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# Role of Laser Produced Silver Nanoparticles in Reversing Antibiotic Resistance in Some Multidrug-Resistant Pathogenic Bacteria

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***Abstract*—Silver nanoparticles (Ag NPs) were produced through nanosecond laser in deionized water. These nanoparticles were characterized by UV–VIS spectrometer and transmission electron microscopy. VITEK®2 compact system was used to identify *Escherichia coli* (ESBL strain) and *Staphylococcus aureus* (MRSA strain) as multidrug-resistance (MDR) bacteria. The antibacterial activity of Ag NPs, ampicillin (AMP), and their combinations was tested against both bacterial isolates through standard microbiological culturing techniques. Our data show that both of *E. coli* and *S. aureus* were highly resistant to AMP. Ag NPs alone reduced growth in both bacterial isolates considerably. Growth declined drastically in both bacteria when AMP was used in combination with Ag NPs. The minimal inhibitory concentration of combined agents for *E. coli* was 20 μg/ml Ag NPs + 1 mg AMP/ml and for *S. aureus* was 10 μg/ml Ag NPs + 1 mg AMP/ml. The results show that the Ag NPs have great potentials in enhancing the antimicrobial activities of drugs that used to be ineffective against MDR bacteria. Administering combinations of antibiotic(s) with Ag NPs may help in treating patients suffering from infections caused by MDR bacteria. Further *in vivo* and *in vitro* investigations are required to evaluate the side effects of these combinations.**

***Index Terms*—Ampicillin, Antibacterial activity, Laser ablation, Nanosecond laser, Silver nanoparticles.**

## I. Introduction

The unnecessary prescriptions of antibiotics have increasingly made these drugs less effective in treating pathogen-associated diseases. As a consequence, antimicrobial resistance (AMR) spreads worldwide and becoming life-threatening problem (Al-Naqshbandi, Chawsheen and Abdulqader, 2019; WHO, 2020). To overcome this dilemma, physicians and researchers have tried various approaches and formulated different therapeutics for treatment (Weber-Dąbrowska, et al., 2006; Opal, 2016; Greenberg, et al., 2018). For their unique properties, nanoparticles (NPs) were thoroughly investigated in this regard (Hajipour, et al., 2012). Since silver NPs (Ag NPs) showed antibacterial effects against wide range of pathogenic bacteria, they were also studied for this purpose for many years now (Chaloupka, Malam and Seifalian, 2010). Ag NPs antibacterial effects have proven to be in a dose dependent manner (Marassi, et al., 2018) and presented as a promising agent for medical, industrial, and food packaging applications (Haider and Kang, 2015, Simbine, et al., 2019). The mechanisms by which the NPs eliminate bacteria are through: Attachment and penetration of bacterial cell wall, generation of reactive oxygen species (ROS), alterations in bacterial signaling pathways, and eventually cytolysis and leakage of proteins and carbohydrates (Rajesh, Dharanishanthi and Kanna, 2015; Dakal, et al., 2016).

As a strategy to eradicate multidrug-resistant (MDR) bacteria, scientists tried to promote the antibacterial effects of Ag NPs either by combining them with other NPs, such as $TiO_2$ (Hamad, et al., 2015c), or through applying different combinations of these NPs with antibiotics (Brown, et al., 2012; Allahverdiyev, et al., 2011). Furthermore, it has been confirmed that the purity of NPs modulates their antibacterial effects, the more pure NPs are the more bactericidal effects they have (Perito, et al., 2016).

Ag NPs are synthesized either by biological, chemical, or physical methods. Each of these methods has its own advantages and disadvantages (Zhang, et al., 2016). Laser produced Ag NPs, in deionized water, have considerable advantage over those produced through other methods. This is mainly due to their high purity as these NPs without any reductants, stabilizing and capping agents (Sportelli, et al., 2018). (Perito, et al., 2016) generated Ag NPs through pulsed laser ablation in liquid with a small average size in diameter and a narrow size distribution. It was concluded that the Ag NPs are significantly effective against Gram-positive bacteria (GPB) and Gram-negative bacteria (GNB). It was also reported that the minimal inhibitory concentration (MIC) values for laser produced Ag NPs at least comparable or lower

