Chapter 1

General Introduction

Part of the work presented in this chapter has been published as per the following details:

1. Pareek et al. (2018) *Material Science & Engineering C* 90: 739-749.
2. Pareek et al. (2017) *Advanced Science, Engineering and Medicine* 9: 527-544.

1.1 Nanotechnology: a general introduction

"Nano" is a Greek word and mathematically means one billionth part of meter (1×10^{-9}). Materials, substances or particles with at least one dimension in the size range of 1-100 nm are called nanomaterials, nano-substances or nanoparticles (NPs). Nanotechnology is an interdisciplinary branch of "Nanoscience" that involves the study of fundamental principles of molecules and structures at nano level. It is an application-based technology which implies the use of nanostructures into nanoscale devices valuable for different scientific fields including biology, chemistry, medicine, physics, material science, engineering etc. (Jain et al., 2008). The foundation stone of nanotechnology was put up long back in 1959 when the well-known physicist and Noble Laureate Dr. Richard Feynman delivered his visionary lecture entitled "There is Plenty of Room at the Bottom" in the meeting of American Physical Society, where he envisioned and proposed to manipulate individual atoms to create very tiny structures with different properties and potential (Feynman, 2011). The recent breakthrough in the nanotechnology have already sparked a flurry of inventions which are increasing at tremendous pace in the field of fast-moving consumer goods (FMCGs). Plenty of products are currently available in the market which utilizes the principle of nanotechnology. The enthusiasm for utilization of nanotechnology is sweeping the planet, generating several billion dollars of investment annually in research and development (R&D). Governments of several countries including India have initiated programmes and schemes related to development in the field of nanotechnology in cross scientific disciplines. Many organizations foresee the global nanotechnology market crossing $1 trillion by the year 2020.

1.2 Properties of nanoparticles

NPs are made up of large number of atoms or molecules bonded with each other. They can be synthesized by assembling atoms/molecules or by breaking the bulk materials to achieve nanometer dimensions ranging from 1-100 nm. NPs possess several distinctive and unique physico-chemical properties which are normally not encountered in their corresponding bulk materials. These properties and behavior of NPs majorly depend on their size, shape, composition and surface properties (Pareek et al., 2018). These unique properties are provided by the fraction of atoms present on the surface of NPs, which increase the surface area to volume ratio as well as thermodynamic stability (phase transition) of NPs, whereas in case of bulk material less atoms in cluster show less defined phase transition upon temperature gradient (Roduner, 2006). It has been observed that as this ratio increases, the dominant behavior of atoms on the surface of nanoparticle (NP) also changes, which is a major contributing factor behind their unique performance. The high surface area to volume ratio also provides sufficient sites for binding of substrates during a reaction, which confer remarkable chemical reactivity (Cuenya, 2010).

By varying the precursor and modifying the synthesis protocol, NPs of diverse origin (organic or inorganic) can be synthesized. Due to the unique properties of metallic inorganic NPs such as resistance to corrosion and oxidation, non-reactiveness, unusual high reduction potential, high melting point and high ionization energy, they have attracted much attention of the scientific community towards their functioning in both basic and applied research. Metals also constitute a large portion of periodic table and have a huge range of chemical activity, which controls their reactivity toward living cells. In this regard, use of inorganic molecule as NPs has been known since long back. The physico-chemical properties of metal and metal oxide NPs are dictated by various attributes like size, shape, architecture, crystallinity and composition (Li et al., 2015). Due to their exceptional properties, metal NPs have been widely applied in fast moving fields like environmental remediation, water purification, antimicrobial agents and in various industrial sectors involving consumer goods such as cosmetic products, toothpaste, soaps, shampoos, detergents etc. (Pradeep, 2009; Hajipour et al., 2012; Lemire et al., 2013).

