Chapter 1
General Introduction
Nanotechnology deals with the materials which exhibit remarkable physical, chemical and biological properties because of their nanoscaled size. Nanoparticles usually have a size range of 0.1 to 1000 nm in each spatial dimension. The word “nano” is derived from a Greek word meaning dwarf or extremely small (Rai et al., 2008). The concept of nanotechnology was given by Professor Richard Feynman (Feynman, 1959) and the term nanotechnology was introduced by Tokyo Science University Professor Norio Taniguchi in 1974. Nanoparticles (NP) exist in a variety of forms, from nanowires to magnetic, noble and transition metals and nonmetal water soluble nanocrystals or quantum dots (QD’s). Some of the characteristics that distinguish nanomaterials from bulk materials include: large fraction of surface atoms, high surface energy, spatial confinement and reduced numbers of imperfections (Cao, 2004). In addition to this, nanoparticles can act as a scaffold and there by allow the attachment of biomolecules like antibodies, peptides and DNA through a number of methods including covalent and hydrogen bonding (Vinogradov et al., 2002).

Nanoparticles provide a platform for new developments in various fields like biosensors, biomedicine and bio nanotechnology. Among the various nanomaterials, metal nanoparticles have received considerable importance due to their significant applications in cell labeling and imaging (Parak et al., 2005), biosensing (Niemeyer, 2001), drug delivery (Langer, 2003), optoelectronics (Jackson et al., 2003), non-linear optical devices (Maier et al., 2001) and surface enhanced Raman spectroscopy (Li et al., 2004). In addition to this, they also have various biomedical applications due to their unique biological properties.

1.1. Methods of Synthesis of Nanoparticles

Various physical, chemical, biological, and hybrid methods are available to synthesize different types of nanoparticles (Liu et al., 2011; Luechinger
et al., 2010; Tiwari et al., 2008; Mohanpuria et al., 2008). The top-down (physical) approach deals with methods such as thermal decomposition, diffusion, irradiation and arc discharge. The bottom-up (chemical and biological) method involves those based on seeded growth, polyol synthesis, electrochemical synthesis, chemical reduction and fabrication of nanoparticles by biological entities.

The major physical methods used for the synthesis of nanoparticles include thermal decomposition, laser irradiation, electrolysis, condensation and diffusion. In thermal decomposition method, fatty acids are dissolved in hot NaOH solution and mixed with metal salt solution to form metal precipitate (Yang and Aoki, 2005). While in diffusion, crystals and short wires of copper are enclosed in glass ampoules and sealed at low pressure; further, the ampoules are annealed at 500°C for 24 h. The crystals are then removed from the ampoules and cooled on a metallic plate at room temperature (Rodriguez-Perez et al., 2006). In UV irradiation technique, polycarbonate films are cut and placed on glass microscope slide and exposed to UV radiation which results in the formation of hydroxyl groups on polycarbonate films. Further, these polycarbonate films are silanized with 3-(aminopropyl) triethoxysilane in ethanol for 2 h and rinsed with deionised water which results in the formation of silver film on the polycarbonate film (Aslan et al., 2006). The arc-discharge method involves use of two graphite electrodes which act as cathode and anode and are immersed in metal salt solution. The electrodes are brought in contact to strike an arc and separated immediately to sustain arc inside salt solution. The synthesis of nanoparticles is carried out at an open circuit and an optimized direct current (Fernandez-Pacheco et al., 2006).

The chemical synthesis of nanoparticles by chemical reduction involve use of various reducing agents like sodium borohydride or sodium citrate (Cao and Hu, 2009) N, N-dimethyl formamide (Pastoriza-Santos and Liz-Marzan, 2000),
poly(N-vinyl pyrrolidine), ethyl alcohol (Kim, 2007a), tetra-n-tetrafluoroborate, and CTAB (Hanauer et al., 2007). In seeded growth method, reduction of metal ions with reducing agent result in formation of seed particles which are further added to growth solutions containing metal ions and additives like L-ascorbic acid and hexadecyltrimethylammonium bromide (CTAB) (Hanauer et al., 2007). In the polyol method the metal precursor is dissolved in methanol or ethyl alcohol and PVP (Polyvinylpyrrolidone) is used as a protective and capping agent (Kim, 2007a). In addition, electrochemical synthesis method induces chemical reactions in an electrolyte solution with the use of an applied voltage.

Even though physical and chemical methods for nanoparticle synthesis are more popular, the use of toxic chemicals in the synthesis is a major challenge to its biomedical applications. In addition, the chemical method involve use of three main components such as a metal salt, a reducing agent and a stabilizer or capping agent for the control of growth of the nanoparticles and prevent agglomeration (Ledwith et al., 2007). But in biosynthesis of nanoparticles, the function of reducing agent and the stabilizer are provided by molecules produced of biological origin (Fig. 1.1). These reducing and/or stabilizing compounds can be present in bacteria, fungi, yeasts, algae, or plants (Gade et al., 2008; Narayanan and Sakthivel, 2010). The reduction may happen enzymatically or non-enzymatically and the stabilizer or capping agent can be biomolecules like protein. But agglomeration of chemically produced nanoparticles have been reported to decrease their surface area and there by the catalytic and antimicrobial activities (Mafune et al., 2000). Also biogenic nanoparticles can be stable over large periods of time. Moreover, biogenic stabilizers of nanoparticles provide easy platform for further functionalization of nanoparticles with other desired molecules (Hennebel et al., 2009). Therefore, use of eco- friendly biological method for the synthesis and assembly
of nanoparticles is of considerable importance (Babu and Gunasekaran, 2009). Thus, biological methods are much remarkable because of its effectiveness, flexibility and ability to generate nanoparticles with defined size and morphology (Raveendran et al., 2003; Narayanan and Sakthivel, 2010; Pugazhenthiran et al., 2009).

