Chapter 1

General Introduction

1.1 Global scenario of Antimicrobial Drug resistance

The evolution of multi-drug resistance (MDR) in pathogenic microorganisms, especially fungi and bacteria, has led to the clinical challenge of unmanageable infections **(Ventola 2015 and Michael et al., 2014).** Multidrug-resistant (MDR) microorganisms, particularly bacteria, pose a global health challenge, with dramatic increases in morbidity and mortality rates among infected individuals; MDR microorganisms are associated with significant clinical consequences in a broad group of patients, including those admitted to the ICU, those undergoing surgery, organ transplantation recipients, and cancer patients **(Michael et al. 2014 and CDC 2019).** Annually, the United States records at least 2.8 million annual cases of MDR bacterial or fungal infections; the deaths of more than 35,000 patients per year are attributed to these infections **(CDC 2021).** In addition, the global surveillance system of the World Health Organization highlighted antimicrobial drug resistance among microorganisms (AMR) as a global health crisis (**WHO 2017)**. The estimated average financial burden of treating an AMR infection is around US $ 50,000 per patient or around ~US $20 billion **(Naylor et. al., 2018).** Engineered (surface-modified or decorated) nanoparticles have the potential to counter the MDR challenge by serving as a clinical alternative for treating a broad spectrum of infections, particularly those caused by MDR microorganisms **(Makvandi et al., 2020).** Among various engineered nanoparticles used in antibacterial treatments, noble metal, and non-metallic nanoparticles have been widely studied as antibacterial agents because of their broad-spectrum antimicrobial properties, effectiveness, and enhanced efficacy **(Makabenta et al. 2021).** This review considers the current insight into the antimicrobial mechanism of the metal and metal-

based engineered nanoparticles against planktonic and biofilm inhabitant multi-drug resistant bacteria.

1.2 **The root cause of the evolution of antibiotic resistance among microbes**

Over the last few decades, antibiotics have saved millions of lives from potentially deadly microbial infections. Unfortunately, the clinical treatment of microbial infections has become challenging due to the evolution of multi-drug resistance in many human pathogens. The fundamental reason behind the evolution of antibiotic resistance is the excess application of antibiotics, both in the healthcare and farming sectors **(Figure 1.1).** Based on their drug resistance magnitude and clinical significance, the "ESKAPE" pathogens have received attention from the research community and clinicians in terms of the development of new antimicrobials **(Cassini et al. 2019, Gajdács et al. 2019 a & b, Sheu et al. 2019 and Ahmed et al. 2018).** Planktonic bacteria may acquire resistance genes from similar organisms; the magnitude of resistance relies on the microbial species and the type of acquired genes (**Figure 1.2 A) (Martinez 2014).** Further, other environmental substances, such as heavy metals may trigger drug resistance **(Romero et al., 2017)**.

Bacteria also can develop biofilms that physically and biologically prevent the host immune cells and antibiotics from eliminating the pathogens. Moreover, biofilms also protect persister cells that tolerate antibiotics, which complicates the treatment of infections **(Mulani et al. 2019).** When bacteria reside in biofilms, biofilm-associated resistance becomes a clinical challenge, which requires physical removal through aggressive debridement or high doses of antibiotics (Wu et al. 2019). These strategies are associated with long-term and expensive treatments and an increased probability of undesirable side effects and adverse outcomes.

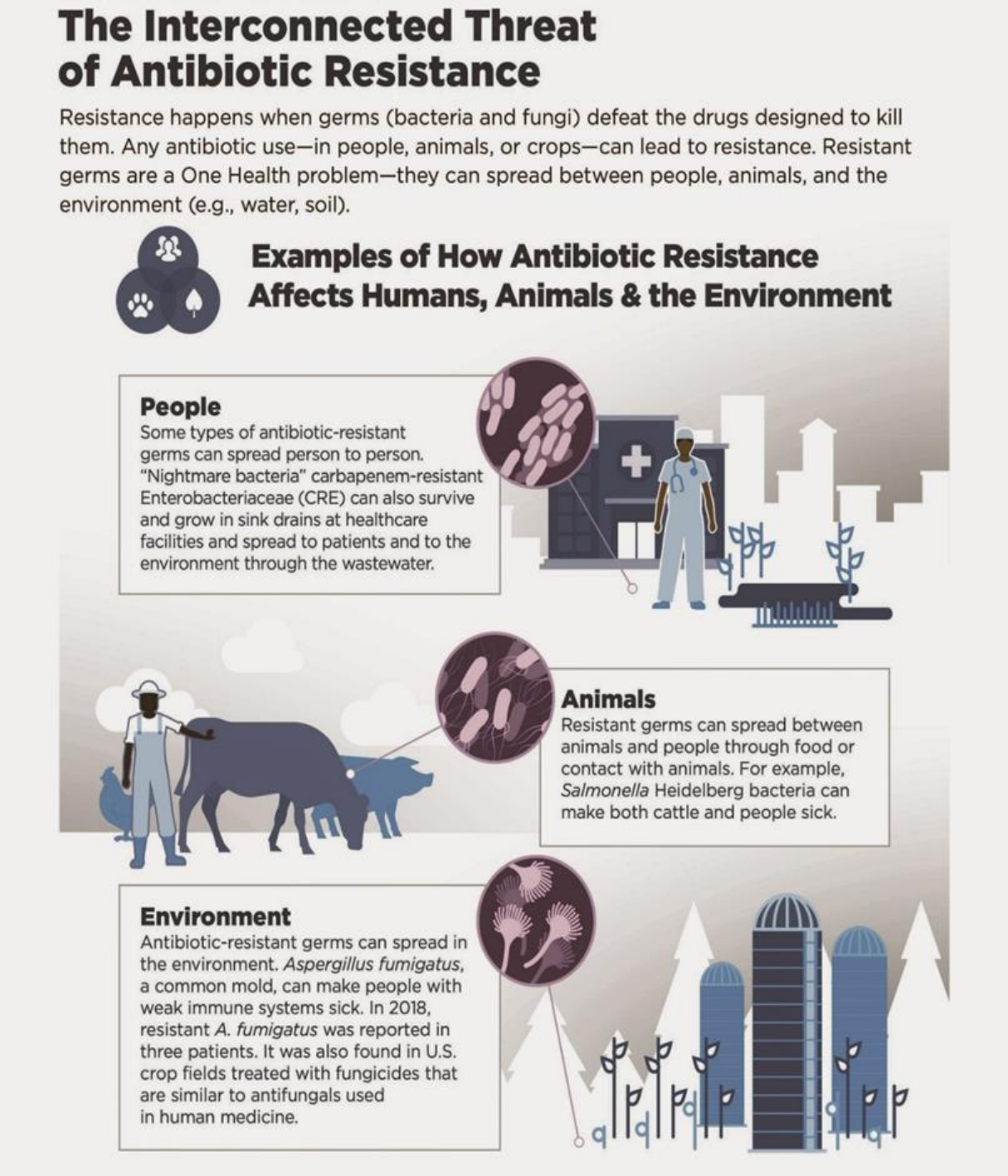

Figure 1.1 Infographic of the interconnected threat of antibiotic resistance to a different domain of life. Reproduced with permission from CDC under Common Creative attribution license.

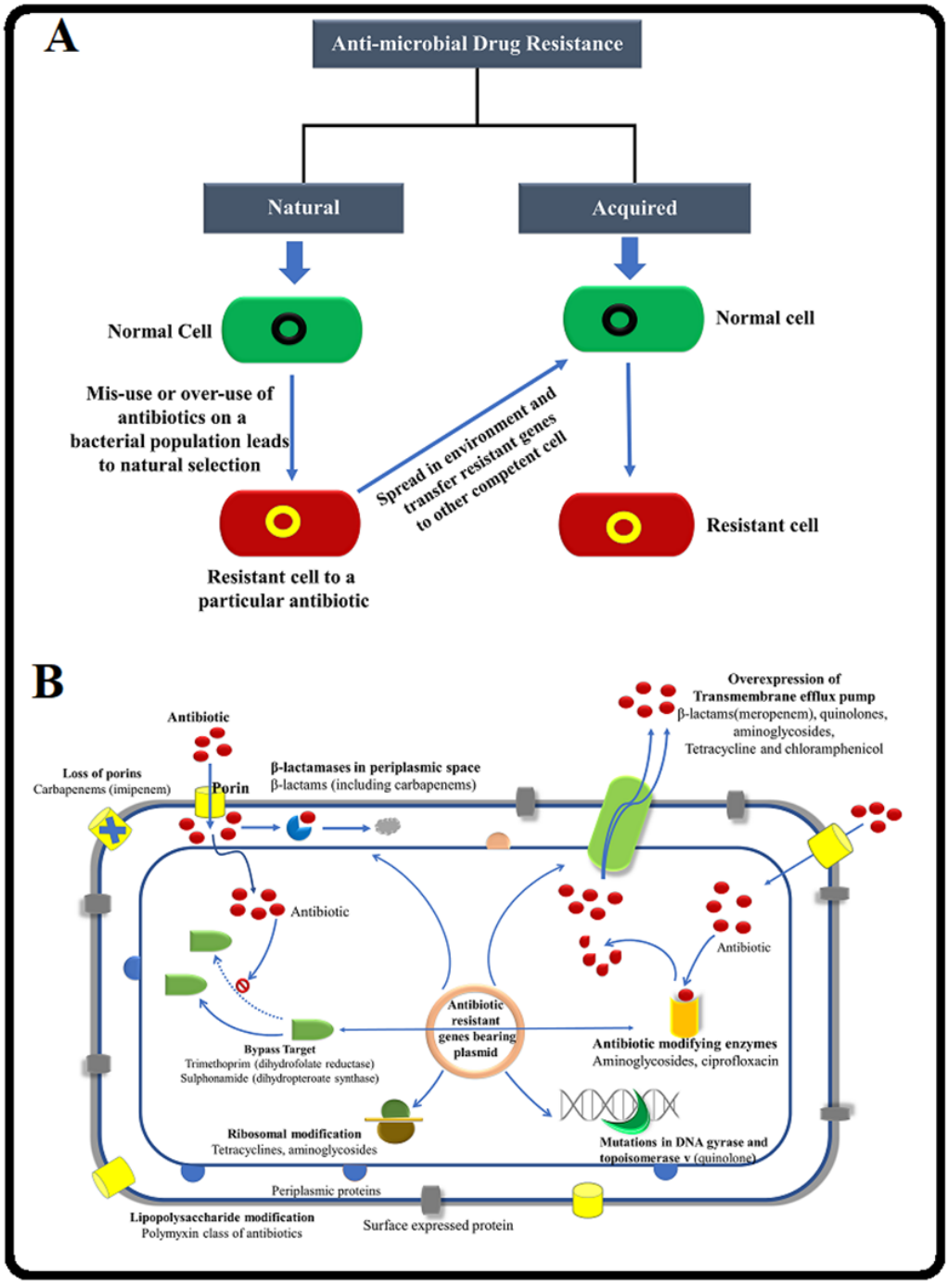

Figure 1.2 A) Bacteria may become antibiotic resistance genes naturally or from the environment. **(B)** These drug-resistance molecular mechanisms include: i) active efflux of antibiotics outside of the cellular environment through overexpression of efflux pumps, ii) utilization of alternative metabolic pathways to those choked by the drug, iii) decreased permeability of the bacterial cell wall, which blocks the access of antimicrobial agents to target

sites, iv) enzymatic cleavage of the antibiotics, v) enzymatic functional group modification of the antibiotic, vi) modification of antibiotic binding targets, vii) overproduction of the target enzyme to increase the active site competition, and viii) horizontal transfer of resistance genes via quorum sensing signaling within other biofilm-residing bacteria.

1.3 Synthesis and physicochemical properties of metal nanoparticles

Chemically, the metals used for nanomaterial synthesis are commonly heavy metals, having a density of greater than 5 g/cm^3. These metals are categorized as transition metals whose d orbital is electron deficient; this is an important property because an electron-deficient metal is chemically more redox-active, facilitating nanoparticle nucleation and maturation (Mukha et al. 2013; Jeevanandam et al. 2018). A "bottom-up" method of synthesizing these nanoparticles is called chemical reduction, which requires respective metal cations and a potent reducing agent (Figure 1.3). The reducing agent can be sodium borohydride, 3-GPTMS, formaldehyde, cyclohexanone, as well as the plant- and microorganism-based biological extracts (Pandey et al. 2020; Khan et al., 2022; Chauhan et. al., 2022; Prema et. al., 2022; Habibullah et al., 2021). The chemical reduction process reduces the salt cation to an electronically neutral state, establishing a nucleation site for the aggregation of metal atoms and starting nanoparticle maturation (Tiwari et al., 2020 and Pandey et al., 2020; Habibullah et al., 2021). Inorganic nanoparticles can tolerate harsh physiological conditions and have been considered efficient drug delivery vehicles for medical use**.** The antimicrobial properties of metal nanoparticles depend on their engineered physicochemical properties such as shape, size, surface charge (ζ-potential), ligand capping, doping, pH stability, roughness, and crystal structure (Zazo et al., 2016).