1.3 Synthesis of nanoparticles

The development of rapid and reliable experimental protocols for the synthesis of NPs is one of the key aspects of current nanotechnology research. As discussed earlier, the physico-chemical properties of NPs depend on their size, shape, surface topology and composition. In the last two decades, synthesis of metal NPs has received enormous attention because of their increased use in various applications. Considerable efforts have been made towards developing new and promising methods for the synthesis of NPs. In general, the methods for the synthesis of NPs can be classified into “top-down” and “bottom-up” approaches (Figure 1.1). In top-down approaches, bulk material is broken, molded or sliced to synthesize nano sized particles. Whereas, in bottom-up approaches, atoms or molecules are assembled step by step to construct NPs. The main advantage of top-down approach is that it can synthesize nanomaterial in bulk quantity within a short span of time. While, the major advantage of bottom-up approach is that it can synthesize homogenous nanostructures with perfect crystallographic and surface structures.

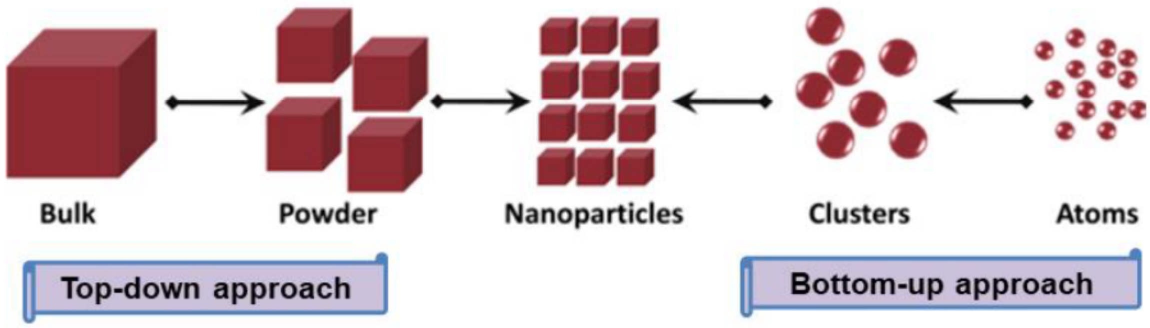

Figure 1.1: Top down and bottom up approach for the synthesis of nanoparticles (Pareek et al., 2017).

The synthesis methods can also be categorized as chemical, physical and biological methods. The existing protocols for the synthesis of NPs suffers from at least one or more drawbacks like homogeneity of the NPs, imperfect crystallographic structure, etc. Thus, the pursuit for development of novel process to fabricate NPs with controlled and tunable properties is still challenging and open for research.

With understandable motives, bottom-up approaches are reported to be more beneficial due to the presence of covalent bonds between single molecules which are comparatively stronger then their weak interactions found in the top-down approach. In contrast to top-down approaches, the bottom-up approaches utilizes the concepts of molecular self-assembly and/or molecular recognition for the synthesis of NPs. The foremost advantage of the bottom-up approach is that it can synthesize homogenous nanostructures with perfect crystallographic and surface structures (Cao et al., 2002). In addition, the synthesized particles possess appreciable stability which is highly desirable for fulfilling their applications. Considering the advantages and limitations of existing synthesis methods, one should choose an ideal method which is ecofriendly, cost-effective, easily scalable and have controlled & monodisperse synthesis of NPs with minimum steps and energy requirement to least batch to batch variation.

In line of above facts, in the present study, the bottom-up approach was selected for the synthesis of NPs. Among different kinds of bottom-up approaches viz. microemulsion, chemical reduction, laser ablation, microwave assisted, biological synthesis etc., we selected chemical reduction (modified) and fungi mediated biosynthesis (myco-synthesis) for the synthesis of NPs. These methods promise high productivity of NPs with ease in upgradation to industrial scale without using hazardous capping materials and generation of toxic by-products (Gericke and Pinches, 2006).

1.4 Characterization of nanoparticles

Characterization of synthesized NPs is an essential step to understand and attest the effectiveness of synthesis protocol. The NPs are generally characterized for various physico-chemical properties viz. size, shape, surface properties, phase constitution and microcrystal structure. The expansion of research for development of novel instruments and characterization technique is one of the utmost challenge in the field of nanotechnology. General techniques used for the characterization of NPs are ultraviolet-visible (UV-vis.) spectroscopy, dynamic light scattering (DLS), powder X-ray diffraction (XRD), fourier transform infrared (FTIR) spectroscopy, energy dispersive spectroscopy (EDS), thermogravimetric analysis (TGA), transmission and scanning electron microscopy (TEM and SEM) & nuclear magnetic resonance (NMR). Recently, a new technique namely nanoparticle tracking analysis (NTA) has been reported which allows direct tracking of NPs based on their Brownian motion, allowing estimation of individual NPs in solution. Table 1.1 summarizes various techniques used for the physicochemical characterization of NPs.