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than those reported for chemically produced Ag NPs. Brasil, et al., 2018, reported synergism in the antibacterial activity of ternary mixtures involving Ag NPs, chitosan and the antibiotics azithromycin, levofloxacin, or tetracycline, against both GPB and GNB strains. The antibacterial activities were performed by *in vitro* antimicrobial susceptibility testing and checkerboard assays. Enhancement of the antibacterial activity was observed in the most combination and the MIC of the drugs was reduced to 97% from 37%. Hwang, et al., 2012, investigated synergistic combination effects between Ag NPs and the common antibiotics such as ampicillin (AMP), kanamycin, and chloramphenicol against different representative pathogenic bacteria. The antibacterial susceptibility and synergistic effects were confirmed through MIC and fractional inhibitory concentration index.

In this study, we tried to uncover the impact of laser produced Ag NPs on pathogenic MDR GPB and GNB. We also aimed to investigate the outcomes of applying different combinations of these NPs and AMP against the same types of bacteria.

## II. Experimental Set-Up

### A. Materials

Ag NPs were generated from a pure Ag bulk plate with a purity of 99.99% and dimensions of 25 mm × 25 mm × 2 mm. The sample was cleaned and sonicated before performing laser ablation in deionized water.

### B. Ag NPs Production

Ag NPs were produced by placing the target on the bottom of a Pyrex glass dish, containing about 15–20 ml of deionized water. A 7.22 W Green marker nanosecond laser (Semiconductor laser: Laserline-Laserval Violino) was used to produce the NPs with the following beam parameters: Wavelength $\lambda$ = 532 nm, frequency $f$ = 30 kHz, laser power $P$ = 7.22 W, pulse width $\tau$ = 5 ns, spot size $D$ = 50 μm, scan speed $v$ = 250 mm/s, laser pulse energy $E_{pulse}$ = 241 μJ, and laser fluence $F_{laser}$ = 12.2 J/cm$^2$. The water level above the sample target was about 2 mm. The ablation process continued for 10 min. The effects of water level on the laser beam intensity and focal length were considered. The experimental setup is shown in Fig. 1.

### C. Material Characterization

The colloidal NPs were characterized using a UV–VIS spectrometer (Analytic Jena, SPECORD 250, dual beam) and Transmission Electron Microscopy (TEM) (JEOL 2000 FX AEM + EDX model). A copper microgrid mesh was used for sample preparation for the TEM analyses. After placing a drop of colloidal NPs onto the mesh, the substrate was allowed to dry at room temperature. This process was repeated several times to deposit sufficient amounts of NPs on the copper microgrid mesh. A microbalance scale (Sartorius BL 210S, with readability $d$ = 0.1 mg) was used to indicate the concentration of the colloidal NPs by weighing the bulk targets before and after the production process of colloidal NPs. A hair dryer was used to dry the target samples after the NPs generation to record the weight of the ablated materials with greater accuracy.

### D. Antibacterial Activity Analysis

The MIC of bacterial strains, antibiotic, Ag NPs, and their combinations was determined using macrodilution method (Dakal, et al., 2016). The Ag NPs concentrations, antibiotic, and their combinations in 5 ml of nutrient broth were inoculated with 0.5 ml of tested bacteria at a concentration of $10^8$ colony-forming units (CFU)/ml. The values of MIC were determined as the lowest concentration of NPs that inhibited bacteria after 24 h of incubation at 37°C using McFarland DensiCHEK plus (bioMérieux, France) (a turbidimetric device for measuring suspended bacteria based on standards optically mimicking bacterial suspensions) (Zamora and Perez-Gracia, 2012). MDR strains of both *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) bacteria were identified using VITEK®2 compact system protocols.

## III. Results

### A. Generation of Ag NPs

Fig. 2a shows absorption spectra of laser fabricated Ag NPs by a nanosecond laser in deionized water. The Ag NPs have a weak and a strong absorption peak at 250 and 400 nm, respectively. The strong absorption peak formation is due to surface plasmon resonance of the Ag NPs, and the weak absorption peak formation is due to interband transitions (Hamad, Li and Liu, 2015a). Fig. 2b shows the size distribution of the NPs. The size of laser produced Ag NPs was between few nanometers to about 60 nm, and some of them were up to 120 nm. The average size of laser