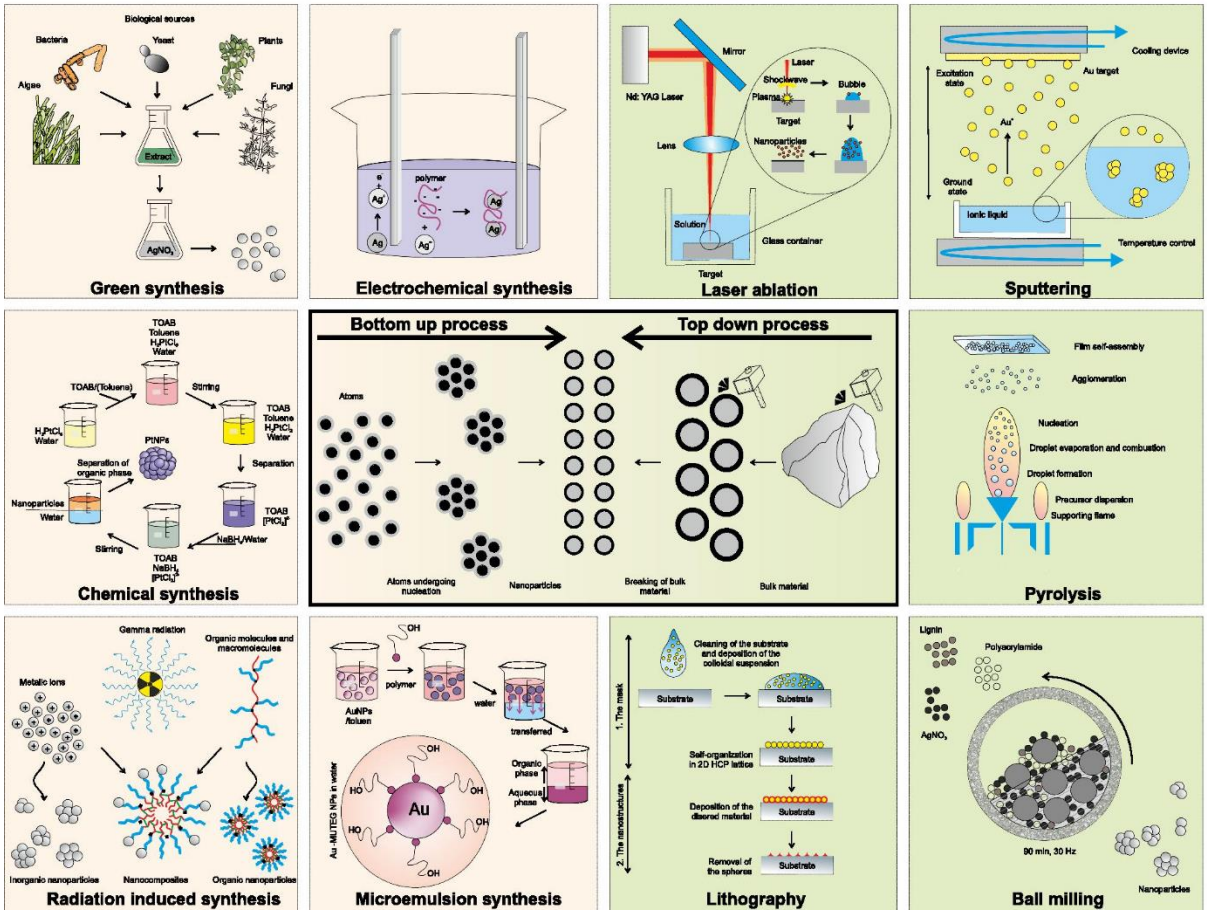

Figure 1.3 Schematic representation of the top-down (images with the green background) and bottom-up (images with pale yellow background) approaches of nanoparticle synthesis, the image was adapted from Habibullah et al., 2021.

1.4 **Antimicrobial mechanism of metal nanoparticles: A detailed account**

Metallic vessels and other products have been applied for many centuries because of their antimicrobial properties. For example, pots made from copper and silver were used for water decontamination and food preservation in Persia (Alexander 2009). North Americans and the Japanese used silver coins for the disinfection of water as well as the preservation of milk, wine, and vinegar to prevent diarrhea and dysentery (Alexander 2009, Borkow and Gabbay 2009)**.** Antimicrobial metallic substances have also been employed in agriculture. For example, the Bordeaux mixture, a suspension of copper sulfate and calcium hydroxide has been used to manage downy mildew disease of grapes, potato blight, peach leaf curl, and apple

scab (Ayres 2004; Dixon 2004). In developing countries, copper salts remain in use in the agricultural sector; they are used in Europe and North America to a lesser extent for tomatoes and other crops. Antimicrobial elements have also been widely used in history for medicinal purposes. For example, Ag is used in sutures for vaginal tears after childbirth, silver cations are used to manage gonorrhoeal eye infections in newborns, and silver foil wrapping is used in surgical wounds to prevent infection (Silver et al. 2006). Te, Mg, and their oxides and Cu and Hg salts have been utilized to manage infections such as tuberculosis, leprosy, gonorrhea, and syphilis over several centuries (Frazer and Edin 1931).

Antibiotic therapeutics are typically used against bacterial infections caused by planktonic communities, which are prone to form biofilms or detach from the biofilm microenvironment; however, antibiotic therapeutics are ineffective in reducing or eradicating established biofilms. The reduced ability of an antibacterial agent to enter and destabilize the biofilm niche contributes to the ability of the biofilm to acquire antibiotic resistance (Ikuma et al., 2015). Metal nanomaterials have an advantage over antibiotics because of their high relative surface areas and nonspecific cell interactions. Small-sized nanoparticles can easily penetrate and destabilize the biofilm niche of a microorganism. For example, silver nanoparticles below six nm can penetrate and destabilize the biofilm architecture (Zhang et al. 2014). Mechanistically, the interaction between bacteria and nanoparticles at the nano-bio interface causes down expression of capsule-forming exo-polysaccharide and membrane-associated proteins, leading to reduced bacterial biomass and irreversible inhibition of the cell viability. For example, Ag nanoparticles immobilized polymer coatings restrict biofilm formation on human dentin (Besinis et al. 2014). Similarly, TiO2 nanoparticles have shown hydroxyl group binding potential in the extracellular polysaccharide matrix of *in vitro-developed* biofilms (Sahle-Demessie and Tadesse 2011).

The antimicrobial mechanism of metal nanoparticles can be generally assigned to at least four pathways: (i) stress or nanoparticle-induced reactive oxygen species (ROS) generation, (ii) metallic cation release and binding with enzymes, (iii) accumulation of nanoparticles in the immediate environment of microbes, and (iv) biomolecule damage by nanoparticle internalization (Djuriši'c et al., 2015) The different modes of antibacterial activity of nanoparticles are illustrated in (Figure 1.4).

1.4.1 Mechanism of cell membrane destruction and effect on membrane potential

The bacterial cell envelope actively inhibits the entry of antimicrobial agents into the cellular environment and acts as a physical barrier. A Gram-positive bacterial cell wall contains teichoic acids. In contrast to Gram-positive bacteria, Gram-negative exhibit an outer covering containing lipopolysaccharide. The phosphate groups linked to these molecules make bacterial surfaces highly negatively charged. This highly negatively charged interface repels the entry of hydrophobic antimicrobial agents across cell membranes **(**Muzammil et al. 2018; Gupta et al. 2019). Compared to the eukaryotic cell potential, bacterial cell surfaces are more negatively charged, resulting in strong electrostatic interactions with cationic nanomaterials (Matsuzaki 2009). The controlled alterations of nanoparticles' surface charge density and hydrophobicity are crucial parameters for selectively disrupting the bacterial membrane architecture (Palermo and Kuroda 2010; El Badawy et al. 2011; Romero-Urbina 2015).

The chemical functionalization of nanoparticles' surfaces plays a fundamental role during interactions at the nano-bio interface (Fleischer and Payne 2012; Albanese et al. 2012; Cho et al. 2009; Setyawati et al. 2016). Cationic nanoparticles with a balanced amphiphilicity can exert potent antimicrobial effects with lower hemolysis and cytotoxicity levels (Palermo and Kuroda 2010).

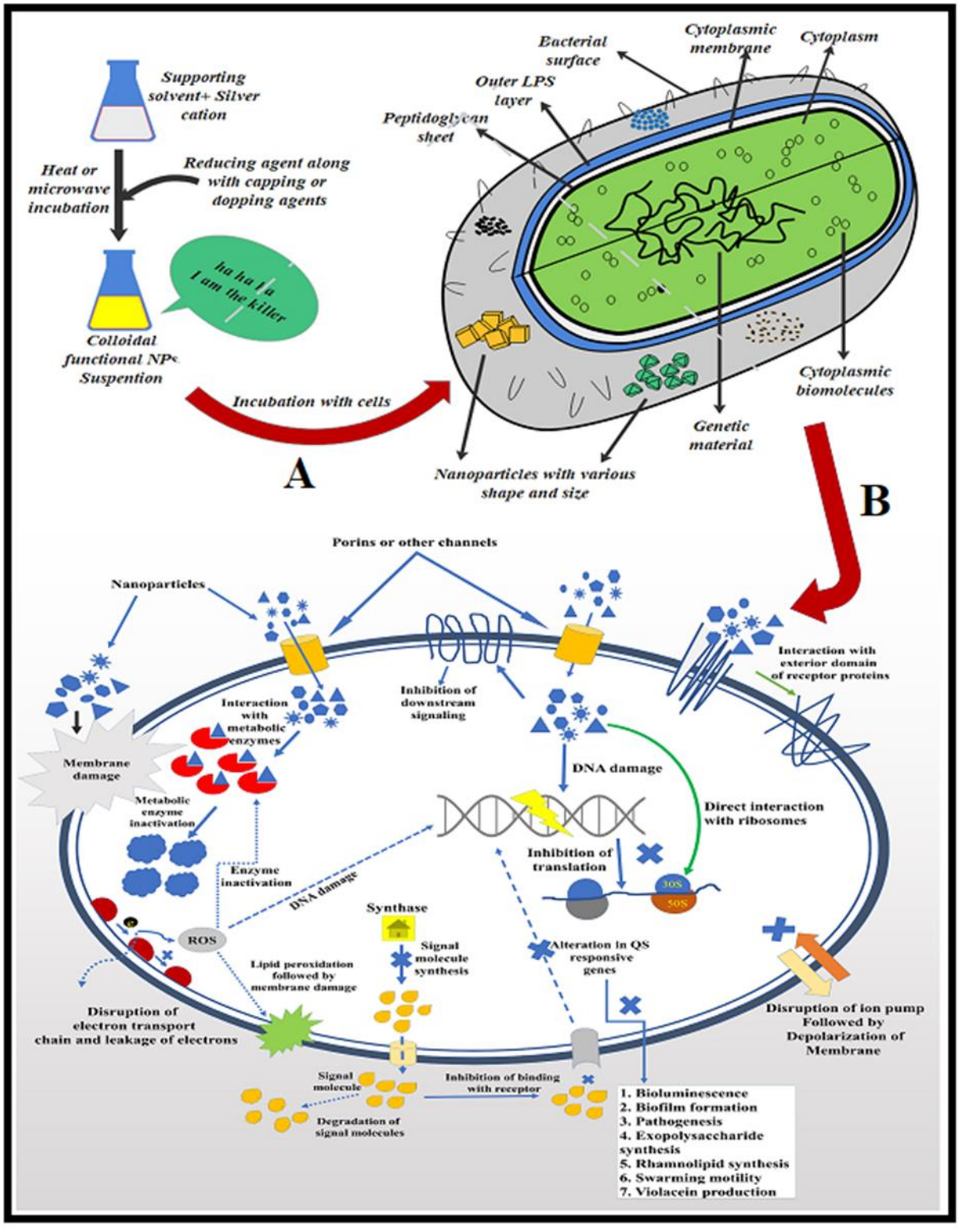

Figure 1.4 A) The nano-bio interface and **(B)** different mechanisms or modes of action of metal nanoparticles against multidrug-resistant microorganisms.

A well-defined range of engineered metal and metal oxide nanomaterial-based antimicrobial agents exhibit selective targeting of the negatively charged surface of microbes **(Table 1).** It is now well established that metal nanoparticles exhibiting cationic functional groups act more on bacterial cells than those exhibiting neutral or negatively charged groups (Goodman et al., 2004; Arvizo et al. 2010; Fleischer and Payne 2014; Giljohann et. al., 2007). At the same time, cationic nanoparticles induce cytotoxicity in various organisms. A molecular-level interaction study, which involved five different cationic molecule-functionalized diamond nanoparticles with different molecular structures and conformations, demonstrated that spatial charge distribution plays a crucial role in controlling the interaction with the bacterial cell membrane (Zhang et al. 2020).