Table 1.1: Techniques used for characterization of various parameters of NPs (Pareek et al., 2017).

Parameter	Characterization method
Nature of nanoparticles	Ultraviolet–visible (UV-vis.) spectroscopy X-ray Diffraction (XRD) Energy Dispersive X-Ray Analysis (EDX)
Particle Size	Transmission electron microscopy (TEM) Dynamic light scattering (DLS) Laser differectiometry Atomic force microscopy (AFM)
Charge determination	Zeta potentiometer X-ray photoelectron spectroscopy (XPS)
Chemical analysis of surface	Fourier transform infrared spectroscopy (FTIR) Scanning electron microscopy (SEM) Thermogravimetric analysis (TGA) Static secondary ion mass spectrometry

1.5 Applications of nanoparticles

Due to their versatile properties, NPs have many potential applications in the field of electronics, information technology, energy, catalysis, automobile, defense and security, cosmetics, biosensor, food and packaging, ecology, environment and agriculture. Among different kinds of organic and inorganic NPs, metal and metal oxide NPs are known to gain special attention due to their exclusive and imitable properties and widely applied in sensing and visualization of biomolecules, diagnostics, therapeutics, gas/vapor sensing, water sterilization, antimicrobial agent etc. (Pareek et al., 2017).

Among various kind of metal/ metal oxide NPs, silver NPs (Ag NPs) have received considerable attention due to their unique properties such as conductivity, chemical stability, relatively lower toxicity, and outstanding therapeutic potential viz. anti-inflammatory and antimicrobial activities (Marambio-Jones and Hoek, 2010). Due to their unique antimicrobial and catalytic properties, Ag NPs have been widely used in the fast-moving consumer goods such as cosmetics, toothpaste, soap, shampoos, detergents, washing machines, water purification systems as well as in the field of environmental remediation. The antimicrobial potential of silver has been well recognized since ancient era. Hippocrates, the father of modern medicine, believed that silver powder has beneficial healing and anti-disease properties (Lansdown, 2002). Silver vessels have been utilized for the preservation of perishable items as well as for disinfections of water since long back (Pradeep, 2009). Considering the antimicrobial properties of silver, its varak has been used for garnishing sweets in Indian cuisine since very long time. However, due to emergence of antibiotics as an economical and efficient alternative as well as lack of proper understanding of mechanism of toxic effects of silver on microorganisms, for a long period of time modification and development of new silver antimicrobial formulations have been neglected (Pareek et al., 2018).

1.6 Silver as an antibacterial agent

The unprecedented increase in antibiotic resistance in this era has resuscitated the attention of scientific community to exploit silver and its various species (bulk, ion and nano forms) as antimicrobial agents. Compounds that locally kill or inhibit the growth of microorganism without affecting the nearby tissues are called as antimicrobial agents. In current era of science and technology, Ag NPs are an auspicious contrivance towards the improvement of biomedical field because of its vast and valuable applications like drug delivery, biosensing, antibacterial agent, etc. (De Jong and Borm, 2008; Rai et al., 2009; Doria et al., 2012; Pareek et al., 2017). Ag NPs are now-a-days used as an antimicrobial agent in wound dressings, textiles, food storage containers and personal care appliances (McQuillan et al., 2012). Need of Ag NPs as a potential antibacterial agent raised due to the worldwide escalation and augmentation of multi-drug resistance (MDR) in microorganisms (Fischbach and Walsh, 2009). Microorganisms that acquired resistance to more than two drugs are called as MDR microbes. The major reason behind the development of MDR microorganisms is the unadvised and excessive use of antibiotics. According to an estimate, over 50% of the antibiotic prescriptions given by doctors in developing countries are without clear evidence of infection or adequate identification of pathogens. Hence, it contains broad-spectrum antibiotic instead of narrow-spectrum drugs. The situation becomes worse when the patients do not take complete course of medication (Ling et al., 1983, Aslam et al., 2018).

