Considering the unprecedented increase in the antibiotic resistance in recent years, there is a constant need to search new and more effective antibacterial agents. The knowledge of antimicrobial properties of metals lead to turn researchers to exploit the antibacterial potential of metal and metal oxide nanoparticles (NPs) and development of "nano-weapons" against bacteria.

Among various metals, silver has a very rich history of its use as an antimicrobial agent. The increase in antibiotic resistance has resuscitated the attention of scientific community to exploit various silver species (bulk, ionic and nano forms) as antimicrobial agents. In current era of science and technology, silver NPs (Ag NPs) have been reported as an appealing contrivance for the improvement in the biomedical field because of their vast and valuable applications like drug delivery, biosensing, antibacterial agent, etc. However, the precise molecular mechanism of their mode of action remained unclear. In addition, the effect of various physico-chemical properties of Ag NPs on their interactions with biological media, bacterial cells and cytotoxic action mechanism has not been explored in-depth. Hence, systematic studies are required to understand the detailed mechanism behind antibacterial action of Ag NPs, which would open new avenue for the development of target specific silver based new generation antibiotics.

In the present study, chemical and biological methods were used to synthesize Ag NPs with varied size (5, 10, 20, and 50 nm) and surface capping (citrate, L-fucose, lysozyme and fungal proteins). UV-visible spectroscopy, TEM, EDS, XRD and FTIR analysis were performed to confirm the size, elemental composition, crystallinity and surface capping of synthesized Ag NPs. The antibacterial potential (MIC) of all the synthesized Ag NPs was determined against Gram-negative non-pathogenic model organism (*E. coli* K12) in comparison to silver ions. A negative correlation was observed between the size of the Ag NPs and their dissolution kinetics & antibacterial potential. Among all the tested NPs,

lysozyme coated Ag NPs (L-Ag NPs) showed the best MIC value of 9 µg (Ag) mL^{-1}. Hence, L-Ag NPs were selected for further mechanistic studies in comparison to silver ions.

In order to understand the possible mechanism behind the antibacterial activity of L-Ag NPs, biochemical and selective differential gene expression studies were performed by exposing the *E. coli* K12 cells to sub-MIC concentrations of silver ions and L-Ag NPs for 5, 30, and 60 min. ROS generation and membrane damage assays were undertaken to study the mechanistic aspects at biochemical levels. A concentration dependent increase was observed in ROS and membrane damage with both the tested silver species. Higher extent of damage was observed in bacterial cells exposed to silver ions in comparison to L-Ag NPs which confirmed that L-Ag NPs act as reservoir and slowly release silver ions.

Differential expression of selected genes was evaluated by the quantitative real-time polymerase chain reaction (qRT-PCR) analysis. The obtained results showed enhanced expression of stress related genes at 5 min. time point, which got down-regulated as the silver exposure time proceeds. Up-regulation of genes related to efflux pump machinery was observed at later time points. The DNA repair machinery gets activated after the completion of one replication cycle. The *suf* machinery was found to be involved in maintaining homeostasis under the oxidative stress of silver species treatments. Maximum changes in the gene expression profile were observed at MIC_{75} concentration of tested silver species.

Further, whole transcriptomic profile of non-pathogenic (*E. coli* K12) and pathogenic (*Klebsiella pneumoniae* MGH78578) Gram-negative bacteria were studied by RNAseq analysis to understand the effect of L-Ag NPs on all the metabolic pathways, signaling and molecular machinery. Differential regulation of total 4344 and 5357 genes were observed in *E. coli* K12 and *K. pneumoniae* MGH78578, respectively. The down-regulation of genes related to porin proteins confirmed their role in the entry of silver ions released through L-Ag NPs which was further confirmed by TEM-EDS analysis which showed no sign of membrane damage in both the bacterial species. RNAseq data confirmed the generation of ROS in the form of hydrogen peroxide radicals and showed active involvement of SoxRS system to sense the oxidative stress and in turn activate the defense mechanism in *E. coli* K12. Whereas, in addition to hydrogen peroxide, superoxide also contributes to generate ROS in *K. pneumoniae* MGH78578. For the sensing of silver, both OxyR and SoxRS systems were found to be involved in *K. pneumoniae* MGH78578. To remove the surplus silver ions from the bacterial cytoplasm, Cu^{+}/Ag^{+} specific efflux machineries (CusCFBA and Cue systems) become

activated in *E. coli* K12. In contrast to *E. coli* K12, silver specific *sil* system was found to get activated in *K. pneumoniae* MGH78578.

Overall, this study discloses the complete mechanism behind the action of silver species in Gram-negative bacteria. To the best of our knowledge, this is the first study where the effect of silver nanoparticles (lysozyme coated) was studied on *E. coli* K12 and *K. pneumoniae* MGH78578 by high throughput RNAseq analysis. The present study suggests that uncontrolled, unregulated and overuse of silver based products can result in the development of resistance in bacterial system and advocates the cautious use of Ag NPs capped with tested capping agent(s) for the controlled release of silver ions, which can reduce the chances of development of resistance in bacterial species against silver.