Several studies suggest that cationic nanomaterials significantly influence lipid membranes via electrostatic interactions; however, other studies indicate that the opposite phenomenon occurs (Liu and Liu 2020). Amphiphilic molecules can induce membrane leakage, generating pores or dissolving membrane lipids (London and Brown 2000). Several cationic nanomaterials, including peptides, polymers, dendrimers, and inorganic nanoparticles, have been studied (Leroueil et al. 2008; Hong et al. 2004; Mecke et al. 2005; Leroueil et al. 2006). Studies on synthetic liposomes loaded with dye demonstrated that cationic nanoparticles induce more rapid membrane leakage than anionic materials (Moghadam 2012; Lai et al. 2017). A thermodynamic model has considered the molecular interactions between functionalized nanoparticles and cell membranes; it indicates that both anionic and cationic nanoparticles can effectively interact with lipid membranes (Ginzburg and Balijepalli 2007). However, it is unclear why cationic nanoparticles are more effective in inducing membrane leakage. It is also reported that many nanoparticles act to stabilize the lipid membrane and prevent leakage (Zhang and Granick 2006). For example, latex beads decreased phosphatidylcholine membrane leakage by inducing local gelation (Wang et al. 2008). However, it was observed that when

the nanoparticle-membrane interaction was too strong, a transient leakage took place (Wang and Liu 2016; Wang and Curry 2015). In addition, the capping of different spacer agents can cause electrostatic interactions to segregate the nanoparticle core from the membrane surface (Liu et al. 2018; Yu et al. 2007). In another study Tiwari et al. reported that Polyethyleneimine-capped cationic silver nanoparticles (three sizes of Ag nanoparticles as a function of molecular weight of Polyethyleneimine) were shown to strongly interact with surface-anchored or membrane-expressed proteins of *A. baumannii* cells (Tiwari et al. 2020; Tiwari et al. 2021); the denaturation of the protein quaternary structure, exposing the core tyrosine residue, was confirmed by 2D and 3D fluorescence spectroscopy (Tiwari et al. 2020; Tiwari et al. 2021). However, a detailed molecular mechanism associated with this activity has not been elucidated. Some transient metal nanomaterials can interfere with membranous redox potential, including iron oxide. The impact of these NPs could be on redox potential, proton flux, and membrane-associated respiratory enzyme inactivity in bacteria (Chen et al. 2020; Miyazawa et al. 2018; Nie et al. 2020) (Figure 1.5). For example, Fe_3O_4 nanoparticles repressed energy-dependent proton efflux in K-12 and kanamycin-resistant pARG-25 strains of *E. coli* by ~ 3.0 and ~ 1.3-fold, respectively, compared to Ag nanoparticles (Miyazawa et al.; 2018; Nie et al. 2020). Another study has shown that nanoporous gold (NPG) negatively hyperpolarizes the membrane of *E. coli* cells. The hyperpolarized membrane leads to structural changes in the ion channels (Miyazawa et al.; 2018). Thus, the electrostatic interaction of the bacterial cell with nanoparticles may facilitate enhanced antibacterial efficiency. However, the electrostatic environment at the nanoparticle-cell interface is unclear; it is necessary to understand how a particularly charged nanoparticle interacts with a cell membrane.

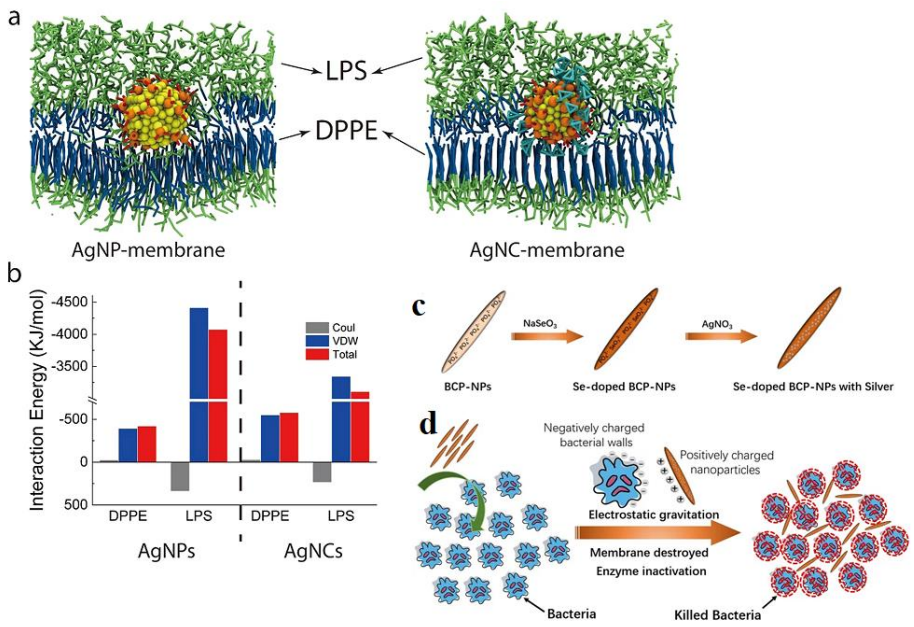

Figure 1.5 Interfacial interaction model of the Ag-NPs, Ag-NCs, and the bacterial cell membrane. The head groups of the Lipopolysaccharides and DPPE are represented as green sticks; the tails of the Lipopolysaccharides and DPPE are represented as blue sticks. The carboxyl groups and adamantanes are represented as red and cyan sticks. The core Ag and S atoms are represented as yellow and orange beads. (**b)** Showing the interaction energy between Ag-NPs/Ag-NCs and the LPS/DPPE molecules. The negative value represents the attraction, and the positive value indicates the repulsion. Coulomb shows electrostatic interaction however, VDW represents van der Waals interaction. Adopted with permission (Chen et al. 2020); (**c)** showing steps of the development of selenium-doped biphasic calcium phosphate nanoparticles with the doping of silver nanoparticles (Ag-SeB-NPs). **(d)** Representing the antibacterial mechanism of Ag-SeB-NPs. Adopted with permission (Nie et al. 2020).

1.4.2 Mechanism of nanoparticle-induced ROS generation and cellular damage

ROS generated as by-products of cellular oxidative metabolic processes has a disastrous effect on fundamental cellular physiology such as cell differentiation, signaling, and survival (Lemire et al. 2013). The accumulation of excessive ROS in the cellular environment causes cell death

due to oxidative stress. ROS can destroy intracellular components through different pathways; superoxide and hydroxyl radicals can react with the thiol group in proteins, enzymes, and membrane receptors, leading to permanent inactivation (Memar et al. 2018). Miller et al. noted that nanoparticles could induce ROS generation through i) direct nanoparticle-induced conversion or leached ions, ii) via respiratory enzymes, and iii) via oxidation of redox-active NADPH oxidases (Miller et al. 2015).

To understand metal-catalyzed oxidation in the cellular environment, we should focus on the role of Fe. During aerobic respiration, the generation of partially reduced H_2O_2 and $O^{2}\bullet-$ can initiate Fe-catalysed autoxidation (Valko et al. 2005). This Fe-catalysed autoxidation is leaden by the Fenton reaction (Valko et al. 2005; Stohs and Bagchi 1995). Essentially, Fe enhances oxygen toxicity by transferring electrons from a donor biomolecule to H_2O_2 (Faulkner and Helmann 2011). Several mechanisms have previously been proposed for intracellular ROS generation. Except for Fe, some other redox-active metals, such as Cu, Cr, Co, and V, can catalyze the Fenton reaction *in vitro* (Valko et al. 2005; Stohs and Bagchi 1995; Strlič et al. 2003). It has also been reported that Cu can catalyze hydroxyl radical formation under an *in vivo* environment (Macomber et al. 2007). However, the *in vivo* monitoring of the Fenton reaction is challenging because of several associated factors such as pH, coordinating ligands, and relative reduction of potential transition metals.

There is a considerable challenge in detecting and identifying metal-induced generation of short-lived reactive oxygen species. Several studies have reported that higher doses of particular metal ions such as chromium (VI), arsenic (III), iron (II), and copper (II) induced a lethal concentration of intracellular ROS (Warnes et al. 2012). For example, adding exogenous hydrogen peroxide in *E. coli* culture-induced superoxide production ($O2\bullet-$) leads to DNA damage and the inhibition of indispensable enzyme activities (Imlay 2003). Another study showed that the release of Cu^{+} from copper iodide (CuI) nanoparticles resulted in ROS

generation followed by damage to DNA and intracellular vital proteins and enzymes in *E. coli* and *B. subtilis* **(**Pramanik et al. 2012). Similarly, silver–zinc oxide nanocomposites release Ag^{+} and Zn^{+} that exhibited biocidal activity against *S. aureus* and MDR *E. coli* through ROS production (Matai et al. 2014). AuNPs have shown oxidase-peroxidase mimicking behavior; further, enhanced catalytic activity can be obtained by immobilizing the Au nanoparticles in mesoporous silica (Lopez and Nørskov 2002; Bernardos et al. 2019). These Au nanoparticles adsorb on the surface of bi-functionalized mesoporous silica nanoparticles (MSNPs). These adsorb AuNPs have shown bactericidal effects against Gram-positive and Gram-negative bacteria through ROS production (Tao et al. 2015). Copper nanoparticles immobilized in a mussel-inspired dendritic polyglycerol (MI-dPG) coating on medical implants were shown to exhibit antimicrobial activity (Li et al. 2017). These MI-dPG mimic foot protein-5 and adsorb tightly to any surface through catechol anchoring and crosslinking (Figure 1.6 a, b). The copper nanoparticles can be immobilized within the MI-dPG coatings by in situ reduction during the coating process or reduction of $CuCl_2$ solution after the formation of the coating (Figure 1.6 c). This coating has shown enhanced antibacterial activity against *E. coli* and *S. aureus* through an "attract-kill-release" strategy via the release of Cu^{+} and ROS production (Figure 1.6 d) (Li et al. 2017)

1.4.3 Inactivation of cellular proteins and enzymes

Proteins (polymers of amino acids) are important macromolecules in living organisms. Changes to the structure (tertiary and quaternary folding) and function can lead to cell death. Several reports indicated that metal nanoparticles, especially silver and gold, directly interact with cellular proteins and inactivate them. Studies involving *E. coli* indicate that only a few amino acid residues adjacent to metal-binding sites in proteins are prone to metal-catalyzed oxidation (Stadtman and Levine 2003; Stadtman 1993). Metal-catalyzed oxidation of side chains of histidine, arginine, lysine, and proline leads to the formation of carbonyl derivatives.

Increased carbonyl products can be used as markers for oxidative protein damage (Stadtman and Levine 2003). Furthermore, the metal-catalyzed oxidation of amino acid side chains affects enzymatic activity and initiates a thermodynamically favorable protein degradation (Stadtman 1993). In principle, metal-catalyzed site-specific oxidation of cellular enzymes is responsible for metal toxicity and cell death.

Many studies have reported that bacterial Fe–S dehydratases are specifically vulnerable to site-specific inactivation by toxic metals (Xu and Imlay 2012). For example, metals such as Ag (I), Hg (II), Cd (II), and Zn (II) damage the Fe–S dehydratases *in vitro* and *in vivo* independently of ROS-initiated bacteriostatic (Xu and Imlay 2012). In addition to the inactivation of the enzymes containing the Fe–S cluster, another mechanism is ionic mimicry.

The active site metal atom is displaced with metal, leading to site-specific enzyme inhibition. For example, the active site of δ-aminolaevulinic acid dehydratase (ALAD) contains Zn (II); the displacement of Zn (II) from Pb (II) leads to potent inhibition of enzyme activity and antimicrobial toxicity (Erskine et al. 1997; Ogunseitan et al. 2000). Similarly, incubation of *S. cerevisiae* with $AgNO_3$ results in the displacement of Cu(I) and Zn from Ag (I) at the Cu (I)-active binding site of Cu and Zn superoxide dismutase. However, this metal displacement does not affect the growth kinetics of *S. cerevisiae* (Ciriolo et al. 1994).

Some metal nanomaterials, notably Ag, negatively influence the bacterial electron transport chain (Bragg and Rainnie 1974). For example, membrane-anchored NADH: quinone oxidoreductase (NQR) is a respiratory chain component in some bacteria that generates a redox-driven transmembrane Na+ potential by translocating Na^+. For example, Ag (I) inhibits the activity of NQR in *Vibrio harveyi*; some reports suggest Ag (I) also dissipates the chemiosmosis potential of the membrane through proton leakage in an NQR-independent mechanism (Fadeeva et al. 2011; Dibrov et al. 2002).