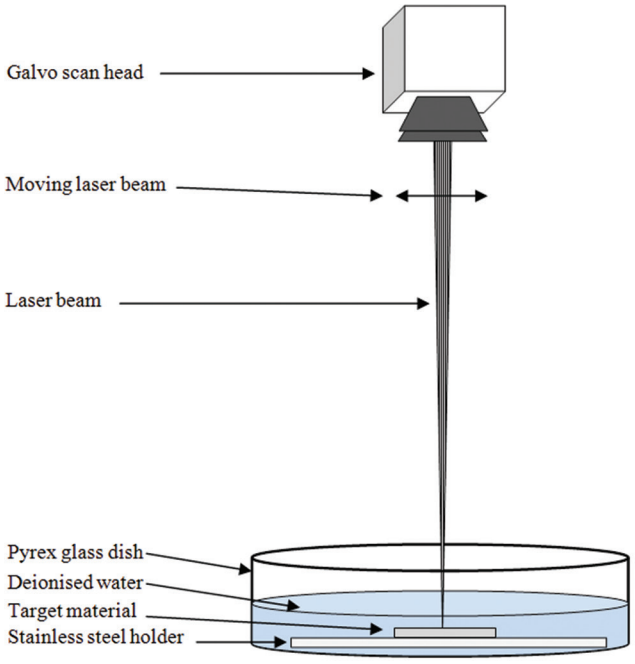


Fig. 1. Experimental set-up to produce Ag NPs in deionized water.

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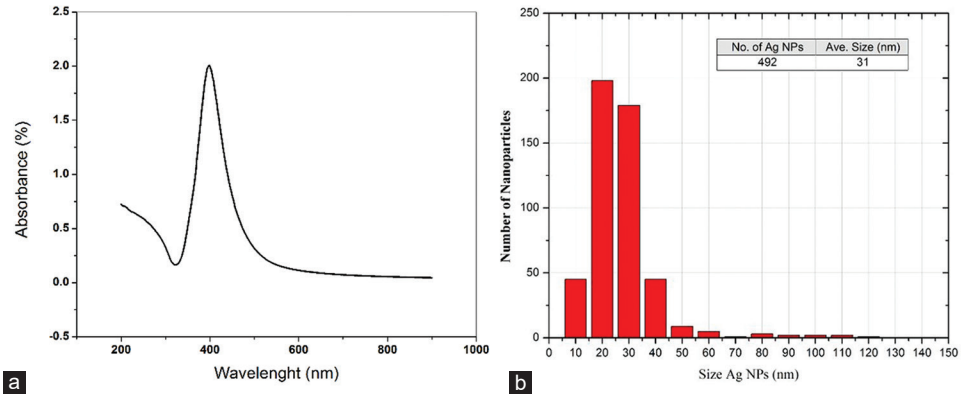


Fig. 2. (a) Absorption spectra of the Ag NPs and (b) size distribution histogram of the Ag NPs.

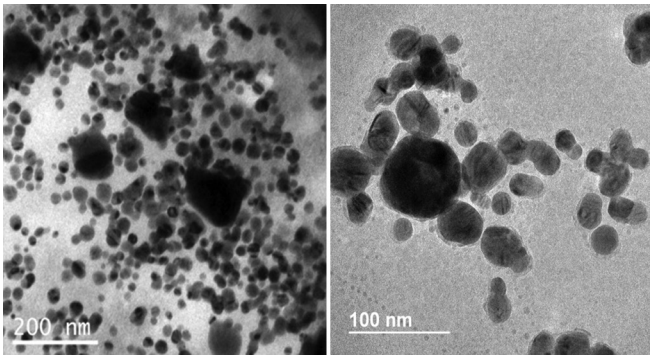


Fig. 3. TEM images of laser-generated Ag NPs in deionized water.

produced Ag NPs is about 31 nm.

Fig. 3 shows the TEM images of laser produced Ag NPs and their semi-spherical shapes. A big and few ultrafine Ag NPs can be seen in these images.

### *B. Antibacterial Activity of the Ag NPs With and Without Antibiotics*

Fig. 4 shows survival rates of *S. aureus*, GPB, and *E. coli*, GNB, after treating them with AMP and/or Ag NPs. Both *S. aureus* and *E. coli* were highly resistant to AMP. Our data show synergetic effects of the Ag NPs and AMP combinations in comparison with the control, Ag NPs, and AMP-treated groups. As shown in Fig. 4a, different combinations of Ag NPs + AMP inhibited the growth of *S. aureus* bacteria completely, even with the lowest Ag NPs concentration (20 μg/ml). In contrast to *S. aureus*, *E. coli* were somehow resistant to the first combination (Fig. 4b).