The knowledge of biological mechanisms involved in the use of antibiotics can be helpful to overcome the resistance problem. Bacterial resistance against antibiotics basically depends upon their envelope structure. 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 (Delcour, 2009). Figure 1.2 represents the general comparison between the cell envelope structure of Gram-positive and Gram- negative bacteria.

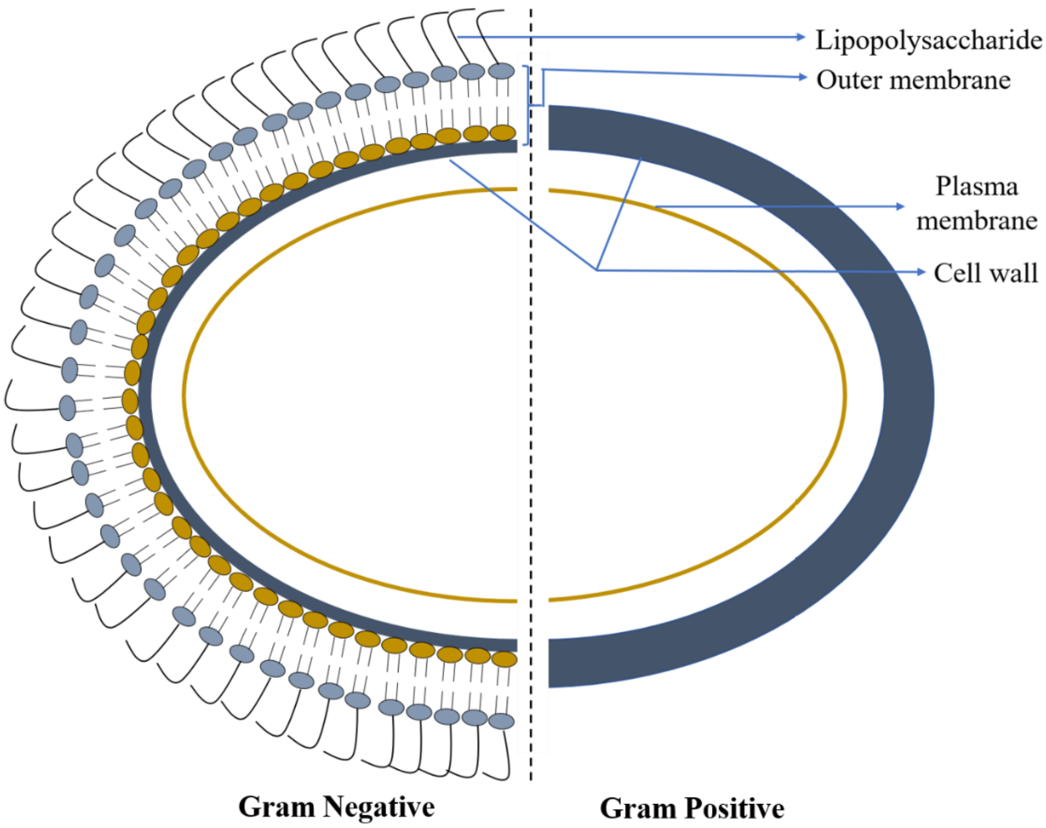

Figure 1.2: Difference between the Gram-negative and Gram-positive bacterial cell envelope.

In general, cell wall/ cell membrane synthesis, protein synthesis, nucleic acid synthesis and intermediary metabolism are the common targets exploited for the development of various antibacterial agents (Neu, 1992; Blair et al., 2015). Bacteria can use single or multiple resistance mechanisms to overcome the effect of antibiotics. The various drug resistance mechanisms (natural/ intrinsic or acquired) present in bacteria (Figure 1.3) have been discussed below (McManus, 1997, Koonin et al., 2001; Chuanchuen et al., 2003; Tenover, 2006, Zhu et al., 2010; Wright, 2011; Panáček et al., 2018):