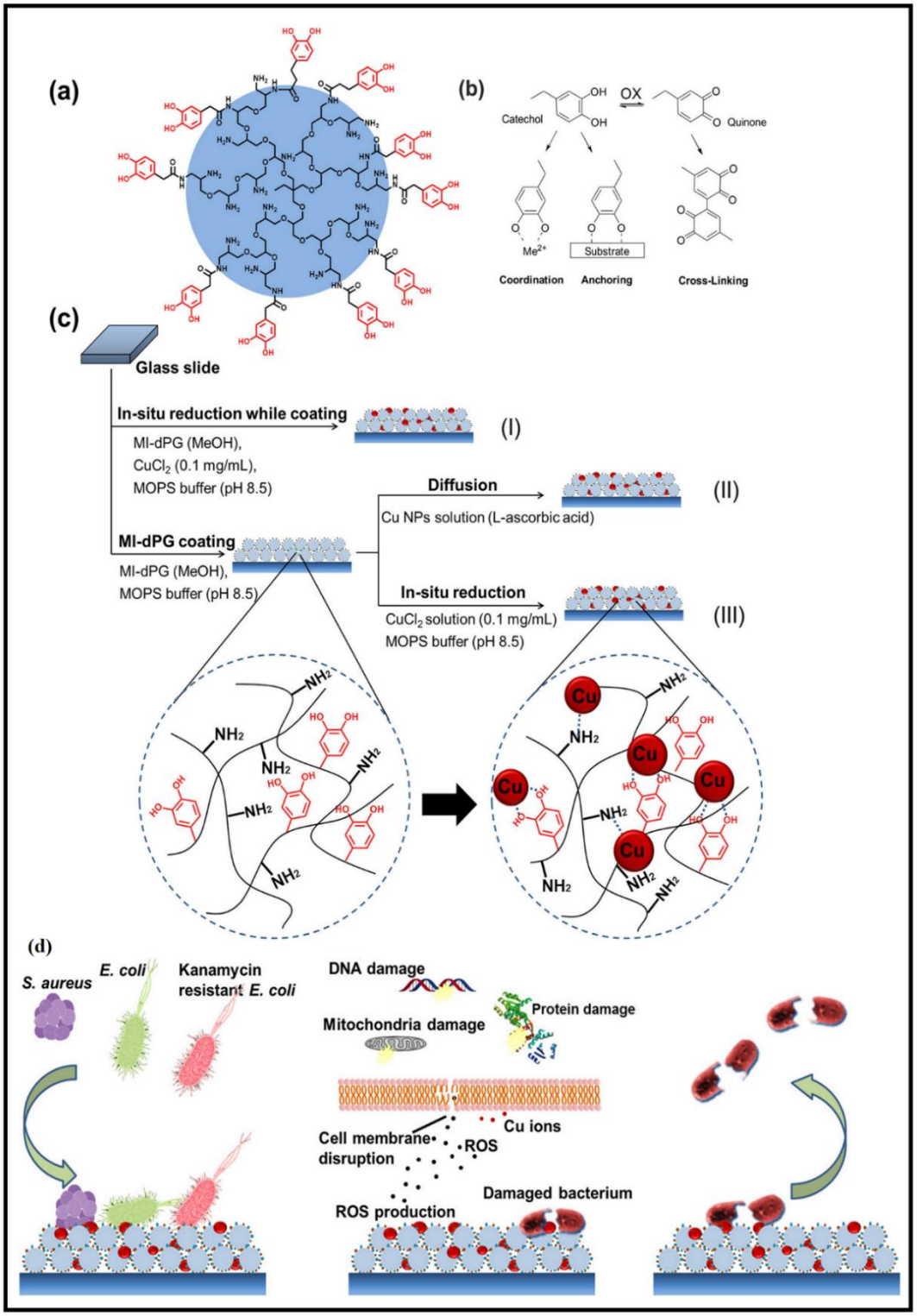

Figure 1.6 In situ synthesis of copper nanoparticles incorporated functional coating on mussel-inspired dendritic polyglycerol (MI-dPG) has shown long stability and broad-spectrum antimicrobial activity. a) Chemical structure of mussel-inspired dendritic polyglycerol (MI-

dPG); b) Mechanism of catechol anchoring and cross-linking. c) Preparation of Cu nanoparticle-incorporated MI-dPG surface coatings using three different methods. MOPS: 3-(N-morpholino)-propane sulfonic acid. d) Schematic contact killing of bacteria on a Cu nanoparticle-incorporated MI-dPG surface coating via an "attract-kill-release" mechanism. Reproduced with permission (Li et al. 2017) American Chemical Society.

1.4.4 Molecular mechanism of DNA damage by metal nanoparticles.

Several studies reported that metal nanoparticles could serve as antibiotic agents due to their oxidative stress induction, metal ion release, and non-oxidative activities. Due to these multiple mechanisms, nanoparticles effectively regulate the gene mutation of bacterial cells (Wang et al. 2017). Reactive oxygen species (ROS) generation has been associated with the antibacterial behavior of ZnO and Ag nanoparticles. In addition, the electrostatic charge of the nanoparticles can impart antibacterial activity. It has been noted that positively charged nanoparticles generate greater amounts of ROS, affecting ubiquinone biosynthesis-related gene mutation (Padmavathy and Vijayaraghavan 2008; Ivask et al. 2012). Positively charged Ag-Polyethyleneimine (PEI) nanoparticles show more bactericidal behavior than negatively charged Citrate-Ag nanoparticles (Ivask et al. 2014). For Al_2O_3 and TiO_2 nanoparticles incubated with *E. coli* cells, the concentration of nanoparticles and the ion release mechanism were indicated as dominant factors that impart antibacterial activity (Wang et al. 2014; Morones et al. 2006; McQuillan et al. 2012; Nezhad et al. 2014; Tamayo et al. 2014). Metals ions interact with bacterial cell walls and continuously release ions, causing cell toxicity and death. Several studies reported DNA damage in *E. coli* and *S. aureus* species due to Ag nanoparticles, including nuclear fragmentation or physical attachment nanoparticles to the DNA because of the high affinity of Ag^+ to phosphates that are abundant in the DNA molecule (Kumar et al. 2011; Choi, and Hu 2008; Jahnke et al. 2016; Holt and Bard 2005). It has been noted that Ag^+ binds to the bacterial DNA and restricts cell proliferation. This phenomenon is

attributed to the detection by the bacterium of a disturbance in the cell membrane; this action causes the DNA to condense, which protects it from potential incoming damage (Feng et al. 2000).

An alteration in the genomic and proteomic profiles due to exposure to Ag nanoparticles has also been reported in *E. coli*; upregulation of 161 genes and downregulation of 27 genes were observed when bacterial cells were exposed to Ag nanoparticles and Ag^+. In addition, 309 and 70 genes were exclusively regulated by Ag nanoparticles and Ag^+, respectively (McQuillan and Shaw 2014). Furthermore, it has been reported that Ag nanoparticles exposed *E. coli* show upregulation of many genes, which are associated with functions such as the citric acid cycle (sdhC), membrane structure and biofilm formation (bolA), cellular transport (mdfA), electron transfer (sdhC), protein efflux (fsr, yajR, emrE), and DNA repair (recN, uvrA, ybfE, yebG, ssb, sbmc, and nfo) (Gou et al. 2010). The Ag^+ in Ag nanoparticles affects the ATP production/recycling for *E. coli* cells by inactivating the expression of ribosomal subunit proteins and other cellular proteins and enzymes essential to ATP production (Yamanaka et al. 2005). In addition, Ag^+ reduces S2 protein expression; the S2 protein is a subunit of the 30S ribosome that coordinates for proper base pairing between the codons and anticodons. Furthermore, it disturbs the function of the ribosome and leads to denaturation; in addition, it suppresses the expression of other proteins, such as succinyl-CoA synthetase, which is necessary for the catalysis of intracellular ATP production (Yamanaka et al. 2005). Another study showed that dnaX and holB genes involved in DNA replication were downregulated in *E. coli* bacteria that were exposed to TiO_2 nanoparticles **(**Sohm et al. 2015). TiO_2 nanoparticle exposure was associated with the downregulation of genes that regulate the induction of pyrimidines, purines, and gluta-redoxin are involved with DNA synthesis (Sohm et al. 2015).

Table 1. Metal nanoparticles and associated antimicrobial mechanisms**.**

Nature of Engineered Nanoparticles	Shape/size	Targeted bacteria	Elucidated mechanism	References
1. Cell wall/membrane damaging metal nanoparticles				
Cationic Ag Nanoparticles	≥1 nm	*S. aureus*	Increased cell membrane permeability and collapsed peptidoglycan layer	(El Badawy 2011)
Anionic silver nanoparticles	10.2±2.3 nm Spherical	*E. coli*	perturb the bacterial respiratory chain by binding with the bacterial membrane, thus raising intracellular ROS generation and the corresponding lipid peroxidation	(Long et. al., 2017)
Gold particles conjugated with anti-protein A antibodies	10-, 20-, and 40-nm spherical	*S. aureus*	localized killing of *S. aureus* in vitro	Zharov et. al., 2006
silver-carbon (Ag-C) nanocomposites	4.6 nm	*E. hirae, E. coli*	ruptured the bacterial cells directly	Sabavath et al., (2020)
Polymyxin B-capped silver Nanoparticles	~10-20 nm Spherical	*V. fluvialis*	Destabilization of cell membrane	(Limbadi et.al. 2015)
Glycol lipoprotein-capped silver Nanoparticle	~10 nm, Spherical	*V. cholerae*	Damage of the membrane which leads to intracellular ion efflux and membrane potential collapse	(Gahlawat et.al. 2016)
Squilla chitosan silver Nanoparticles	~6–9 nm	*S. aureus* and *P. aeruginosa*	Nanoparticles interacted electrostatically with the cell wall followed by pit hole formation	(El-Naggar et.al 2016)
Benign ε-polylysine/Ag nanoparticle nanocomposite	~7 nm, spherical	*S. aureus* and *P. aeruginosa*	Leads cell membrane damage	(Dai et.al. 2016)

Silver nanoparticles	~20 nm, Spherical	*B. pseudomallei*	Leads to cell membrane damage.	(Siritongsuk et.al. 2016)
Nano-silver	~55.2 nm	*S. aureus*	Damage of cell membrane.	(Anuj et.al. 2019)
Silver nanoparticles adsorbed on graphene oxide with tobramycin	~5 nm	*E. coli (BL21DE3)*	Damaging cell wall.	(Ullah et.al. 2018)
Silver nanoparticles	~60 nm, Triangular	*S. aureus* and *E. coli*	This leads to the Disruption of cell walls and collapsed membrane integrity	(Qiao et.al. 2018)
6-Amino-penicillanic acid-coated gold nanoparticles	~3 nm. Nanofibers	*E. coli, K. pneumoniae*	Disrupted cell membrane	Yang et.al. 2017
N-heterocyclic molecule-capped gold nanoparticles		*K. pneumonia* and *S. aureus*	Disrupted cell membrane	Feng et.al. 2016
Antibiotic-capped gold nanoparticles	~30 nm	*K. pneumonia, E. coli, S. aureus*	Intracellular accumulation via cell membrane penetration	Pradeepa et.al. 2016
Cationic gold nanoparticles	~2 nm	*E. coli, E. cloacae, P. aeruginosa, S. aureus*	Interacted with cell Membrane followed by surface aggregation Formation and cell lysis	Li et.al. 2014
Quercetin and acetylcholine linked Selenium nanoparticles	~ 23 nm	*S. aureus*	Compromised membrane integrity	Huang et.al 2016
Silver nanoparticles	~15 nm, Nanocrystal	*A. baumanii*	cell wall damage	Singh et.al. 2018
PEI functionalized silver nanoparticles	5-20 nm, Spherical	*A. baumanii and C. albicans*	Surface protein interaction and subsequent cell membrane damage	(Tiwari et. al., 2020; Tiwari et. al., 2023)
Functional alkoxysilane functionalized silver nanoparticles	5-10 nm Spherical	*A. baumanii and C. albicans*	Thin film-based solid-state interaction and damage of cell architecture.	(Tiwari et. al., 2022)

