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Essay topic: Antimicrobial metals and metal nanoparticles with focus on copper

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1.0 ABSTRACT

Metals have been in commercial use since ancient times, be it as a precious commodity or a building material. The Ebers Papyrus in 1500BC mentions that metals such as copper have certain medicinal properties and have been used as a treatment for pathogenic diseases for a long time. Gradual advancements in the usage of metals as antimicrobials, paved the way for the modern scientific community to explore the depths of antimicrobial properties of certain metals in order to tackle various microbial threats to humanity. Antimicrobial metals such as silver, gold, copper, platinum and also heavy metals have the capacity to inhibit the growth of several pathogens with the release of their respective ions into the cell causing severe damage to the cell wall or membrane, production of reactive oxygen species (ROS) which damages the DNA, denatures proteins and in turn causing cell death. Metal nanoparticles (NPs) can be synthesised and used as effective drug molecules synergistically with antibiotics to tackle drug resistant pathogens. The redox capacity of copper makes it a very common antimicrobial and is highly effective as a surface contact induced pathogen killing and has applications in healthcare industries, water disinfection and agriculture. Copper can be used as a NP for drug delivery as well.

KEYWORDS: Antimicrobial metals, reactive oxygen species (ROS), Metal nanoparticles (NPs), synergism.

2.0 INTRODUCTION

Several prokaryotic and eukaryotic cells have a high complexity when it comes to their reactivity mechanisms related to metals. Metal ions are present as necessary trace elements in the cell membrane. Proteins along with metal atoms contribute to the structural integrity of cells, the electron transport chain and other biocatalytic reactions in the cell [1]. Metals, when present in excess quantities prove to be toxic to the cell. Non essential metals such as silver (Ag), mercury (Hg) and arsenic (As) are extremely poisonous to the cell [2]. Copper, silver and iron have been used since ancient times to treat pathogenic diseases as mentioned in Ebers Papyrus (1500BC). Copper is one of the most abundantly used metal antimicrobials since a long time to treat *Escherichia coli*, multidrug resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas* infections. Silver, gold and zinc, although less common, has also been used to treat infections originating from *Streptococcus pneumonia, Campylobacter jejuni, Vibrio cholerae* & enterotoxic *Escherichia coli* [3]. The advent of antibiotics in the twentieth century led to the decrease in usage of metals as antimicrobials. Despite warnings from Alexander Fleming, what the world did not realise was that along with the treatment of diseases using antibiotics, there was growth and spread of antibiotic resistant strains of pathogenic bacteria [4]. Using metal based antimicrobials in the form of nanoparticles and nanomaterials and administering them to counter antibiotic resistant strains of pathogens was one of the major turning points in the field of pharmaceuticals. Combining one or more metals with each other and with antibiotics and applying them to tackle resistant pathogens is one of the breakthroughs found in the field of metal antimicrobials [5].

Toxic doses of metals like the ones shown in **Figure 1**, when administered to target cells disrupt cellular processes, enzyme activity, membrane function and damage the DNA mainly due to the production of reactive oxygen species (ROS). The toxicity also depends on the absorption capability of the cell and on the chemical reactivity properties of metals and donor ligands in the cell that interact with the metals to form complexes [6].

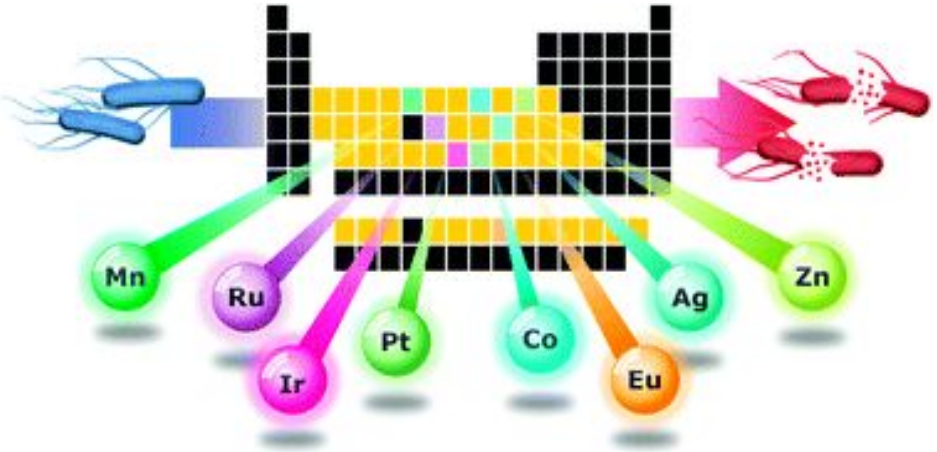


Figure 1: Various metals with antimicrobial properties

Source: [7]