Biological methods make use of wide range of resources including microorganisms, plants, proteins, polypeptides and nucleic acids for nanoparticle synthesis (Rai et al., 2008; Thakkar et al., 2010; Devi and Joshi, 2012). The ease of culture handling and the occurrence of synthesis at ambient temperature and pressure make microbial synthesis of metal nanoparticles as highly promising (Ingle et al., 2008; Gade et al., 2010; Mukherjee et al., 2008). Also this provides opportunities to scale up the synthesis process to generate large quantities of nanoparticles to explore its potential applications.

1.2. Microbial Synthesis of Metal Nanoparticles

Microorganisms synthesize metal nanoparticles due to their varying interactions with metals and resistance towards metals. These properties can vary among various microorganisms due to the differences in mechanisms that it use to manage metal. This include processes such as enzymatic oxidation or reduction (Prakash et al., 2010), accumulation of metallic NPs outside the plasma membrane (Pugazhenthiran et al., 2009), metal ion excretion across permeable membranes (Bruins et al., 2000), binding of metal ions to peptides (Krumov et al., 2007) and efflux pump systems (Nies, 1999).
Fig. 1.1. Silver and gold nanoparticle synthesis using bacteria

The microbial synthesis of nanoparticles can take place either extracellularly or intracellularly or both (Ahmad et al., 2003; Rai et al., 2008; Mukherjee et al., 2008). In intracellular method, negatively charged cell wall of microorganisms play major role in the synthesis. They interact electrostatically with the positively charged metal ions and the enzymes present within the cell wall reduce the metal ions to nanoparticles. Even though the method is simple, additional steps such as ultrasound treatment or reaction with suitable detergents are required to release the intracellularly synthesized nanoparticles for further studies. The mechanism of extracellular synthesis involves reduction of metal ions in the presence of the cell wall reductive enzymes or soluble secreted enzymes (Zhang et al., 2011a). Enzymes like hydrogenase and NADH-dependent reductase are mostly reported in the reduction of Ag$^+$ ions to silver.
NPs (AgNPs) (Mukherjee et al., 2002; Bansal et al., 2005). So the extracellular synthesis method is having the added advantage of obtaining large quantities of nanoparticles in a relatively pure state (Balaji et al., 2009).

Among the different microbes studied for the biosynthesis of nanoparticles, bacteria have received the most attention and are preferred due to the ease of culturing, manipulation and downstream processing (Vaidhyanathan et al., 2010). The biosynthesis of AgNPs was first reported for the bacterium *Pseudomonas stutzeri* AG 259 isolated from silver mine. The study demonstrated the intracellular synthesis of silver nanoparticles with distinct morphology (such as equilateral, triangles and hexagons) and size (upto 200nm) (Haefeli et al., 1984). A large number of bacterial species from diverse sources have been exploited for its nanoparticle biosynthetic potential. Zaki et al. (2011) have reported the AgNP biosynthetic potential of five bacterial strains belonging to species of *Escherichia coli* (S30, S78), *Bacillus megaterium* (S52), *Acinetobacter* sp. (S7) and *Stenotrophomonas maltophilia* (S54). Members of Enterobacteriaceae like *Klebsiella pneumoniae*, *Escherichia coli* and *Enterobacter cloacae* are known to form silver nanoparticles with a size range of 28.2 nm to 122 nm by extracellular reduction of Ag$^+$ to Ag$^0$ (Shahverdi et al., 2007a). Also the metal-reducing property of *Micrococcus luteus*, *Shewanella oneidensis* and *Marinobacter pelagius* was utilized for the synthesis of the gold nanoparticles (AuNPs) (Arunkumar et al., 2013; Suresh et al., 2011; Sharma et al., 2012). In addition, *Brevibacterium casei* and *Geobacillus stearothermophilus* have been reported with the potential to synthesize both silver and gold nanoparticles (Kalimuthu et al., 2010; Fayaz et al., 2011a).

Microorganisms have the ability to synthesize ZnS and PbS nanoparticles also. Bai et al. (2006) observed the ability of *Rhodobacter sphaeroides* to form ZnS NPs intracellularly. Similarly sulfate-reducing bacteria have also been used for the synthesis of ZnS NPs (Labrenz et al., 2000).
Interestingly, Bai and Zhang, (2009a) have also reported biosynthesis of PbS NPs from Rhodobacter sphaeroides. Additionally, a biological route for the synthesis of monoclinic Selenium (m-Se) nanospheres by Bacillus subtilis is also known (Wang et al., 2010a). Biosynthesis of palladium nanoparticles using sulfate reducing bacterium, Desulfovibrio desulfuricans has previously reported by Lloyd et al. (1998). DeWindt et al. (2005) reported the ability of Shewanella oneidensis MR-1 to precipitate palladium Pd(0) nanoparticles on the cell wall and inside the periplasmic space by the reduction of soluble Pd(II). Other reports show the potential of Shewanella algae to deposit platinum nanoparticles in the periplasmic space by reducing PtCl(6)(2-) ions (Konishi et al., 2007). Enterobacter sp. have shown to have the ability to synthesize uniform sized spherical mercury nanoparticles intracellularly under the culture conditions of pH 8.0 and lower concentration of mercury (Sinha and Khare, 2011).