Bio-CuNPs	29.11–78.56 nm.	*Acidovorax citrulli*	inducing oxidative stress, biofilm inhibition, and cellular integrity disruption	Noman et. al., 2023
CuONPs	11–53.8	*K. oxytoca ATCC 51,983, E. coli ATCC 35,218, S. aureus ATCC 25,923, and B. cereus ATCC 11,778.*	higher CuO-NPs found inside bacterial cells, anti-biofilm ability	Shehabeldine et. al., 2023
2. Intracellular ROS-generating metal nanomaterials				
Glutathione-stabilized silver Nanoparticles	~50 nm	*Campylobacter Strains* (MDR)	Increasing ROS	Silvan et.al 2018
Ag nanoparticles	11 nm, spherical	*P. aeruginosa, S. aureus* (MDR)	ROS generation	Yuan et.al. 2017
Ag nanoparticles	10 nm	*P. aeruginosa* (MDR)	Inhibition of enzymatic systems in the respiratory chain and altering DNA synthesis	Salomoni et.al 2017
Ag–Ti nanoparticles	47 nm	*S. aureus*	ROS generation, glutathione reduction, increase membrane permeability, DNA damage	Korshed et.al. 2018
Cu impregnated mesoporous SiO2 nanoparticles with Curcumin	10 nm, photodynamic inactivation	*E. coli*	ROS production by curcumin, adhesion to cell membrane by electrostatic attraction	Kuthati et.al. 2017
Cu_7S4 Nanosheets with (304) and (224) exposed facets	NA	*B. subtilis, E. coli P. aeruginosa,*	synergetic photodynamic and photothermal therapy under near-infrared light (808 nm) irradiation	Mo et. Al., 2022
copper nanoparticles (CuNPs)	20 and 60 nm	*E. coli*	reactive oxygen species generation and genomic DNA damage	Lai et. al., 2022

copper nanoparticles (CuNPs)	44.6 ± 2.7 nm	*S. aureus, E. coli, P. chrysogenum and F. oxysporum*	NA	Alahdal et.al., 2023
Ultra-small Au NCs	~1 nm	*S. aureus, E. coli*	generate ROS to oxidize bacterial membranes and disturb bacterial normal metabolism	Zheng et. Al., 2021
Ag-coated Au nanorods	104.9 nm	*S. aureus*	ROS generation	Kim et.al. 2018
Concanavalin-A directed dextran capped Au nanoparticles with methylene blue	43.3 ± 3 nm, Hexagonal	*E. coli, K. pneumonia, E. cloacae*	ROS generation, disrupt cell membranes, DNA breakdown	Khan et.al. 2017
Metal nanoparticles with anti-QS activities				
Silver nanoparticle	~32 nm Spherical	*S. aureus*	Quorum quenching	Masurkar et.al. 2012
Silver nanoparticle	5-30 nm Spherical	*P. aeruginosa*	inhibition of virulence factors such as protease activity, elastase, pyocyanin, pyoverdine, pyochelin and rhamnolipid of *P. aeruginosa*	Singh et.al. 2015
Silver nanowires	3-4 μm in length	*P. aeruginosa NCIM 2948, C. violaceum ATCC 12472*	Inhibition of violacein production, inhibition of biofilm in *P. aeruginosa*	Wagh et.al. 2013
Honey polyphenol carrying selenium nanoparticle	~13.5 nm Spherical	*P. aeruginosa PAO1*	Inhibition of violacein production, inhibition of elastase, exo-protease, pyocyanin,	Prateeksha et.al.2017
ZnO nanoparticles			Inhibition of violacein production, inhibition of elastase, pyocyanin production and exopolysaccharide Production.	Al-Shabib et.al. 2016

β-cyclodextrin functionalized silicon dioxide nanoparticles		*V. fischeri*	Inhibition of bioluminescence of Vibrio fischeri, down-regulation of luxA and luxR gene of V. fischeri	Miller et.al. 2015
Silver coated CNTs		*S. aureus*	Down-regulation of *sdiA* (a quorum sensing gene) and many virulence genes (safC, ychP, sseA and sseG) of *S. aureus*	Chaudhari et.al. 2015
Ag-Ti nanocomposite		*C. violaceum*	Inhibition of violacein synthesis, inhibition of biofilm evolution and degradation of AHLs	Naik et.al. 2014
Ag-Cur nanocomposite		*P. aeruginosa and S. aureus*	Inhibited biofilm formation	Loo et.al. 2016
Fe/Ag-EPS NPs	5 -50 nm	*P. aeruginosa, S. aureus*	capability of Ag+ release and anti-biofilm agents	Cusimano et. al., 2020

1.5 Metal nanoparticle-mediated eradication of bacterial biofilms

The bacterial species residing in biofilms are challenging to eradicate and pose a severe threat to human health. As such, the prevention of biofilm establishment on tissues and implanted medical devices is an important research activity. Some of the chemical-based strategies being adopted to combat biofilm establishment include preventing bacterial accumulation on the substratum, the discovery and formulation of biofilm-disrupting compounds, and the destabilization of mature biofilms. Conventional antimicrobial agents are usually used to treat infections on the surfaces of medical devices. Antibiotic doses are administered based on standardized susceptibility tests on planktonic cells; these often fail to eradicate biofilms, leading to surgical removal of the implants. To inhibit bacterial adhesion on the medical device

and dismantle biofilms, alternative treatment approaches are needed. Metal nanoparticles have shown promising activity as anti-biofilm agents. However, a detailed investigation of the molecular mechanism of this anti-biofilm activity is lacking. The following sections consider the structural and functional dynamics of metal nanoparticle-mediated eradication strategies involving bacterial biofilms, including potential molecular mechanisms.

1.5.1 The biological dynamics of bacterial biofilms

Bacteria can survive through two different life strategies: (a) in a planktonic state and (b) in biofilm form. Biofilms are a self-induced aggregation of microorganisms that can be established on biotic (living) or abiotic (non-living) surfaces. The development of bacterial biofilms on the surface of medical devices such as vascular catheters, prosthetic joints, and cardiac pacemakers is a significant clinical challenge (Percival et al. 2015). Bacterial colonies in biofilms are submerged within a self-secreted polymeric substance (EPS) that contains DNA, complex polysaccharides, and functional proteins**.** Biofilm-submerged bacterial colonies are resistant to the host immune system and exogenous antibiotic therapy. Once a biofilm matures on the substratum, it can be challenging to destabilize the architecture with conventional therapeutic approaches. For such cases, invasive procedures such as removing the infected device are required (Costerton et al. 1999; Melchior et al. 2006; Vlastarakos et al. 2007; Kania et al. 2008). The biofilm development steps can be divided as; attachment of bacterial cells to the substratum, accumulation of surrounding cells in a community (maturation), and detachment (dispersal of cells), as shown in Figure 1.7. After implantation of a medical device, the host secreted extracellular proteins adhere to the implant surface. Such host-specific proteins act as a ligands that can be recognized by bacterial cells and establish interaction with bacterial surface receptor proteins, which leads to the colonization of cells on the surface. The biofilm development begins around bacterial cells by secretion of extracellular polymer

substances until a three-dimensional biofilm architecture is reached (Pinto et al. 2019). External environmental factors, along with host physiology, may stress the dispersal of biofilm inhabitant cells from the matrix, enabling colonization on other sites (Beitelshees et al. 2018).

The self-secreted extracellular polymeric matrix establishes an intercellular interaction among bacterial cells and protects cells from a hostile environment. Thus, the EPS matrix contributes to antimicrobial drug resistance in biofilms compared to planktonic cells. The biofilm EPS matrix formation is a dynamic process that costs high energy for bacteria and involves synthesis and secretion steps. In most bacterial biofilms, the EPS constitutes around 90% of biofilm biomass (Fulaz et al. 2019). Polysaccharides are the main constituents of the EPS matrix of a bacterial biofilm. Most of these carbohydrate molecules are hetero-polysaccharides, composed of a mixture of charged and neutral sugar residues. The chemical nature of these polyanionic and polycationic exo-polysaccharides depends on the bacterial species and strain. Structurally, exopolysaccharides are indispensable for biofilm development and serve as a protective barrier. In addition, these complex polysaccharide residues can hold water molecules within the biofilm to enable the movement of cells (Fulaz et al. 2019 and Flemming et al. 2016). As a result, biofilms are considered porous structures surrounded by water-filled voids.

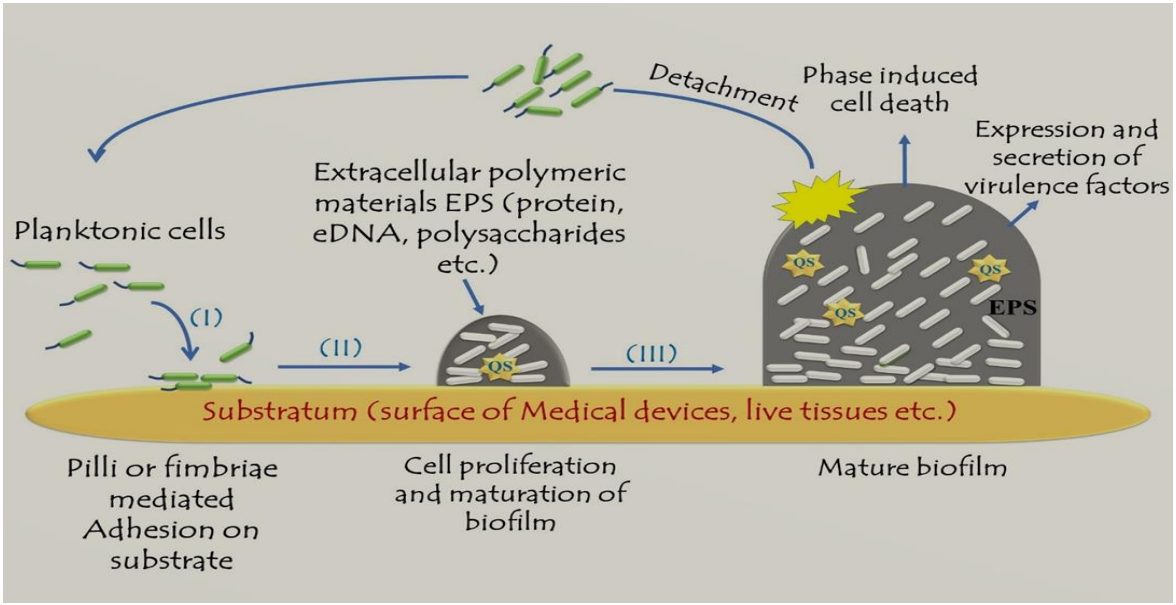

Figure 1.7 Representative structure of bacterial biofilm: Bacterial biofilm is formed due to complex coordinated interactions of microorganisms with the surface. The sequence of events of typical biofilm formation is as follows; (i) Initial surface attachment to the surface through movement appendages like fimbriae, through Pilli and it can be extracellular carbohydrate matrix, (ii) Formation of a monolayer, movement along the surface with formation of micro colonies and (iii) Biofilm maturation and formation of a three-dimensional structure. The whole event is coordinated by the Quorum sensing (QS) system. In this system, a signal molecule is secreted by a cell and responds against the type of signal molecule. The chemical nature of secreted molecules depends on the class of bacteria.

Extracellular matrix proteins and enzymes are important molecules for the biofilm. Most proteins provide physical stability to biofilm by connecting cells with other constituents (Fong and Yildiz 2015). EPS enzymes actively degrade matrix biopolymers (e.g., polysaccharides, proteins, and eDNA) and convert them to monomers within the biofilm, providing nutrients to bacterial cells and promoting biofilm reorganization (Fong and Yildiz 2015). In addition to protein and polysaccharides, extracellular DNA also plays a fundamental role in stabilizing biofilm (Fulaz et al. 2019). The structural role of extracellular DNA in biofilm differs significantly among species. For instance, extracellular DNA plays a key structural role in *Pseudomonas aeruginosa* biofilms; however, it is less significant for *S. epidermidis* biofilms. In addition, to providing strength to biofilm structure, eDNA also facilitates the transfer of genetic information between bacterial cells.

The complex architecture of the biofilm limits the penetration of many antimicrobial drugs in the biofilm core and acts as a diffusion barrier (Srivastava and Bhargava 2016). Biocidal drugs get inactivated by interacting with EPS components resulting in decreased activity and efficacy due to enzymatic degradation and complex formation associated**.** In addition, biofilm

microenvironments maintain low oxygen and pH, influencing antibiotic efficiency (Srivastava and Bhargava 2016). As such, biocidal drugs usually reach the deeper layer at sub-lethal concentrations, enabling the evolution of drug resistance among cells. This emergence of resistance may lead to genetic mutations that can be transferred to other cells through intercellular communication **(**Smirnova et al. 2010). Thus, intra-matrix phenomena contribute to the emergence of antibacterial drug resistance of the biofilm.

1.5.2 Anti-biofilm mechanism of metal nanoparticles

Nanomaterials may support biofilm control via entirely different antibacterial mechanisms than available chemical antimicrobial agents. Several researchers have reported nano-formulations that provide sustained release of drugs, increased physiological stability, and reduced host cell toxicity (Sun and Li 2012). In addition, due to their smaller (~10 nm) size, metal nanoparticles can penetrate microbial cell membranes and deeper biofilm layers. However, a detailed mechanism of the anti-biofilm effects of metal nanoparticles is still not understood. Several properties of nanoparticles that are associated with anti-biofilm properties are discussed in the following sections.