3.0 Mechanisms of metal transport in cells:

Metals are transported into a cell in one of two ways. Metals with affinity to water need the assistance of membrane transporters to get into the cell and metals which are lipophilic are directly transported into the cell allowing ROS to react with them and cause toxicity in different ways as shown in **Figure 2**. Excess metal concentrations would result in it's efflux from the cell. The direct transport is facilitated by general bacterial protein transporters and trimeric β-barrel proteins known as porins present on the outer membrane of gram negative bacteria. Although the mechanism of transport of non essential metals is still unclear, these transporters catalyse energy independent movement of polar solutes like metal ions across the cell membrane into the cytoplasm causing metal toxicity to the cell [8]. The extent of transport can be tested by gene knockout or overexpression experiments for these proteins. Certain membrane transporters are involved in the direct uptake of non essential metals which works similar to the uptake of inorganic substrates [9]. Indirect transport in which metals are taken up by the cell through its co-transport with low molecular weight ligands. Metals when bound to ligands such as phosphate, amino acids, peptides and organic acids are easily taken up by the cells. A few examples include citrate dependent transport of Fe(Ⅲ) in *Escherichia coli*, phosphate transporter Pho84 in *Saccharomyces cerevisiae* and PitA in *Escherichia coli* and mercury-cysteine complexes that promote uptake of Hg(Ⅱ) in *Geobacter sulfurreducens* [10,11,12,13]. Siderophores can be used to chelate metals like iron

and iron siderophores can chelate metals other than Fe(Ⅲ) such as cadmium and gallium which are readily taken up by the cell [14].

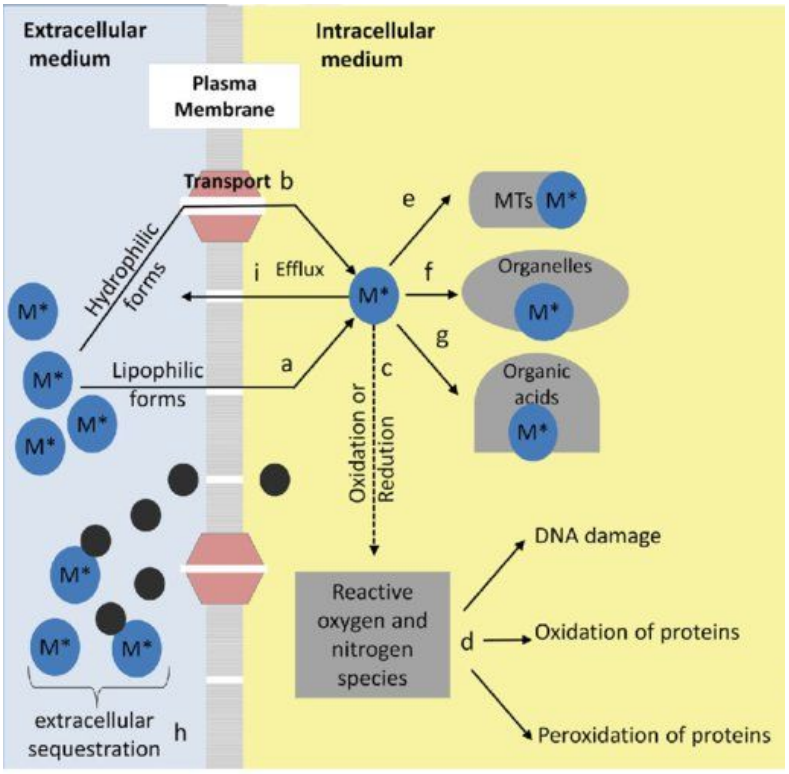


Figure 2: a and b represent direct and indirect transport of metal ions (M*) from an extracellular environment into the bacterial cell respectively. Path c and d shows the ways in which ROS cause cell toxicity upon oxidation or reduction of metal ions which includes DNA damage and oxidation of proteins. Once inside the cell, metal ions can also be stabilized by proteins like metallothioneins (MTs) (e), chelated by proteins and stored in organelles (f) or are linked to organic acids (g). They can also form complexes extracellularly (h) and if the cell detects excess metal ions, they are flushed out (i).