Baesman et al. (2007) have reported the ability of B. selenireducens and S. barnesii to synthesize tellurium nanoparticles, in which B. selenireducens formed nanorods of ~10 nm in diameter and 200 nm in length and S. barnesii formed small irregularly shaped nanospheres of 50 nm diameter. Titanium nanoparticle synthesizing property of Lactobacillus sp. is also known (Prasad et al., 2007). Also Brevibacterium casei is reported to have the ability to extracellularly synthesise cubic spinel-structured single-crystalline ferromagnetic Co₃O₄ nanoparticles functionalized with proteins in the size range 5–7 nm (Bansal et al., 2012). Serratia marcescens isolated from the Caspian Sea in the north of Iran has been studied for the intracellular bioreduction of bismuth subnitrate (Bi(NO₃)₃) to elemental BiNPs (Nazari et al., 2012). Also a mild biological procedure was reported for the synthesis of intracellular antimony sulfide (Sb2S3) NPs by S. marcescens (Bahrami et al., 2012). Magnetotactic bacteria (MTB) which consist of fastidious and mostly aquatic prokaryotes have the well
known potential to form magnetic NPs (Faramarzi and Sadighi, 2013). Also *Escherichia coli* have been reported for the synthesis of a mixture of copper and copper oxide quasispherical nanoparticles of size range 10-40 nm in presence of aqueous copper precursors (Singh et al., 2010).

Microorganisms can expect to use various mechanisms for the nanoparticle synthesis and only limited information is available with respect to the molecular basis of nanoparticle synthesis. The role of nitrate reductase enzyme in the reduction of Ag\(^+\) ions to silver nanoparticles has been studied by Kalimuthu et al. (2008) using *Bacillus licheniformis* isolated from municipal waste. Kalimuthu et al. (2010) have also reported the importance of α-amylase from *Brevibacterium casei* in biosynthesis of AuNPs (10-50nm). A study on the biosynthesis of gold nanoparticles using *Micrococcus luteus* has demonstrated the role of extracellular α-amylase and cell wall teichuronic acid (TUA) in the synthesis of monodispersed and spherical AuNPs (Pichaimani et al., 2012). Presence of silver-binding proteins in the organic matrix of microbes and its role in providing amino acid moieties to serve as the nucleation sites for the formation of silver nanoparticles has also been suggested (Narayanan and Sakthivel, 2010). Recently Elbeshehy et al. (2015) have reported the role of peptides in the formation of silver nanoparticles in *Bacillus pumilus*, *Bacillus persicus* and *Bacillus licheniformis*.

1.3. **Silver nanoparticles (AgNPs)**

Nanotechnology is an evolving field with promising biomedical applications (Paul and Robeson, 2008). The application of nanoparticles as alternative antimicrobial agents in medicine has significantly increased and the most popular nanoparticle for clinical application is silver nanoparticles (AgNPs) (Silver et al., 2006). Advantages with the use of AgNPs include weak ability of bacteria to develop resistance toward silver ions and the non toxic
nature of AgNPs to human cells in low concentration (Lansdown, 2006). Silver nanoparticles have been known to have broad antimicrobial activities due to its binding with DNA, interaction with enzymes that control respiration and other cell functions or with chemical functionality or receptors on the cell membrane (Ellis, 2007). At the same time it has also been reported to induce bacterial cell death due to the formation of free radicals (Prabhu and Poulose, 2012). These features increased the development of various silver nanoparticle based formulations for both clinical and nonclinical applications. In addition mammalian cells are known to phagocytose these nanoparticles, followed by degradation through lysozomal fusion and thereby reducing toxicity and free radical damage (Arbab et al., 2005). Also AgNPs offer promising opportunities to re-explore the biological properties of already known antimicrobial compounds (Rai et al., 2009). Thus various studies have emerged in recent years focusing on efficient synthesis of silver nanoparticles.

The antibacterial activity of silver has long been known and water and wine were stored in silver vessels in ancient times to prevent spoilage (Silver et al., 2006; Rai et al., 2009). In the 17th century, the silver ion was used to heal ulcers and chronic wounds (Klasen, 2000). In the 19th century, it was further used for the treatment of burns and the prevention of ophthalmic diseases in newborns. During the first decades of the 20th century, silver nitrate was used to treat wounds and burns, but around the Second World War, antibiotics treatments were developed and the interest in silver become faded (Edwards-Jones, 2009). Around that same time it was recognized that silver nitrate was mordant for the eyes of newborns and invented argyrol, a protein-stabilized silver colloid (Silver et al., 2006). A similar colloidal silver solution was also commercialized as collargol. These colloidal silver preparations were early nanosilver formulations (Nowack et al., 2011). Furthermore, other distinct properties such as conductivity, chemical stability, catalytic activity, nonlinear optical behavior along with
its bactericidal properties make silver nanoparticles to have diverse applications (Rosarin and Mirunalini, 2011; Roe et al., 2008).