1.5.3 Effect of physical properties of the metal nanoparticles on their anti-biofilm activity

Several studies have revealed that the smaller-sized (~10 nm) nanoparticles have a greater antimicrobial effect than larger-sized particles. This phenomenon is based on the fact that smaller nanoparticles can directly pass through bacterial cell membranes via water channels. Direct cellular entry may lead to membrane damage. Similarly, smaller nanoparticles can also achieve the disruption of mature biofilms. Smaller-sized nanoparticles can pass through the water channels in the core of the biofilm matrix. The biocidal activity of metal nanoparticles also depends on their shape. Different shapes of nanoparticles such as triangular, rod-shaped,

and spherical shapes have shown noticeable alterations in the cell membrane architecture. For example, triangular silver nanoplates with a basal lattice plane were more biocidal than spherical and rod-shaped silver nanoparticles (Pal et al. 2007). The variation can explain the morphology-dependent biocidal activity of the Ag nanoparticles in Ag ion release kinetics. Therefore, it may be possible to control the biocidal activity of silver nanoparticles by controlling their shape (Cheon et al. 2019). Metal nanoparticles' surface charge (zeta potential) may also affect their antimicrobial activity (Shaoying et al., 2022; Chen et al., 2021; Qiao et al., 2020). An increase in zeta potential forms the strong electrostatic interaction between nanoparticles and negatively charged bacterial cell walls leading to cell membrane disruption and membrane depolarization (El Badawy et al. 2011). The surface charge of nanoparticles can be tuned by capping or doping active molecules to increase the possibility of electrostatic interactions between the nanoparticles and the biofilm.

1.5.4 The nanoparticle–biofilm interface

Interactions between nanoparticles and biofilms are complex interfacial phenomena. They can be considered as three sequential processes: (i) channeling of nanoparticles to the biofilm–water interface, (ii) adsorption on the biofilm surface, and (iii) penetration within the biofilm (Ikuma et al. 2015). Biofilms are dynamic structures; their physical and chemical properties are continuously influenced by the local microenvironment (Petosa et al. 2010). The complex nature of nanoparticle transport into biofilms relates to the dynamic nature of biofilms. Generally, the relative self-diffusion coefficient is proportional to the size of the nanoparticle (Peulen and Wilkinson 2011). However, the penetration of nanoparticles in the biofilm matrix is also affected by the EPS viscosity, bacterial cell density, internal liquid flow, exogenous mass transfer resistance, chemical interactions of the nanoparticle with the EPS, and the characteristics of intra-biofilm water channels **(Miller et al. 2014).** A detailed understanding

of nanoparticle interaction with biofilm matrix components is essential to develop improved biocidal efficacy of nanomaterials. Most published studies have considered the biocidal efficacy of drug delivery nanocarriers; only a few have examined nanoparticle transport phenomena' fundamentals in biofilms.

Initially, a biomolecular corona or a protein corona occurs when nanoparticles encounter complex matrix biomolecules within the biofilm (Mu et al. 2014; Ke et al. 2017; Docter et al. 2015). A detailed mechanism of protein corona formation related to biofilms has not been fully elucidated. However, several reports have indicated that the composition and development of the corona depend on the physicochemical properties of nanoparticles such as size, shape, zeta potential, and surface functionalization; furthermore, the biological characteristics of the interacting medium depend on the concentration and topology of the nanoparticles (Docter et al. 2015). While protein corona formation and its effect on nanoparticle interactions have been reported in other environments (e.g., blood plasma), a comprehensive investigation of the nanoparticle–protein corona interactions in biofilms is needed (Docter et al. 2015).

1.5.4.1 Mechanism of nanoparticle-mediated inhibition of bacterial quorum sensing cascades.

Bacterial colonies exhibit a collaborative behavior in response to drastic environmental changes. This adaptive behavior is governed by a signaling process known as quorum sensing (QS). The QS system is bacterial density-dependent; it initiates cell-to-cell communication by producing and secreting small signal molecules, which are referred to as autoinducers. Once a threshold concentration of signaling molecules is achieved, the transcription of specific genes is induced, which ultimately activates cellular processes in a coordinated manner. One of the main autoinducers responsible for QS signaling in Gram-negative bacterium is N-acyl-l-homoserine lactones (AHLs) (Whiteley et al. 2017; Turan et al. 2017). An understanding of

the effect of metal-based nanoparticles on QS signaling remains limited. Most studies have been focused on nanoparticle biocidal activity for specific bacterial strains, whereas the studies involving bacterial populations and cooperative behavior have been limited. Recently, some efforts to utilize nanomaterials in bacterial QS research have been undertaken to evaluate metal nanomaterials as tools against bacterial infections (Naik and Kowshik 2014). Several studies have used *C. violaceum* as a living bacterial model to understand QS systems. In brief, the interference of cell-to-cell communication can occur at three different levels: (i) by inhibiting the synthesis of signaling molecules, (ii) by limiting the accumulation, exchange, and transport of the QS signal, and (iii) by disturbing signal perception and response (Kalia et al. 2019**;** Grandclement et al. 2016). As such, the bacterial QS system can be inhibited at several steps**.**

In search of novel and efficient microbial quorum sensing inhibitors, researchers have evaluated several classes of natural, synthetic, and semisynthetic compounds, including phyto-compounds. In addition, anti-QS activities of nanoparticles have been recently documented. Some of the results involving the anti-QS activities of nanoparticles are summarized in Table 1. Silver nanoparticles have been shown to inhibit QS-induced virulence in Gram-positive and Gram-negative bacteria (Wagh et al. 2013). Singh et al. demonstrated that biosynthesized Ag nanoparticles inhibited the complete violacein production in *C. violaceum* 12472. This study indicated that Ag nanoparticles interfered with QS via attenuation of AHL production. Many QS-mediated virulence factors in *Pseudomonas aeruginosa* PAO1 were inhibited by Ag nanoparticles such as pyocyanin (18– 96% suppression), Las a protease activity (15–86% inhibition), Las B elastase (22–86% inhibition), and pyoverdine (14–95% suppression) (Singh et al. 2015). In addition, the expression of QS-regulated virulence genes was significantly reduced. The expression of las A, las B, phzA1, and rhl A were repressed by 79, 84, 68, and 72%, respectively. In another study, silver nanowires (SNWs) synthesized by the polyol

process were shown to inhibit quorum sensing; the nanowires were shown to inhibit violacein synthesis by 60 and 80% in a dose-dependent manner in the *C. violaceum* CV026 strain (Wagh et al. 2013). It should be noted that the chemical composition of signal molecules differs based on bacterial class and species. Additional efforts are needed to understand the interaction of signal molecules from several bacterial species with nanoparticles.

In addition to silver nanoparticles, other metallic nanoparticles have been explored for their anti-QS activity, including ZnO, TiO_2, Se-Ni, and Au nanoparticles. Prateeksha and colleagues demonstrated that a selenium nano-scaffold containing selenium nanoparticles and honey polyphenols exhibited enhanced anti-QS activity and anti-biofilm efficacy as compared to selenium nanoparticles and honey polyphenols (Prateeksha et al. 2017). It was shown that surface-conjugated selenium nanoparticles with honey polyols (SeNPs@HP) interfered with the QS system by interacting with AHL molecules and their receptors. At a dose of 4.5 μg/ml of SeNPs@HPs, there was a decrease in virulence factors such as elastase (52.7%), exoprotease (60.2%), pyocyanin (49.6%), and rhamnolipid (59.6%) from *Pseudomonas aeruginosa* strain PAO1. The molecular docking results revealed that the interaction of honey polyphenols with the N-(3-oxododecanoyl)-l-homoserine lactone binding site of Las R might inhibit the virulence of *Pseudomonas aeruginosa* PAO1 (Singh et al. 2017). The nanoparticles significantly affected violacein production; in the QS system, ZnO nanoparticles mainly disrupted the QS steps associated with signal perception and response. TiO_2 nanoparticles and Ag nanoparticles affected the autoinducer biosynthesis. Ag nanoparticles with the smallest size and citrate capping agents produced the most significant effect; the impact of TiO_2 nanoparticles was not altered by UV irradiation (Gómez-Gómez et al. 2019). Heavy metals are typically required as enzyme co-factors for cellular metabolism in nanomolar concentrations; higher concentrations are generally toxic (Scheller et al. 2010). Heavy metals

have also been shown to stimulate the production of AHLs; for example, the las system in *Pseudomonas aeruginosa* is upregulated by copper as a stress response.

1.5.4.2 Molecular interaction of metal nanoparticles with polymer matrix of biofilm.

The extracellular polymeric substance supports intercellular interactions in biofilm communities and protects bacterial cells from a harsh environment. Thus, the EPS matrix contributes to antimicrobial drug tolerance and enhanced antimicrobial resistance compared with planktonic cells. The main matrix components are extracellular DNA (eDNA), structurally complex polysaccharides, and proteins. Extracellular DNA plays an indispensable role in bacterial adhesion within the biofilm, bacterial aggregation within the biofilm, biofilm formation, biofilm structure maturation, maintenance of biofilm integrity, as well as intercellular communication or QS for transfer of genetic information **(**Srivastava et al. 2011; Kassinger and Hoek 2020). Therefore, eDNA is potentially a target molecule for eradicating bacterial biofilms. Gold and silver nanoparticles have a strong affinity toward biofilm extracellular DNA; they show different types of interactions depending on the microenvironment of the biofilm (Kassinger and Hoek 2020). When a nanoparticle penetrates a biological system, it rapidly acquires a corona of biomolecules over its surface in either monolayer or multilayer form (Joo and Aggarwal 2018). As such, interactions involving Au nanoparticles and Ag nanoparticles with biofilm components are also affected by this corona (Joo and Aggarwal 2018). Moreover, it has been shown that a change in the concentration of salt leads to changes in the kinetics of the interaction of eDNA with Au nanoparticles and Ag nanoparticles (Zhang et al. 2012).

As eDNA has a polyanionic nature, electrostatic interactions play a significant role in the case of Au nanoparticles and Ag nanoparticles that are coated by positively charged molecules. Carnerero et al. showed that gold nanoparticles form covalent and non-covalent interactions

with a polyanionic DNA backbone. Additionally, Jiang et al. indicated that the gold and silver ions emanating from nanoparticles also interact with nitrogen and oxygen atoms in the DNA bases through short-range hydrophobic and Van der Waals forces (Carnerero et al. 2017; Jiang and Ran 2018). However, electrostatic interactions are stronger than Van der Waals and hydrophobic interactions (Figure 1.8) (Joshi et al. 2020). Recently, femtosecond spectroscopy showed that leached gold ions inhibited biofilm formation in Gram-negative bacteria (Radzig et al. 2019). It has also been shown that phosphor-thiolation (PT) of bacterial DNA protects it against oxidative damage**.** Treating biofilms containing phosphor-thiolated DNA with Au nanoparticles would result in strong adsorption due to favorable Au-S chemistry (Carnerero et

al. 2017). It is believed that strong Au-S interactions play a fundamental role in the inactivation of extracellular DNA.