To uncover the impact of lower concentrations of Ag NPs (≤20 μg/ml) on the outcomes of combined treatments (AgNPs + AMP) against both types of bacteria, another experiment was carried out and the data are shown in Fig. 5.

Accordingly, it can be noted that the Ag NPs combined with AMP were more effective against *S. aureus* than *E. coli* bacteria. In addition, our data show that the impact of Ag NPs on the combinations (AgNPs + AMP) was in a dose-dependent manner (Fig. 5). As shown in Fig. 5a, the fourth treated group (10 μg/ml Ag NPs + AMP) was able to kill all *S. aureus* bacteria, whereas for *E. coli,* even 20 μg/ml Ag NPs in the fifth combination could not kill *E. coli* bacteria entirely and this was consistent with the previous experiment (Figs. 4b and 5b).

For more visibility, Fig. 6 shows the effects of our combinations on both GPB and GNB at concentrations equal or <20 μg/ml of Ag NPs and 1 mg/1 ml AMP. It can be seen that the impact of the combination is similar on both types of bacteria, but still is higher against *S. aureus* in comparison with *E. coli* bacteria.

## IV. Discussion

This study was carried out to address possible roles of Ag NPs in reversing AMP resistance in multidrug-resistant GPB and GNB. We used AMP in our experiments because typically, it is effective against both GPB and GNB. Unfortunately, there is growing evidence suggesting that AMP globally become less effective nowadays in eradicating different types of pathogenic bacteria, which is mainly due to the emergence of AMR in these bacteria as a consequence of excessive use of this drug (Katzung and Trevor, 2012; Krzyżaniak, Pawłowska and Bajorek, 2016; Chawsheen, AL-Naqshbandi and Abdulqader, 2020; Al-Naqshbandi, et al., 2021). In our experiments, bacteria were treated with Ag NPs, AMP, and their combinations. Efficacy of these treatments was evaluated by measuring survival rates of the studied bacteria. Our data show that Ag NPs have the ability to kill *S. aureus* and *E. coli* bacteria but to a limited level that is not exceeding 69%. Our data also show that *E. coli* bacteria are more resilient to Ag NPs than *S. aureus.* Combinations of AgNPs + AMP can effectively eliminate both types of bacteria, especially *S. aureus* that was more sensitive to all combinations than *E. coli.* In spite of the fact that Al-Ogaidi, 2017, used a different method for Ag NPs preparation, but our results came in agreement with theirs regarding different antibacterial activity of Ag NPs, AMP, and their combinations against the targeted bacteria (Al-Ogaidi, 2017).

The antibacterial activity of the Ag NPs can be explained on the basis of metallic silver ($Ag^{\circ}$) and ionic silver ($Ag^{+}$) NPs which being released from the Ag NPs. In other words, the antibacterial activity of the Ag NPs is due to their ability to release Ag ions that are able to bind strongly with electron donor groups such as biological molecules consisting of O, N, and S (Juan, et al., 2010; Hajipour, et al., 2012; Sportelli, et al., 2018).

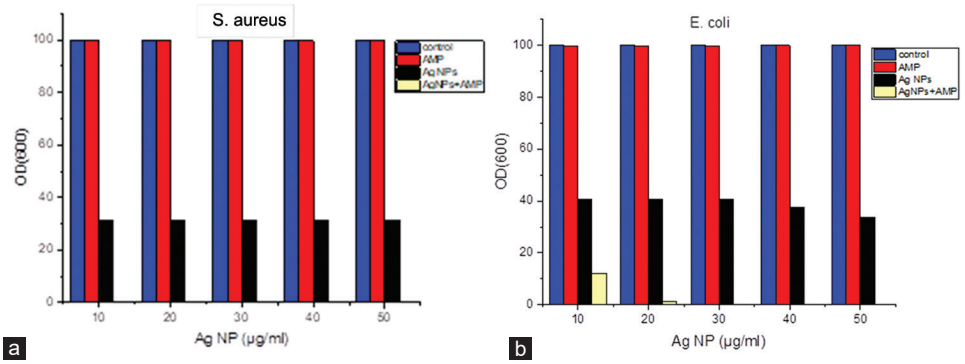


Fig. 4. Survival rate (optical density) of *Staphylococcus aureus* (a) and *Escherichia coli* (b) bacteria after been treated with or without AMP (1 mg/1 ml) and/or ascending concentrations of Ag NPs (10, 20 30, 40, and 50 µg/ml).