1.4. Gold nanoparticles (AuNPs)

Gold is one of the well known noble metals and has been used in medicine throughout the history of civilization (Higbi, 1982). Gold and gold compounds have been used in treatment of rheumatic diseases and discoid lupus erythematosus (Parish, 1999), restorative dentistry (Hickel and Manhart, 2001; Manhart et al., 2004) and various inflammatory skin disorders such as pemphigus, urticaria and psoriasis (Thomas and Pandrea, 1993). Compared to the bulk metal, gold nanoparticles (AuNPs) are very important nanoscale materials as they exhibit completely new and improved properties. Gold nanoparticles are exceptional because of their tunable Surface Plasmon Resonance (SPR); hence they are used in bio-labeling (Lin et al., 2009), biosensor devices for the detection of viruses and bacteria (Xia et al., 2007), drug delivery (Niemeyer, 2001), tissue/tumor imaging, photo thermal therapy and immuno-chromatographic identification of pathogens in clinical specimens (Huang, 2006). In addition, gold nanoparticles are biocompatible, inert and can bind readily to a range of biomolecules such as proteins/enzymes (Niemeyer, 2001), DNA (Alivisatos, 1996) and amino acids (Selvakannan et al., 2004). Moreover, because of their nontoxicity, versatility in surface modification, polyvalent effects and photothermal effects, AuNPs become useful in the development of antibacterial strategies (Giljohann et al., 2010; Boisselier and Astruc, 2009).

Among microorganisms, bacteria have received the most attention in the area of AuNPs synthesis. For the first time microbial synthesis of AuNPs was reported in Bacillus subtilis 168 which revealed the presence of 5-25 nm octahedral NPs inside the cell wall (Beveridge and Murray, 1980). In
Rhodopseudomonas capsulata, spherical AuNPs with 10-20 nm range have been observed (He et al., 2007). Shewanella algae were demonstrated to reduce Au (III) ions under anaerobic conditions with the possible assistance of gold reductase in its cell membrane (Kashefi et al., 2001). By the addition of gold thiolate and gold chloride complexes, the filamentous cyanobacterial strain Plectonema boryanum UTEX 485 was made to synthesize AuNPs (Lengke et al., 2006). Prolonged incubation of Au (III) ions with cyanobacterial cell mass was found to result in the formation of thin triangular and hexagonal sheets of gold particles. In another study, gram negative bacterium Rhodopseudomonas capsulata was shown to reduce gold ions into gold nanoparticles (He et al., 2007). When the biomass of R. capsulata was exposed to gold ions at neutral pH, synthesis of gold nanoparticles of 10 to 20 nm size was observed. Also, when the same reaction was carried out at acidic pH, triangular gold particles were obtained along with the spherical gold nanoparticles.

1.5. Bactericidal activity of nanoparticles against drug resistant bacteria

The mechanisms of nanosilver toxicity against bacteria have thoroughly examined and found that the biocidal activity of silver nanoparticles is mainly associated with its physico-chemical properties like specific surface area, high adsorption ability, chemical reactivity and catalytic activities (Elechiguerra et al., 2005; Choi and Hu, 2008). Three forms of silver may be present in colloidal solution, that is metallic silver Ag\(^0\), free silver ions Ag\(^+\) and silver ions Ag\(^+\) adsorbed on the nanoparticle surface. Continuous release of silver cations from the nanostructured surface proves to be a prominent agent responsible for efficient antibacterial activity (Malina et al., 2010). Silver nanoparticles target the bacterial cell wall which is a protective barrier against various substances. Nanoparticles binding to bacterial cell wall may results in perforation leading to cell death. Silver nanoparticles with average particle size of 12 nm have been reported to form irregular-shaped pits in the Escherichia coli cell membrane.
(Feng et al., 2000). In addition, AgNPs is known for its capacity to work as a catalyst within all the protein structures. The catalytic behavior is mainly manifested by its binding with functional groups of amino acids and the reaction between the –SH groups of neighboring protein amino acids and formation of -S-S- bonds between them. Formation of additional –S-S- bonds may induce molecular changes that lead to protein inactivation and deactivation of enzymes. The thiol groups deprived of hydrogen form disulfide bonds -S-S- in the bacterial cell wall and thereby block the pathways of electron transfer through the respiratory chain (Wzorek and Konopka, 2007).

The catalytic properties of nanosilver and the occurrence of generated reactive oxygen species contribute damage to cell wall peptides and genetic material (Lok et al., 2006; Choi and Hu, 2008). It was also found that gram-positive bacteria are more resistant to AgNPs activity compared to gram-negative ones (Egger et al., 2009). Cell wall of gram positive bacteria is made up of much more murein, peptidoglycan material which is negatively charged. Due to the negative charge of Gram-positive cell wall, more silver cations are kept within the wall and that may prevent their penetration into cells (Wzorek and Konopka, 2007). Fig. 1.2 represents antimicrobial mechanisms proposed for AgNPs from different studies.

Various studies have reported AgNPs as a strong stressogenic agent towards bacteria. *E. coli* showed enhanced synthesis of heat shock proteins and outer membrane proteins, such as OmpA, OmpC, OmpF, OppA, MetQ under the influence of silver. Induction of heat shock proteins evidenced high intensity of stressogenic factors. Also even small amount of silver nanoparticles was found to disturb cytoplasmic membrane potential to generate ATP. Due to defects in energy production, bacterial outer membrane proteins cannot be efficiently transported and as a result they accumulated in the cell cytoplasm (Lok et al., 2006). Another important characteristic of AgNPs is the generation
of reactive oxygen species (ROS), which react with substances in bacterial cell and seriously damage other molecules and cell structures. Under the aerobic respiration, small quantity of ROS may occur in cell where molecular oxygen is reduced to carbon dioxide. Usually, a properly functioning cell produces enzymes like, superoxide dismutase and catalase to scavenge toxic reactive oxygen species. Superoxide dismutase catalyses the conversion of superoxide anion into hydrogen peroxide which is broken down to oxygen and water. But studies on nitrifying bacteria revealed silver nanoparticles to have increasing effect on intracellular ROS level and bacterial growth inhibition (Choi and Hu, 2008). Moreover, many other studies also suggest genetic material of the bacterium as another target site of action of silver nanoparticles (Feng et al., 2000; Kim et al., 2010). The replication capability of bacterial DNA has also been reported to be affected by AgNPs (Morones et al., 2005). Moreover, silver ions also affect gene expression as in *E. coli*, where it was observed to stop expression of S2 protein, which is a component of 30S ribosomal subunit.