a) Components of bacterial envelope (glycocalyx, cell wall or plasma membrane) can prevent the penetration and entry of drug inside, thus making the bacterial cell resistant to a particular drug. For example, presence of outer membrane in many gram-negative bacteria make them resistant to various antibiotics such as rifampin, penicillin etc.
b) Several pathogenic bacteria have non-specific transporter proteins known as “efflux pump” in their plasma membrane, which can expel the drug from cell and make the bacteria resistant.
c) Bacteria can become resistant by inducing modification in the structure of applied drug thus making it inactive. For example, the enzyme β-lactamase present in several *Staphylococci* species hydrolyzes the β-lactam ring of penicillins due to which antibiotic gets inactivated and cannot kill bacterial cell. Similarly, the acetyltransferase enzyme present in certain bacterial species make them resistant particularly to chloramphenicol as it cause acetylation of the amino group present in chloramphenicol which results in its deactivation.
d) Bacteria can also become resistant to a drug by modifying their cellular structures (like peptide bridge in cell wall, ribosome etc.) and enzymes which are targets for the drug. The resulting changes in bacterial metabolism make them less susceptible to the antibiotic.
e) Antibiotic resistance can also be due to the presence of pre-existing genes on the bacterial chromosome or on plasmid. These genes can also be acquired by a bacterial cell due to lateral or horizontal gene transfer.

In order to overcome the drug resistance, there is a constant need to search new and more effective antibacterial agent. For the development of new antibacterial agents, there is a need to study the interaction between bacteria and new antibacterial agent. The knowledge of antimicrobial properties of metals leads to turn researchers to exploit the antibacterial potential of metal and metal oxide NPs and development of “nano-weapons” against bacteria (Pareek et al., 2018). In recent years, Ag NPs have developed a new hope to treat bacterial diseases and replace antibiotics. 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, interaction studies between bacteria and silver would be influential to understand the mechanism behind antibacterial action and would open new avenue for the development of target specific silver-based new generation antibiotics.