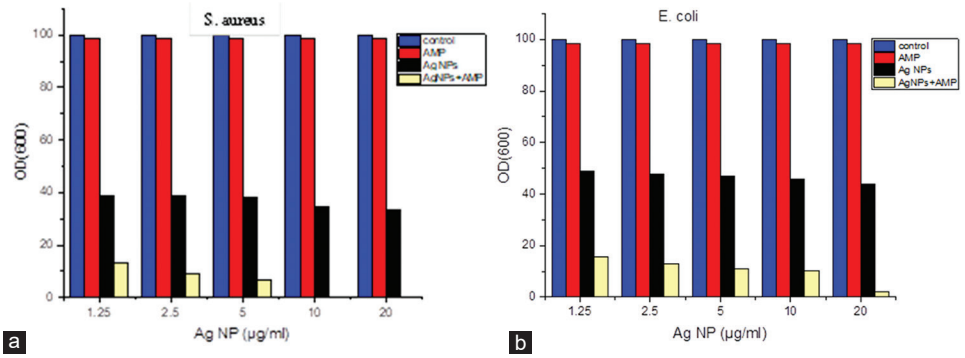


Fig. 5. Survival rate (optical density) of *Staphylococcus aureus* (a) and *Escherichia coli* (b) bacteria after been treated with or without AMP (1 mg/1 ml) and/or ascending concentrations of Ag NPs (1.25, 2.5, 5, 10, and 20 µg/ml).

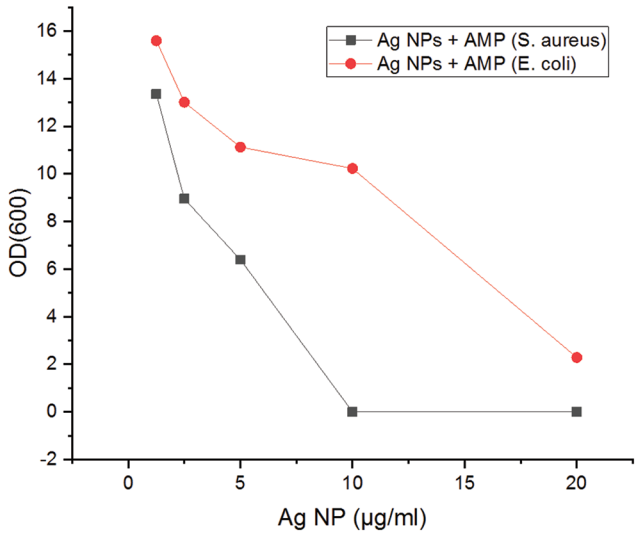


Fig. 6. Effects of (Ag NPs + AMP) on *Staphylococcus aureus* and *Escherichia coli* bacteria. In this experiment, 1 mg of AMP was used in combination with ascending concentrations of Ag NPs (1.25, 2.5, 5, 10, and 20 µg/ml).

### *A. Antibacterial Mechanism of Ag NPs*

There are several proposed mechanisms of action that may explain the impact of Ag NPs, separately or in combination with AMP, on bacterial survival rates. When Ag NPs attach to bacterial cell membrane important functions of this structure such as respiration and permeability will be disrupted (Feng, et al., 2000). Later on, these NPs will penetrate bacterial cells and causes formation of pits in their membranes. Ag NPs may also interact with thiol (organosulfur compound) groups of respiratory chain and transport proteins (sulfur-containing proteins), and eventually preventing them from functioning properly (Morones, et al., 2005). In addition, silver metal or silver ion leads to generation of free radicals and ROS which may damage DNA and denature proteins (Liao, Li and Tjong, 2019). Beside Ag NPs, Ag ions which release either from Ag metals or Ag NPs may interact with nucleic acids and cytoplasmic components, or lead to inhibition of respiratory chain enzymes. These ions may also damage membrane permeability (Russell and Hugo, 1994), thus affecting passive diffusion rate across cell membrane. Furthermore, Ag ions may take role in the adhesion of the Ag NPs to the cell membrane through offering electrostatic interactions between the Ag ions and the negatively charged cell membrane. As a result, structural integrity of the bacterial cell may be compromised through cytoplasmic shrinkage that detaching it from the cell wall and consequently lead to bacterial death (Prasher, Singh and Mudila, 2018). More detail of the antibacterial mechanism of the Ag NPs can be found in our previous review paper (Hamad, Khashan and Hadi, 2020).