Fig.1.2. Diagrammatic representation of antibacterial mechanisms of silver nanoparticles (Modified figure from Hajipour et al., 2012)
Microbial development of resistance mechanisms and escalation of emerging and re-emerging infectious diseases associated with medical procedures have become a serious problem to public health in recent days (Huh and Kwon, 2011). This demands design of new antimicrobial drugs with highly efficient and improved delivery potential by utilization of knowledge from recent advances in nanomaterial research (Turos et al., 2007). The unique physical properties of nanoscale materials have shown to have promises to develop wide array of methods for cost-effective diagnosis, rapid determination of resistance of antibacterial drugs and efficient delivery of antimicrobial agents (Rosi and Mirkin, 2005). Their application as drug delivery vehicles is due to its advantages such as controlled release, improved solubility, specific site-targeted delivery, minimized side effects, and enhanced cellular internalization. Additional advantages of nano-antimicrobial formulations including its protective effect towards antimicrobial drugs and ability to overcome resistance in conjugation with antibiotics (Weir et al., 2008; Mansour et al., 2009; Santos-Magalhães and Mosqueira, 2010) demands studies on exploration of antimicrobial nanomaterials and novel nanosized platforms for clinical applications. Many types of lipophilic and water-soluble antibiotics have been reported to be conjugated inside or on the surface of nanoparticles, or carried via encapsulation (Abeylath and Turos, 2008). Among the nanoparticles used, silver and gold nanoparticles (AuNPs) are considered to have highly useful applications due to its facile and well-studied synthesis, easiness with surface functionalization, biocompatibility, less toxicity and ability to increase drug concentration at infected site (Demurtas and Perry, 2014).

One of the major problems associated with antimicrobial drugs is their failure to fight with bacteria that have the ability to produce biofilms. Biofilms are a complex microbial community formed by their adhesion to a solid surface followed by secretion of a matrix (proteins, DNA and extra-polysaccharide),
which cover the microbial cell community (Hajipour et al., 2012). Thus nanoparticles have been demonstrated to be a promising agent for combating microbial biofilms.

1.6. **Bacterial biofilms and its clinical relevance**

Biofilm formation enables single-cell organisms to become a temporary multicellular lifestyle, in which “group behavior” facilitates survival in adverse environments. The shift from planktonic growth to biofilm occurs in response to environmental changes, and involves multiple regulatory networks, which translate signals to concerted gene expression changes and mediate the spatial and temporal reorganization of the bacterial cell (Lenz et al., 2008; Monds and O’Toole, 2009). This cellular reprogramming alters the expression of surface molecules, nutrient utilization, and virulence factors and enables the bacteria for their survival in unfavorable conditions (Stanley et al., 2003; Lenz et al., 2008; Klebensberger et al., 2009). Within the biofilm, bacteria are protected in a self-produced extracellular matrix, which accounts for ~ 90% of the biomass (Flemming and Wingender, 2010). The matrix is composed of extracellular polymeric substances (EPS), carbohydrate-binding proteins (Branda et al., 2006; Diggle et al., 2006), pili, flagella, other adhesive fibers (Pinkner et al., 2006; Cegelski et al., 2009), and extracellular DNA (eDNA) (Whitchurch et al., 2002; Thomas et al., 2009; Von Eliff et al., 2006). In the matrix, nutrients are trapped for metabolic utilizations by the resident bacteria and water is efficiently retained through H-bond interactions with hydrophilic polysaccharides (Conrad et al., 2003; Flemming and Wingender, 2010). In response to changes in nutrient availability, enzymes secreted by the bacteria modify EPS composition (Gjermansen et al., 2005), thereby tailoring biofilm architecture to the specific environment (Sauer et al., 2004). Thus, these structural components of the matrix give rise to a highly hydrated, robust structure with high tensile strength. This keeps bacteria in close proximity,
enabling intimate cell-to-cell interactions and DNA exchange (Flemming and Wingender, 2010) and also protecting the biomass from desiccation, predation, oxidizing molecules, radiation, and other damaging agents (Jefferson et al., 2005; Flemming and Wingender, 2010).

The flexible nature of biofilms is also partly attributed to the presence of environmental gradients within the biomass, which give rise to community “division of labor” with subpopulations of bacteria showing differential gene expression in response to local nutrient and oxygen availability (Lewis, 2005; Domka et al., 2007). Metabolically inactive nondividing persister cells which are tolerant to a number of antibiotics may also present within biofilm (Lewis, 2005) and this may play important role in reseeding of biofilms on cessation of antibiotic treatment in the clinical setting (Lewis, 2008).

Within the host, the matrix protects biofilm bacteria from exposure to innate immune defenses such as opsonization and phagocytosis and antibiotic treatments (Jefferson et al., 2005; Leid et al., 2005; Cerca et al., 2006). Interbacterial interactions can promote the spread of drug resistance markers and other virulence factors (Vuong et al., 2004). As a result, biofilm-forming pathogens persist, establishing chronic and recalcitrant infections such as upper respiratory infections (Koch and Hoiby, 1993), urinary tract infections (UTIs) (Foxman, 2010), periodontitis (Kuramitsu and Wang, 2011), catheter-induced and other device-associated infections (Jacobsen et al., 2008; Fey, 2010). In immune-compromised patients, the manifestation of infections by opportunistic biofilm-forming pathogens can be devastating, leading to severe symptoms and, in many instances, death.