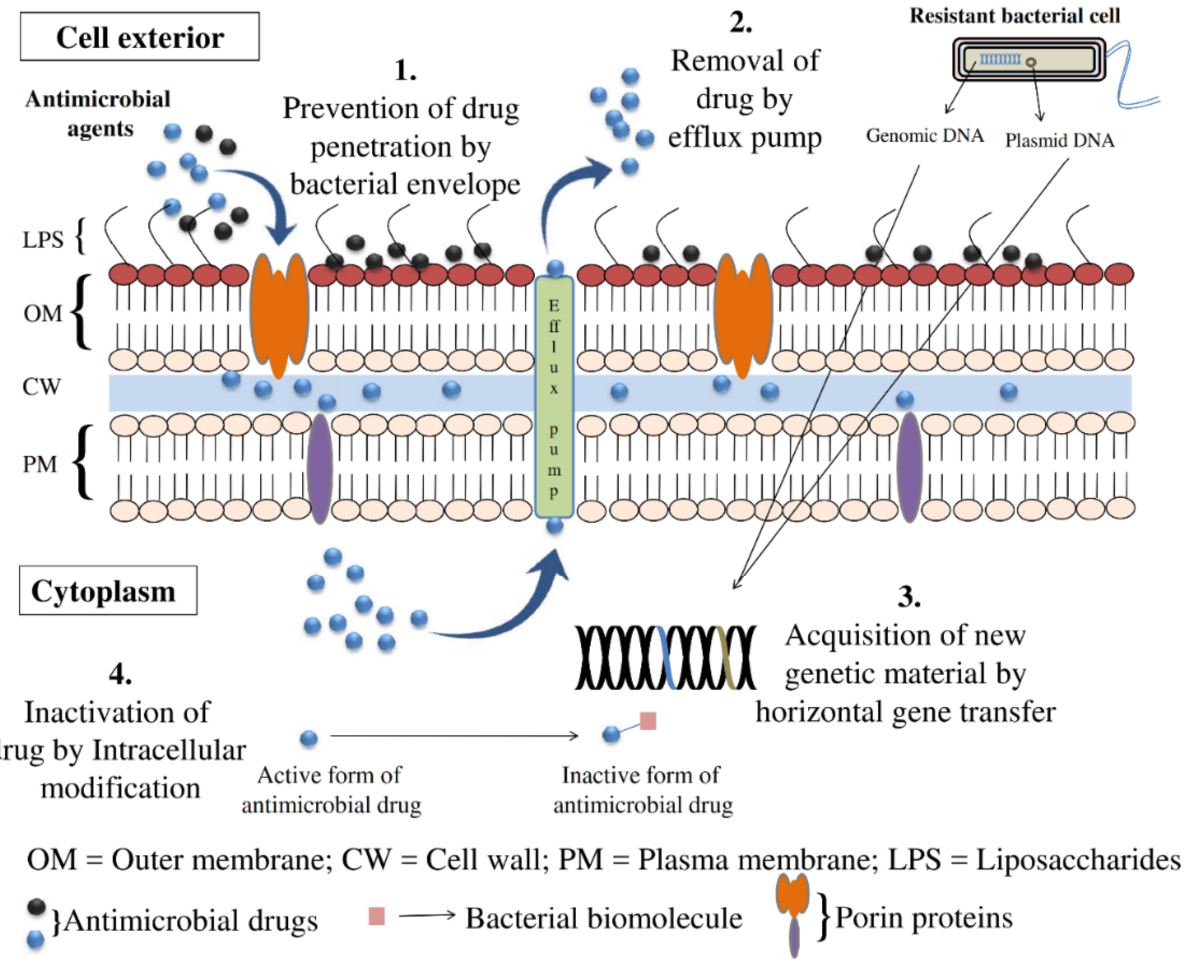

Figure 1.3: Antimicrobial drug resistance mechanisms in bacteria (1) Prevention of antimicrobial drug penetration into the bacterial cell by bacterial cell envelope; (2) Removal of antimicrobial drug from bacterial cell by non-specific efflux pumps; (3) Acquisition of new genetic material from drug resistant bacterial strain and (4) Inactivation of antimicrobial drug by intracellular modification (Pareek et al., 2018).

In this perspective, systematic and in-depth mechanistic studies can help in developing an efficient Ag NPs-based antibacterial system. These studies shall involve two fundamental steps, which can make impact on the effectiveness of NPs antibacterial activity

- First is the proper understanding of the physical and chemical behaviour of NPs in biological media, which will decide its course of action on bacterial cells. It will provide the knowledge regarding the effect of physico-chemical modifications (size, shape, surface coating etc.) of Ag NPs towards the variations in their antibacterial activity.

- The second step is to have in-depth knowledge about the bacterial cell machinery, which can get affected by Ag NPs. These include increased generation or dissipation of the metabolic regulation pathways. This knowledge can be achieved by critically studying their transcriptomic profiles.

1.7 Gaps in existing research

The literature survey reveals that antibacterial action of Ag NPs majorly depends on their physico-chemical properties (majorly size and surface capping) which regulates the entry of silver ions into the bacteria; and bacterial regulatory pathways upon the treatment of Ag NPs. However, systematic studies on the variation in the physico-chemical properties of Ag NPs, their interactions with biological media and subsequently their effect on bacterial cell at transcriptomic levels are not yet established. Although, multiple reports are available on the antibacterial activity of Ag NPs, the precise molecular mechanism of their mode of action has remained unclear, which makes an obstacle towards the generation of potential antibacterial agents against various pathogenic and MDR bacteria.

In order to unveil the precise molecular mechanism behind the antibacterial action of Ag NPs, one must have the complete time-based information about the effect of Ag NPs exposure to the bacterial cells. Bacterial transcriptomic analysis at different time intervals upon Ag NPs exposure can be attempted to unveil the whole bacterial machinery under the influence of Ag NPs. To the best of our knowledge, no study has been reported, which reveals the time-based gene expression profile in bacteria upon the Ag NPs exposure.

1.8 Objectives

Keeping the above-mentioned facts in mind, the present thesis aims to accomplish the following objectives:

1. Synthesis and characterization of silver nanoparticles with varying physico-chemical properties (specifically size and surface capping).
2. Investigations on the antimicrobial effects of silver species at physiological and biochemical levels.
3. Evaluation of bacterial transcriptomic profile with respect to silver species treatments.