### *B. Antibacterial Mechanism of Ag NPs Conjugated AMP*

In the case of Ag NPs conjugated AMP, Allahverdiyev, et al., 2011, reported that the Ag NPs produce a complex with

antibiotics through possible binding sites which include S, N, and OH (Allahverdiyev, et al., 2011). Interactions between Ag NPs and AMP increase the density and concentration of the antibiotic on the surface of the bacteria. In this regard, Ag NPs play the role of the carrier that deliver and facilitate the way in for AMP "hydrophobic compound" to reach inside the bacterial cell. Afterword, the cell wall will be destroyed by AMP through its well-known mechanism of action and then increases its permeability for these NPs (Biggs and Kucers, 1986). Ag NPs prevent DNA from unwinding and consequently interfere with the duplication process in the affected bacteria (Fayaz, et al., 2010; Allahverdiyev, et al., 2011). Furthermore, Fayaz, et al., 2010, reported that Ag-AMP core-shell compound interacts with the bacteria over the cell wall which prevents the generation of cross-links in the peptidoglycan layers, as a result, the cell wall will be lysis (Fig. 7).

Functionalized Ag NPs, capped by stable citrate, were used as a sensing probe for detection of AMP in urine samples based on color changing and shift localized surface plasmon resonance to longer wavelength known as red shift phenomenon. They found out that pH and temperature have a direct impact on hydrolysis of AMP. After the addition of the antibiotic, Ag NPs aggregated and then AMP conjugates with the surface of the these NPs through sulfur bond and electrostatic force ($F_e$), and replacement of citrate ions from the surface of NPs will take place (Shrivas, et al., 2017).

Even though our data showing that *S. aureus* are more sensitive than *E. coli* to Ag NPs, there are some studies suggesting that "at the same concentration," Ag NPs are more effective against GNB than GPB because of their thinner cell wall (Pandey, et al., 2014).

In the case of *E. coli* bacteria, silver ions interact with the respiratory chain enzymes and prevent the respiratory chain at a low potential point (Holt and Bard, 2005). The Ag NPs in the bacterial cell tend to "interfere with the bacterial growth signaling pathway by modulating tyrosine phosphorylation of putative peptide substrates critical for cell viability and division (Shrivastava, et al., 2007)." A low molecular weight area in the bacterial cell will be produced when the Ag NPs enter the cell and then the bacteria aggregate to protect their DNA. After that, the NPs interact with the respiratory chain, which leads to the death of bacterial cell (Rai, Yadav and Gade, 2009). In the case of GPB, *S. aureus*, peptidoglycan layer is 30 nm thick and negatively charged, which makes it less vulnerable against Ag NPs in comparison with the GNB, *E. coli*, where the thickness of peptidoglycan layer is about 3–5 nm. The thickness and negative charge of the peptidoglycan layer renders the Ag ions produced from Ag metals inactive, as a result, makes GPB to be more resistant against antimicrobial drugs. In addition, lipopolysaccharides (LPS) part in cell membrane of the GNB prevents and protects the microorganisms from the chemical reactions and keeps the structural integrity of the cell membrane. However,