1.7. Biofilm formation on medical devices

The use of medical devices is one of the areas of medicine and an increasing source of healthcare associated infections. During the implantation of
a medical device, proteins from the patient’s blood or tissue directly adsorb onto the surface. This will depend on many parameters such as surface hydrophobicity, roughness; porosity and chemical composition (Thevenot et al., 2008; Vroman 1987). The resulting layer of adsorbed proteins has been shown to be important for the adhesion of bacteria. The free swimming bacteria, in the so-called planktonic state, will adsorb to these surface adsorbed proteins (Pavithra and Doble, 2008). The adhered bacteria will increase in numbers by proliferation and recruitment of other bacteria from the immediate environment. Once a fine number of bacteria have formed a colony on the surface, these will change their gene expression pattern. Genes will be activated and expressed that are responsible for the production of extracellular polymeric substances, which results in the formation of biofilm (Hoiby et al., 2010; Hall-Stoodley and Stoodley, 2009).

The morphology of the biofilm has been extensively studied and found that this slime layer is mainly composed of extracellular polymeric substances (EPS). This EPS matrix can have varying composition but mainly consists of polysaccharides. The biofilm also is highly hydrated and contains up to 95% water and the layer has more porous morphology with holes and tunnels (Donlan, 2002a: Sutherland et al., 2001). The biofilm matrix provides proper transport of oxygen and nutrients to the bacteria. The anaerobic conditions in the biofilm also lead to altered gene expression and metabolism in the bacteria. These new metabolic conditions decrease sensitivity of bacteria to a number of antibiotics, which have been designed to hinder with the bacteria’s metabolism. Thus microbes living in a biofilm derive more benefits such as physicochemical advantages, multispecies synergisms and rapid gene transfer which are not available in its planktonic stage (Jefferson et al., 2005).

Biofilm formation on surfaces of medical implants is very difficult to prevent and the post-biofilm formation treatment is difficult to manage because
of its slimy nature. The systemically administered antibiotics will not penetrate and therefore the bacteria will not be killed. The application of biofilm disturbing agents in combination with antibiotics seems a more competent strategy, but this has not been applied frequently because of the aggressive nature of the chemicals involved (Hoiby et al., 2010). The topological and chemical nature of a medical device surface is also important for the rate of microbial adhesion. A perfectly smooth surface will be less likely to be inhabited than a rough surface, where more surface area is available as well as more adhesive force can be generated by the microorganism per surface area. Also the chemical feature is essential for the initial population of a surface by free-swimming pathogens. Hydrophilic surfaces have been shown to be less quickly occupied by free-swimming bacteria than hydrophobic surfaces (Sousa et al., 2009; Katsikogianni et al., 2006). Therefore the modification of implant surfaces may be a more efficient strategy to fight the infection of biomedical implants. The inhibition of initial bacterial colonization of the surface is a prime focus of biomaterial science. This will avoid most implant related infections and reduce the chance of biofilm formation.

The pathogenic bacteria such as *Staphylococcus aureus*, *Enterococcus faecalis*, *Streptococcus* sp., *E. coli*, *Klebsiella* sp. and *Pseudomonas* sp. have reported to be growing on catheters, artificial joints and mechanical heart valves and lead to persistent infections (Donlan and Costerton, 2002b). Even though a variety of gram-positive and-negative bacteria have been involved in device related infections, Staphylococci, particularly *Staphylococcus epidermidis* and other coagulase- negative staphylococci (CoNS) are the major cause of infections in both temporarily or permanently implanted devices (Hugonnet et al., 2004; Donlan, 2001).
1.8. Coagulase Negative Staphylococci (CoNS)

Staphylococci are members of the family *Micrococcaceae* with *Staphylococcus aureus* as the major species involved in human infection. However, the more benign coagulase-negative staphylococci have surfaced more effectively as the cause of human infections (Rupp and Archer, 1994). Thus among the staphylococcal species, *Staphylococcus aureus* and *Staphylococcus epidermidis* are at the first and the second positions in the list of the leading etiologic agents. This is followed by a certain number of CoNS species that are emerging as new pathogens, such as *Staphylococcus hominis*, *Staphylococcus haemolyticus*, *Staphylococcus capitis* and *Staphylococcus warneri* (Arciola et al., 2005; Campoccia et al., 2010). These organisms are either introduced during implantation of a medical device or derived from a temporary bacteraemia, which ultimately form a biofilm.

The well documented CoNS species that most frequently cause catheter associated infections are *S. epidermidis*. Because of their well known role as device contaminants, the change of life style from commensal to pathogens exhibited by *S. epidermidis* is very important. Approximately 75% of patients with coagulase negative staphylococcal bacteremia have indwelling medical devices that are most often intravascular catheters (Rupp and Archer, 1994). The spread of drug resistance in CoNS is also very remarkable and 80% of coagulase negative staphylococcal isolates often show resistance to methicillin and other semisynthetic penicillins (Diekema et al., 2001). The various protective mechanisms present in CoNS provide survival advantages to both its commensal and infectious life styles. The success in the colonization and development of infection has been related to the capacity of these microorganisms to produce biofilm. This also interferes with the treatment of infections, impair the action of host immune cells and compromise action of antibiotics (Bayston and Rodgers, 1990). Since the biofilm formation on
medical devices and the dissemination of cells from this to other parts of the body forms the basis of CoNS infection, use of medical devices with antimicrobial and antibiofilm property to limit CoNS infection is very important.