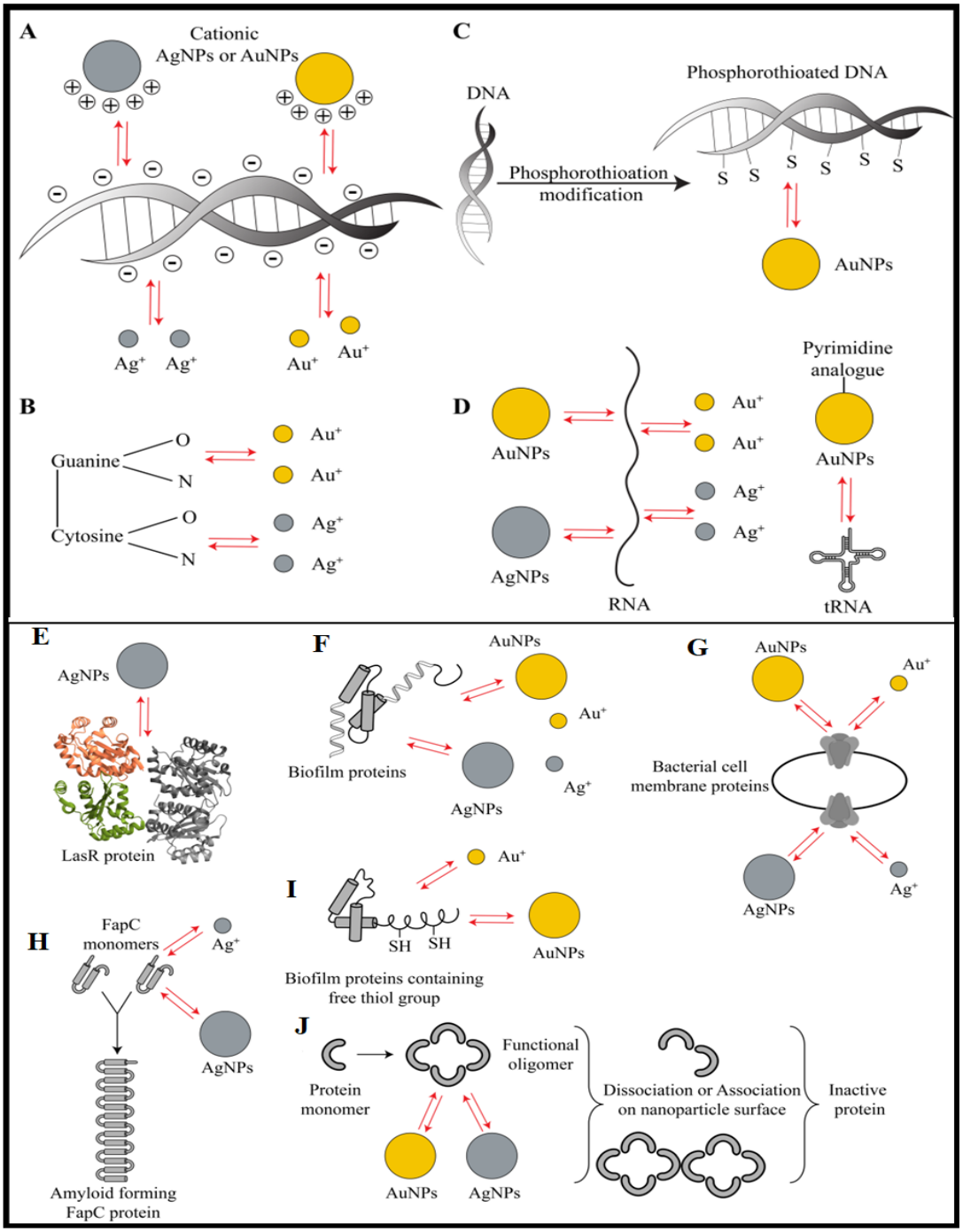

Figure 1.8 Schematic representation of the interaction of gold and silver nanoparticles with extracellular DNA. (A) Cationic gold and silver nanoparticles and their respective metal ions establish electrostatic, hydrophobic, and Van der Waals interactions with poly-anionic

extracellular DNA in the biofilm. (B) In particular, the silver and gold ions form high-affinity interactions with G-C base pairs of eDNA molecules through short-range Van der Waals and hydrophobic forces. (C) To prevent the oxidative damage of DNA, bacterial cell modifies it via phosphor thiolation. In such cases, gold nanoparticles interact through Au-S bonding extracellular DNA. (D) Both silver and gold nanoparticles also can interact with bacterial RNA molecules especially tRNA through electrostatic and Van der Waals interactions. Schematic representation of various molecular level interactions between metal nanoparticles and extracellular matrix proteins. (E) The metallic nanoparticles interact with quorum-signaling protein molecules (e.g., Las R) through electrostatic, hydrophobic, and hydrogen bonding. In this case, nanoparticles selectively interact with ligand binding sites, making them incompetent for cell signaling. (F) metal nanoparticles interact with metabolic proteins and enzymes present in the biofilm through electrostatic, hydrophobic, hydrogen-bonding, Van der Waals, and π–π interactions, causing them to be inactive for metabolism. (G) Metallic nanoparticles interact with membrane proteins such as electron transport systems that collapse the membrane potential and ultimately cell death results. (H) Gold and silver nanoparticles also establish interaction with amyloid-forming proteins such as Fap C and sequester its monomer through electrostatic, hydrophobic, and hydrogen bonding. (I) Gold nanoparticles and their leached ions show strong Au-S bonding with thiol-rich proteins. (J) Gold and silver nanoparticles interact via electrostatic and hydrophobic interaction with functional oligomers of specific proteins. Reproduced with permission (Joshi et al. 2020) under a common creative attribution license.

1.6 Potential risk related to application of metal nanoparticles as antimicrobial agents

Antibiotic-resistant microbes, especially bacterial strains increasing day by day. Among them, *Staphylococcus aureus, E. faecalis, and E. faecium* are resistant to vancomycin, *Streptococcus pneumonia* is resistant to penicillin, *A. baumannii* is resistant to carbapenem and *M.*

tuberculosis, *P. aeruginosa*, *S. enterica*, *V. cholera*, and *Enterobacteriaceae* are considered as multidrug-resistant (Betts et al., 2018). The previous results presented by several groups of researchers prove that some microorganisms may also become resistant to the effects of nanomaterials. However, the acquisition of nano-resistance happens less frequently than with antibiotics due to the multiple-site action of nanoparticles (Zhao et al., 2013). This nano resistance is achieved through multiple mutations in the genes. The evolution of resistance to metals may include the following mechanism, intracellular or extracellular layer sequestration, reduction of cell membrane permeability, enzymatic detoxification/conversion, and metal ion outflow from the cell, unlike the mechanism of resistance to antibiotics as depicted in (Figure 1.9) (Zhang et al. 2020). The development of nano-resistance to nanomaterials is a clinical concern, and several reports have confirmed that bacteria can resist the action of silver and gold nanoparticles even after the first exposure to these metals.

As antibiotic resistance increases rapidly, simultaneously, emerging resistance to nano-antimicrobials, especially nano-silver (Ag), has recently been reported, which reveals a "serious knowledge gap" regarding the safe use of antimicrobial nanomaterials and the potential subsequent generation of resistance mechanisms against these nanoparticles (Zhang et al., 2020). Several scientific and complicated questions arise regarding nano-resistance: (A) What is the exposure level that facilitates prolonged resistance against nanomaterials, and is the underlying key factor in developing resistance? (B) Do nanomaterials' physicochemical properties play a fundamental role in developing nano-resistance? If so, which physicochemical properties are most significant? (C) Can bacteria develop resistance specifically to nanoparticles rather than dissolved ions? (D) Is the resistance genes transferable from one generation to the next? Finally, (E) What are nano-resistance's environmental and clinical implications? To search for the answer to the above questions, we have to take a closer

look at the exposure scenarios that contribute to the survival selection of antibiotic-resistant bacteria, the rapidness of intra-species gene exchange, and the emergence of new resistance traits; these elements could include the following reasons 1) the misuse/overuse of antimicrobials/sanitary products in hospitals and human communities and 2) the continuous flow of drugs in water bodies, soils, and sediments. Similarly, the evolution of nanomaterial exposure to microbes resembles the two causes above. First, there has been much attentiveness to the application of engineered nanomaterials in various fields such as industry, agriculture, medicine, environmental protection, etc. Thus, production and utilization of nanomaterials have increased significantly in the past few years; for example, the annual global production of TiO_2, CeO_2, ZnO, CNTs, nano-Ag, and nano SiO_2 were ~ 3000, 55, 550, 300, 55, and 5500 MT per year in 2012, respectively (Piccinno et al., 2012). The global market for nanotechnology-based products has been growing at a rate of 25 % per year**.** Further, it is estimated that the global production of nanomaterials will increase to 58,000 tons per year in 2020, having a cost of ~ USD 8.0 billion, and is expected to expand at a compound annual growth rate (CAGR) of 14.1% from 2021 to 2028 (Grand view research report ID- GVR-4-68038-565-6). Second, enhancement of production capacity and universal application will inevitably release a considerable quantity of engineered nanomaterials into the environment. Synthetic and natural nanomaterials have multiple pathways through which they can interact with different environmental bodies. These nanomaterials can occur in the air, especially incidental NMs (INMs) are likely to be at higher levels in urban areas. Nanomaterials can also accumulate in different water sources or soils around landfills, industrial discharges, and municipal wastewater or can be generated by natural processes in these environmental compartments (Westmeier et al., 2018; Malakar and Snow, 2020).

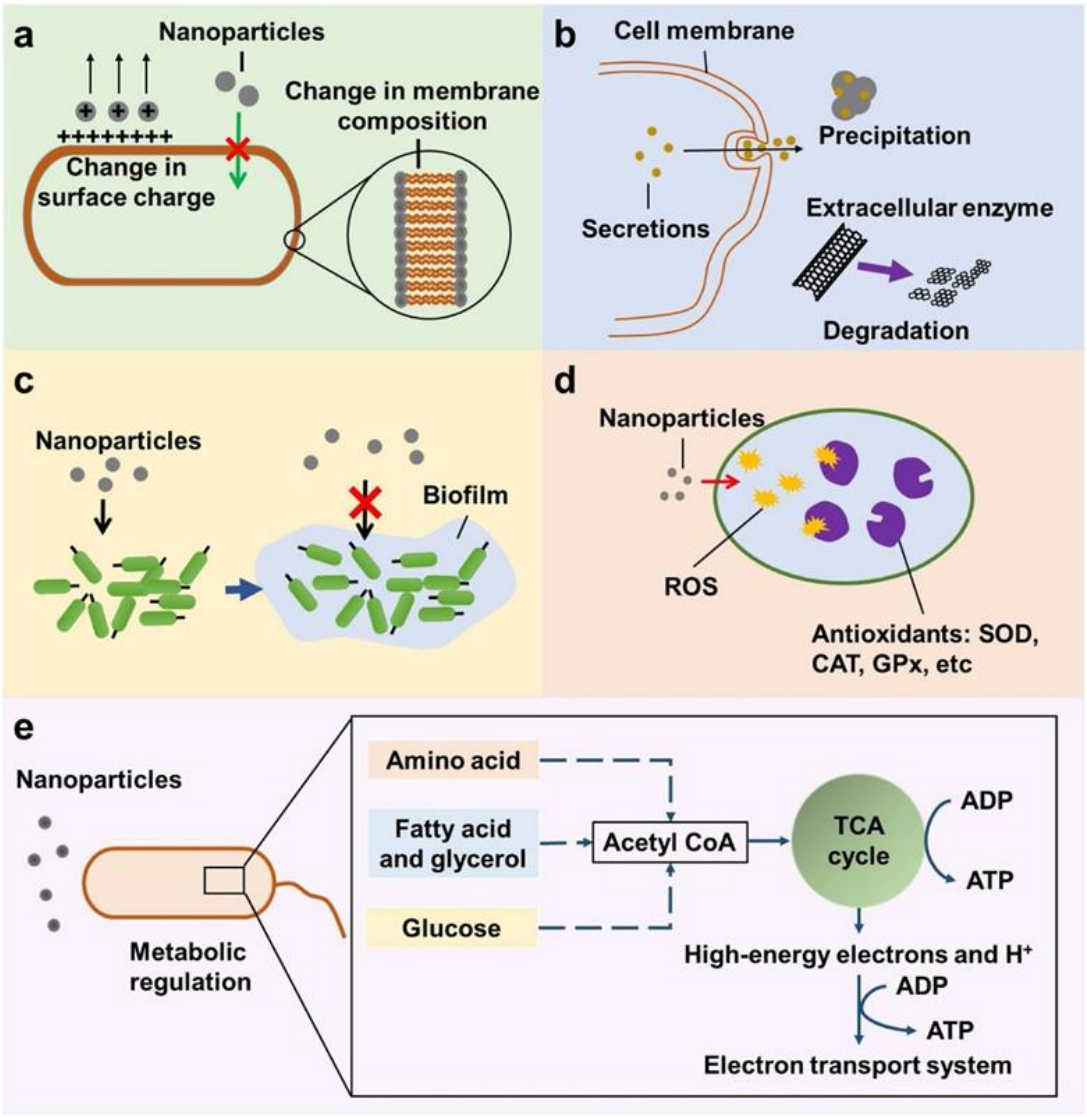

Figure 1.9 represents the molecular Mechanisms of the development of nano-resistance. a) indicating the changes in cell membrane structure and function; b) nanoparticle modification; c) biofilm development; d) induction of the anti-oxidative system; e) regulation of metabolic pathways. Adopted with permission from Zhang et.al. 2020 © Elsevier.