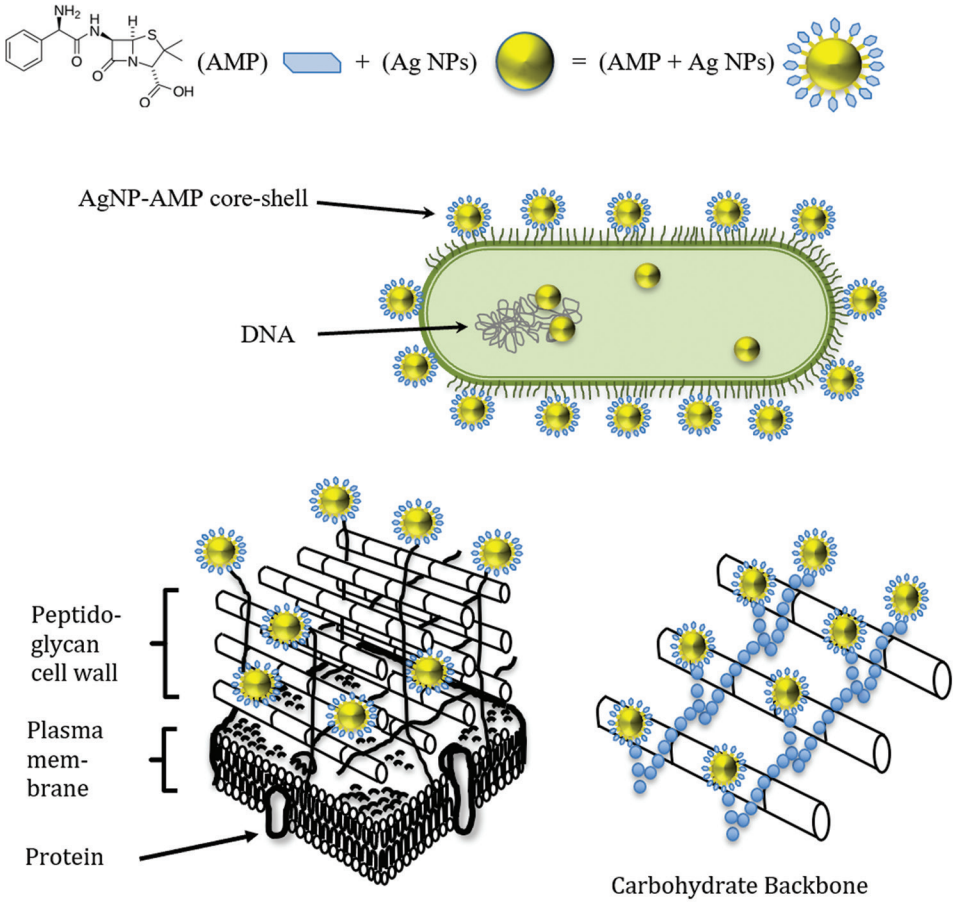


Fig. 7. Possible mechanism of AMP conjugated Ag NPs against microorganisms (Fayaz, et al., 2010).

negatively charged LPS promotes the connection between the NPs and the cell membrane which makes the bacterial cells more susceptible to antimicrobial drugs (Prasher, Singh and Mudila, 2018).

Laser-generated Ag NPs have negative zeta potentials of about −42.3 mV (zeta deviation = 7.65 mV) (Hamad, et al., 2015b), thus, the NPs are negatively charged particles. On the other hand, *S. aureus* bacteria are GPB, they are positively charged bacteria. The electrostatic interaction between NPs and bacteria is the key prominent force facilitating the Ag NPs binding to surface of *S. aureus* bacteria. Due to that, Ag NPs combined with AMP are more effective against *S. aureus* than *E. coli* bacteria. The surface electrostatic interaction changes in accordance with surface charge of the nanomaterials. In the case of the positively charged Ag NPs, strong electrostatic forces help in combining of positively charged Ag NPs to GNB or negatively charged bacterial outer membrane or cell wall (Poh, et al., 2018). Last but least, the surface charge properties of the Ag NPs when conjugated with the AMP should be taken into account, as the surface charge of the NPs is related to the NPs' stability in the aqueous solution (Mafuné, et al., 2000).

## V. Conclusions

The Ag NPs which fabricated using a nanosecond laser in deionized water have the ability to generate bactericidal effects against both MDR GPB and GNB. Different combinations of Ag NPs and AMP are able to reverse antibiotic resistance in these bacteria; this is more likely through compromising the structural integrity of cell membrane, damaging cytoplasmic compartments, interfering with cellular signaling pathways, and blocking DNA duplication process.

## VI. Authors' Contribution Statement

AH, AA, and MC: Evenly participated in designing the research. AA: Carried out biological experiments. AH: Prepared and validate Ag NPs. MC and AH: Evenly contributed in writing the manuscript.

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