The knowledge about the components and the architecture of staphylococcal biofilms has allowed the development of new strategies to disrupt biofilm. Even though bacteria are hidden deeply inside the biofilm and are protected against antibacterial agents, the biofilm matrix is available to the outside environment. In addition, the matrix is a porous network in which fluids run along channels (Arciola et al., 2012). These features make the biofilm matrix a good target for anti-biofilm therapies. The new strategies include modification of the biomaterial surface to give anti-adhesive properties, doping the material with antimicrobial substances and combining anti-adhesive and antimicrobial effects in the same coating. Nanotechnologies and nanomaterials in medical research have created new therapeutic horizons and are rapidly growing. Thus the use of materials coated with antimicrobial nanoparticles appears very innovative and promising.

1.9. Treatment strategies for biofilm inhibition

The preferred treatment strategy for bacterial infections is mainly antibiotics. Conventional antibiotics prevent the bacterial cell division. Although, over the years antibiotics have proven critical in eliminating bacterial pathogens and various evidences indicates their damaging effect on host microbiota, and development of antibiotic resistance (Dethlefsen and Relman, 2010; Ubeda et al., 2010). Moreover, even though prophylactic antibiotic administration prior to surgery is highly successful in reducing infection rate, it has little effects in protection from surgical procedures involving implants or prostheses (Secinti et al., 2011). In most cases, the best treatment for foreign
body associated biofilm infections is the removal of the infected device. However, the device removal is difficult in cases like implantable prostheses, pacemakers, and cardiac implants (Fey, 2010). Biofilm bacteria are predominantly recalcitrant to antibiotic treatments because of increased transmission of resistance markers within the biofilm community, diffusion limitations posed by the extracellular matrix, antibiotic inactivation by high metal ion concentration and low pH, and the presence of metabolically inactive persister cells that survive treatment (Mack et al., 2004; Lewis, 2008). These characteristics make biofilm bacteria up to 1000 fold more tolerant and/or resistant to antibiotics than planktonic cells (Hoiby et al., 2010). Thus, there is an urgent need for more effective antibiofilm treatments.

The most commonly used approach for preventing device-associated biofilms is the impregnation of medical devices with antimicrobial agents (Fey, 2010). Now nanotechnology is emerging as one of the most favorable methodology for the prevention and control of microbial biofilms and the main approach with promising result is with silver nanoparticles. Silver has been used as an anti infective agent for hundreds of years and has been widely used to sterilize wound infections during World War I (Chen and Schluesener, 2008). The positively charged silver ions facilitate electrostatic attractions between the metal and the negatively charged bacterial membrane (Kim et al., 2007b). The biocidal activity of silver to bacteria is partly owing to thiol-group reactions that inactivate enzymes (Chen and Schluesener, 2008). Silver treatment inhibits DNA replication, expression of ribosomal and other cellular proteins, and interferes with the bacterial electron transport chain (Yamanaka et al. 2005). Silver nanoparticles have been shown to inhibit *Pseudomonas aeruginosa* and *Staphylococcus epidermidis* biofilms by > 95% and in vivo studies showed ability of AgNPs coated implants to inhibit *Staphylococcus aureus* biofilm formation without causing silver accumulation in host tissues (Kalimuthu et al.,...
Thus, novel strategies, designed to block biofilm by the use of antibacterial or antiadhesion agents provide exciting avenues for exploration and the development of fast-acting, potent, and bioavailable treatment strategies and nanotechnology approach seem to be the most promising field of research to control or eradicate biomedical biofilms formed by multiresistant microorganisms.

In addition to infection related to implanted medical devices, Coagulase-negative staphylococci have been found to be the most common pathogen involved in wound infection also. Thus the current study was undertaken to develop a nanocomposite membrane for wound healing applications.

1.10. **Electrospun PCL/AgNPs membrane as effective wound dressing biomaterial**

Electrospinning is a technology that has been widely used for the development of nanoscale fibers. Electrospinning process has a great deal of attention because of its ability to produce ultrafine fibers from polymer solutions with diameters ranges from nanometer to submicrometers that exhibit high surface area to volume using electrostatic forces. Also the mechanical, biological and kinetic properties of the scaffold can be easily manipulated by altering the polymer solution composition and processing parameters. Electrospun fibers have various applications such as filtration of subatomic particles, composite reinforcement, multifunctional membranes, tissue engineering scaffolds, wound dressings, drug delivery, artificial organs and vascular grafts (Luu et al., 2003; Yoshimoto et al., 2003; Bolgen et al., 2007).

The complete electrospinning unit was first designed by Cooley and Morton in 1902. They used various collectors to elucidate the effect of the external electric field on fluids. Utilization of high electrostatic potential is the mechanism behind the electrospinning technique to produce nanofibers. It
mainly consists of four parts: a syringe pump to control flow rate, syringe with needle which act as one of the electrode to charge the polymer solution, power supply to generate electric field and collector which act as other grounded electrode to collect fibers (Zhu and Chen, 2013). Under the influence of electric field, charge is developed in the polymer solution. At low electric field strength, a pendant drop emerged out from the tip of needle which is balanced by the surface tension of solution. As the voltage increased, charges on solution repel each other which results in elongation of drop into conical shape due to electrostatic forces in opposite direction. As the voltage reached to its critical value, all equilibrium forces on drop get distorted and the electrostatic forces overcomes the surface tension due to which, a jet is emerged out of the cone and get deposited on grounded electrode (Fig. 1.3).