In contrast to antibiotics, the structural characteristics of nanoparticles are changeable, such as fingerprint traits and physicochemical properties acquired from the surroundings and environmental transformation. Fingerprint traits define various specific intrinsic properties of nanoparticles, such as chemical composition, surface functionalization, shape, size, electrical conductivity, and hydrophobicity/hydrophilicity index. In particular, a nanoparticle's

antimicrobial potency depends on size, shape, and surface property. Therefore, it would be logical to understand whether bacterial cells can develop resistance and mechanisms to nanoparticles with the composition but different structural properties such as shape, size, or surface properties. For example, it is reported that, for large (≥10 nm) nanoparticles, the secretion of extracellular substances was the main resistance mechanism in controlling extracellular agglomeration and deactivation of the antimicrobial activity of nanoparticles. On the other hand, in response to small (≤10 nm) nanoparticles, bacteria adopt different strategies, i.e., via point mutations or controlling the no. of porin in the membrane to restrict the entry of nanoparticles into cells (Niño-Martínez et al., 2019). Moreover, can bacteria differentiate between the core and shell structure and develop adaption/resistance to them? Unfortunately, no such kind of studies have been performed in this area yet. However, in A549 mammalian cells, the core material is regulated by cellular uptake and toxicity under controlled shape, size, and surface chemistries (Bai et al., 2020). It is also reported that Pt-cored nanoparticles showed much lower cellular uptake than Au- and Pd-cored nanoparticles with identical size, shape, and surface ligands. Hence, it is a challenging field to study the effects of nanoparticle structure on the origin and mechanism of resistance. However, such complicated systems are critical to investigate, and well-designed analytical approaches are needed to decipher mechanistic and insightful conclusions.

Before the significant boom in the application of nanoparticles as antimicrobials, ionic forms of metals had been used as biocides. Thus, it has also been investigated that bacteria can develop resistance to metal ions via reduced cellular uptake, increased efflux, intracellular and extracellular sequestration, metabolic bypass, and chemical modification. These mechanisms, as mentioned earlier, play an important role in adapting to metal-based nanoparticles. In addition, ionic and particulate forms are exchangeable in contact with surrounding

environments; thus, the resistance mechanisms show strikingly. The molecular mechanisms of nano-resistance among microorganisms to metal ions and nanoparticles have been systematically reviewed recently (Niño-Martínez et al., 2019).

Additionally, a question arises; can bacteria particularly develop resistance to nanoparticles? The probable answer could be yes; they can (Zhang et al., 2020). Cell envelopes limit the cellular uptake of large-sized nanoparticles and counteract the toxic effects accordingly, gram-negative *E. coli and Ochrobactrum sp*. Increase the levels of saturated fatty acids in the cell membrane in response to the puncture effect of carbon nanotubes, and by reducing the levels of unsaturated fatty acids, cell membrane rigidity increases significantly (Zhu et al. 2014). Analogously, some bacteria, i.e., *Pseudomonas putida* F1, convert cis unsaturated fatty acids into trans-isomers upon short-term exposure to nano-Fe◦ and leads to the counteraction of nanoparticle-induced membrane fluidizing (Kotchaplai et al., 2017). It is also observed that Saccharomyces cerevisiae increases the chitin component in the cell wall as an adaptive strategy to overcome the effect of "nano dart" lead sulfide nanoparticles (Sun et al., 2014).

Interestingly, some gram-positive bacteria, such as S. *aureus* and *B. subtilis*, increase the ratio of branched-chain fatty acids and lower the level of straight-chain fatty acids in the cell membrane in response to carbon nanotube exposure. In addition, cationic nanoparticles exhibit antimicrobial activity by establishing electrostatic interaction with the negatively charged cell surface of bacteria (Radovic-Moreno et al., 2012). According to recent studies, it is confirmed that bacteria can adjust the surface charge and repel bactericidal nanoparticles (Kumariya et al., 2015).

1.7 The potential implication of metal resistance

The major emerging concern over adaption/resistance to metal nanoparticles is the possible coevolution and co-expression of antibiotic resistance genes along with cross-adaption (Figure 1.10). For example, as a defense mechanism of *P. aeruginosa* PAO1 induces overexpression mexW (an efflux pump gene) and marC (associated with tetracycline resistance) against quantum dots; as a result, the MICs (Minimum inhibitory concentration) of respective antibiotics were increased by 50–100% upon incubation with quantum dots (Yang et al., 2012). Nano-ZnO and nano-Ag induced the cell envelope stress response in bacteria, which suggested leading antibiotic resistance via hyper-synthesizing lipopolysaccharides, thus enabling cells to counter a wide range of metal toxicity. Similarly, the exposure of Al_2O_3 and ZnO nanoparticles could expedite the evolution of new resistance genes via de novo mutation. The resulting mutants have shown increased resistance to ciprofloxacin, and chloramphenicol had an increased MIC of 2–3 times compared to wild-type strains. Another report confirmed that nanoparticle-related photo disinfection-induced bacterial stress responses transiently increased antibiotic tolerance. These tolerant bacteria can evolve antibiotic resistance through the subsequent selection of sub-lethal antibiotics (Yin et al., 2020).

Another concern is that the adaption for nanoparticle exposure may enable microorganisms to counter and survive in a wide range of harsh environments and boost their pathogenicity. It is observed that swarming motility increased in *B. subtilis* under Al_2O_3 nanoparticle exposure via stimulated production of the lipopeptide surfactin that exploits water surface tension and mediates motility and colonization (Mu et al., 2016). Similarly, gene clusters linked with the flagella movement were considerably upregulated in *E. coli* as part of the stress response regulation network for cationic polystyrene nanomaterials (Ivask et al., 2012). At the nonlethal level of graphene oxide, the production of the quorum-sensing signal molecule (homoserine lactone) was enhanced in *P. aeruginosa* PAO1, leading to biofilm formation and gradual

adaption to GO (Zhang et al., 2018). Lipopolysaccharides are a major component of the outer membrane of gram-negative bacteria and have been considered the principal molecule for the induction of septic shock in infected patients. It was observed that lipopolysaccharide production was increased in bacteria resistant to polystyrene and other metal nanomaterials (Ivask et al., 2012).

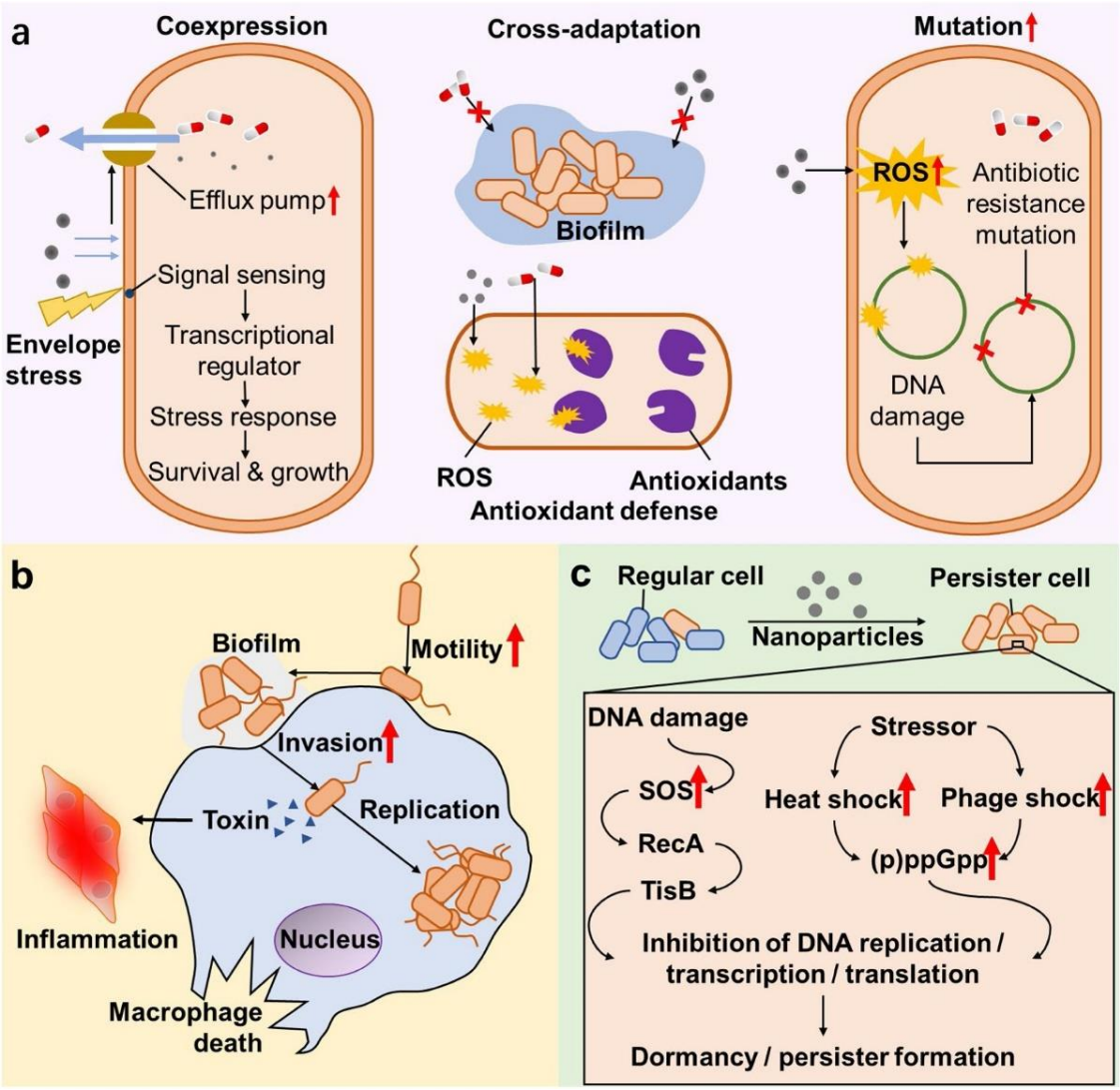

Figure 1.10 Implications of nano-resistance for a) co-evolution of antibiotic resistance; b) enhanced pathogenicity; and c) induction of community persister. Adopted with permission from Zhang et.al. 2020 © Elsevier.

Further concerns are the bacterial ability to survive and replicate inside phagocytic cells. When these bacteria interact with a mammalian host, various substances (such as host-produced surfactants and biocidal peptides) that cause severe cell envelope stress attack the microbial cell. Thus, to counter this stress and bacterial survival, these cells respond to cell envelope stimuli via signal transduction systems, collectively known as envelope stress responses. Finally, metal nanoparticle adaption could derive and contribute to the formation of persister cells, which may be within regular populations and are highly tolerant to several antibiotics and the phagocytic microenvironment. The exposure scenarios, and biochemical, and genetic antibiotic resistance mechanisms indicate the resemblance and divergence between antibiotic and nano resistance evolution. The relationships between specific nanoparticle physicochemical characteristics, recognition, and adaption in bacteria need to be detailed, indispensable for selective applications and understanding of the complex interaction of nanoparticles with microbes.

Motivation of Work and Objective

This thesis highlights the surface interaction and impact of nanoparticles on bacterial as well as fungal cells. Silver and gold Nanoparticles have been prepared via chemical methods with tuneable shape, size, and selective surface properties for various applications. The antimicrobial activity of the nanoparticles has been explored extensively. Although, among all metal nanoparticles, silver cations and nanoparticles have been used as antimicrobial agents for centuries. However, the biomedical applications of metal nanoparticles are limited because of their non-selective reactivity with biomolecules. The research community has explored the antimicrobial activity of metal nanoparticles against eukaryotic and prokaryotic pathogens in recent decades. Several organic and inorganic grafting agents are used to modify the efficacy and spectrum of activity of metal nanoparticles unfortunately the selectivity has to been achieved yet. Second, some issues are also associated with the physicochemical stability of particular nanoparticles over time. On the other hand, the antimicrobial mechanism of metal nanoparticles against the planktonic and biofilm-forming microbes is limited. Therefore, researchers suggested several mechanistic nanoparticle pathways, including antimicrobial activity by ROS generation, protein damage, membrane depolarization, DNA damage, quorum sensing inhibition, and membrane damage. However, the antimicrobial mechanisms for many types of nanoparticles have not been fully elucidated to this point.

Despite advancements in the biomedical research field, some critical issues must be addressed before human application of metal nanoparticles. These challenges are;

- Stability of MNPs at various physico-chemical & physiological environments.
- Biocompatibility.
- Nonspecific interaction of MNPs.

- Metal resistance among microorganisms.
- Limitations in Applicability.

Therefore, it is important to decipher the mechanistic pathways of biocidal activity for these materials to design nanoformulations containing metal nanoparticles as broad-spectrum and efficient antimicrobial nanomaterials. Hence, this thesis is solely dedicated to the synthesis of rapid, stable, and variable amine-functionalized metal nanoparticles such as gold and silver. Further, an approach has been opted for understanding the interaction dynamics of nanoparticles at nano-bio interface on bacterial as well as fungal cells to optimize the system to deliver desirable antibiotics and antifungals against multidrug resistance pathogens. Though, the following objective has been proposed during research work;

- Synthesis, Characterization, and surface functionalization/tuning of MNPs with different amine molecules.
- Understanding the microbial cell surface binding dynamics of functionalized metal nanoparticles to design selective antimicrobial agents against multidrug resistance microbes.
- Development of biocompatible, antibiotic-decorated metal nanoparticles for the delivery of broad-spectrum antimicrobial agents as well as fluorescent sensors for biological and metal analytes of interest.