Fig.1.3. Schematic diagram of the electrospinning unit (Modified figure from Zhu and Chen, 2013)
The electrospinning technology has many applications in the biomedical field because of the simplicity of the procedure in generating the large surface area to volume ratio of the material and the mechanical stability of the fibres (Khan, 2012). One of the main applications of electrospun fibers in biomedical application is drug delivery, where these fibers help to encapsulate the therapeutic agent in the fibers. Moreover, electrospun fibers maintain the integrity and bioactivity of the drug molecules due to the mild processing parameters. Localized inoculation of medicines in wound treatment using electrospun fibers as delivery vehicles can significantly reduce the systemic absorption of the drug and prevent/reduce any side effects from the drugs. Also, the efficacy of the drug would also improve due to localization of the treatment (Cui et al., 2010). The release of the drug is then dependent on the degradation of the polymer fibers and thus can be properly controlled. The core shell electrospun fibers have usually been used in drug delivery applications. This is due to the fiber’s ability to encapsulate the drug molecules until they are needed in the hollow core. These fibers protect the drug and also prevent other molecules such as enzymes and growth factors from denaturing during processing (Wang et al., 2010b; Chen et al., 2010; Okudaa et al., 2010). Thus the therapeutic agents remain unaltered and encapsulated until needed at the site of action.

The skin is the largest organ of the body, covering the entire external surface and forming about 8% of the total body mass. It forms a self renewing and self repairing interface between the body and the environment. Also it acts as an effective barrier against microbial invasion, and protects against mechanical, chemical, osmotic, thermal and photo damage. When damage done at the the most superficial layer of the skin epidermis, wound healing takes places via reepithelialization without any skin grafting. But serious trauma may lead to partial or complete damage to both dermal and subdermal tissues.
(Seals et al., 2001). The dermis underlies the epidermis and provides physical strength and flexibility to the skin, as well as being the matrix that supports the extensive vasculature, lymphatic system and nerve bundles. In addition, it is composed predominantly of an extracellular matrix (ECM) of interwoven collagen fibrils. Fibroblasts, the major cell type of the dermis, produce and maintain most of the ECM. Wounds that extend partially through the dermis are capable of regeneration, but the body cannot heal deep dermal injuries adequately. In such cases, complete reepithelialization takes a long time and is complicated by scarring of the base (Marler et al., 1998).

An ideal wound dressing material should be a good barrier for protection of wound from infection and dehydration. Also it should have the ability to remove the exudates from the wound and have good oxygen permeability (Chellamani et al., 2013). There is increasing interest towards employing electrospinning for scaffold fabrication because of its mechanical, biological and kinetic properties which can be easily manipulated by altering the polymer solution composition and processing parameters. The ability to produce a non-woven nanofibrous structure with morphological and architectural features similar to that of the natural ECM in skin is the another advantage of electrospinning process. Additionally, the scaffold structure changes over time as the polymer nanofibers degrade and allow the seeded cells to proliferate and produce their own ECM (Li et al., 2002).

Many biodegradable or non-biodegradable biocompatible polymers have been used to generate nanofibres. Various natural and synthetic polymers are used for nanofibers manufacturing, resulting in various degradation rates based on polymer choice. Among them Polycaprolactone (PCL) is bioresorbable and biocompatible, and has been considered as a wound dressing material since in the 1970s. Extensive research had been conducted on its biocompatibility and efficacy, in vitro and in vivo, resulting in a number of medical and drug delivery
devices that are composed of PCL with FDA approval. At present, PCL is being regarded as a soft and hard tissue compatible bioreabsorbable material (Yoshimoto et al., 2003).

Nanofibers based drug delivery systems have improved therapeutic efficacy, reduced toxicity and also have controlled delivery rate. Various biodegradable and biocompatible polymeric materials have been electrospun into nanoscale fibers and have been demonstrated for their potential as effective drug carriers. Also, the production and designing of effective antimicrobial materials has become a highly desired objective in maintaining primary health care. In recent years, silver nanoparticles (AgNPs) have been widely used in biomedical research. The unique biological properties of AgNPs such as biocompatibility and antibacterial affinity have led to its wider application in healthcare products and medical applications namely implants, catheters and wound dressing materials. In this context, AgNPs incorporated electrospun PCL membrane with potential broad spectrum of antibacterial property can be used as effective drug delivery system and in developing wound dressings.

The present study is designed to identify bacterial isolates with the potential to synthesize silver and gold nanoparticles. The nanoparticles fabricated by bacteria were further explored for generation of nanoantibiotic combinations by conjugating this with known antimicrobial agents, for surface engineering application on medical device, and also to develop nanoparticles based electrospun nanocomposite to resist wound associated infection against Coagulase Negative Staphylococci (CoNS).
1.11. **Objectives of the study**

The specific objectives include

1. Isolation, screening and identification of bacteria with the potential to synthesize silver and gold nanoparticles
2. Optimization, purification and characterization of silver and gold nanoparticles
3. Antibacterial activity and mechanism of action of silver and gold nanoparticles
4. Effect of silver and gold nanoparticle based antibiotic conjugates on Coagulase Negative Staphylococci causing device related infections
5. Effect of silver nanoparticle fabricated medical device on colonization of Coagulase Negative Staphylococci (CoNS)
6. Development of electrospun polycaprolactone membrane incorporated with biosynthesized silver nanoparticles as effective wound dressing biomaterial against Coagulase Negative Staphylococci