Source: [15]

4.0 Mechanisms of action of antimicrobial metals:

4.1 Reactive oxygen species (ROS) production and antioxidant depletion: Reactive oxygen species such as hydrogen peroxide (H_2O_2) or superoxide ($O_2^{\cdot-}$) are highly reactive compounds interfering with cellular components which are produced in a target organism with exposure to toxic doses of certain metals such as chromium (Cr), cadmium (Cd), arsenic (As), telenium (Te), silver (Ag), copper (Cu) and iron (Fe) [1]. The presence of H_2O_2 in *Escherichia coli* catalyses the production of superoxide and deregulates iron metabolism in

the cell which results in DNA damage and inhibition of enzymes required for cell growth. Iron catalysed auto-oxidation reaction during aerobic respiration produces reduced forms of oxygen such as H_2O_2 or $O_2^{\cdot-}$ which is highly toxic to the cell [16]. Fenton chemistry, as shown in **Figure 3** explains how metals catalyse $OH^{\cdot}$ radical formation which is toxic to the cell. In the presence of toxic metals, a small portion of intracellular iron is available for Fenton reaction which disrupts the [4Fe-4S] (iron-sulphur) cluster in proteins that in turn releases more iron ready for Fenton reaction in the cytoplasm resulting in further ROS production. Intermediate sulphur (S) radical chemistry facilitates thiol mediated reduction generating ROS. Toxic metals causes oxidative stress by diminishing the total cellular thiol and depleting glutathione, a major antioxidant present in microorganisms. This renders proteins vulnerable to attack by metal ions or ROS as shown in **Figure 4** and prevents the repair of these proteins by disulphide exchange enzymes [18,19,20].

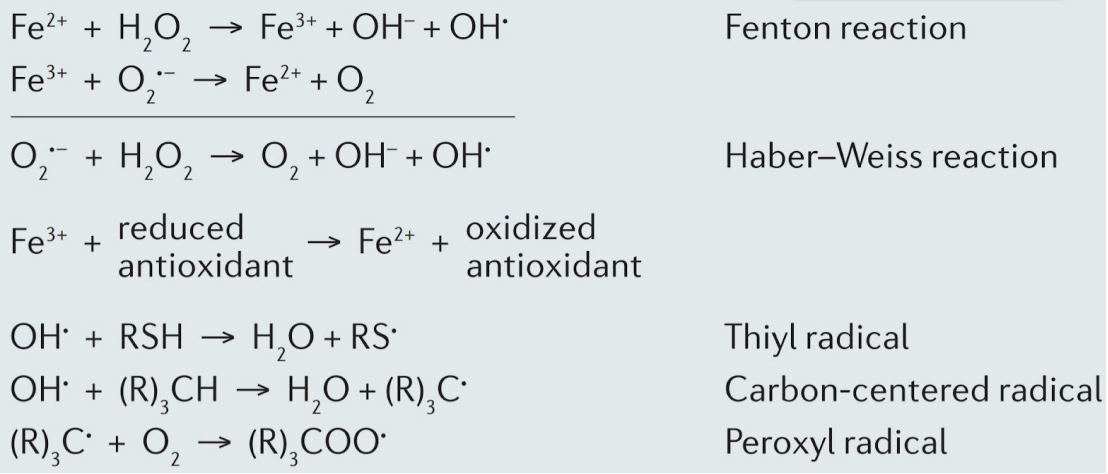


Figure 3: Toxic compounds are produced as a result of ROS interaction with intracellular iron which characterises the Fenton reaction and Haber-Weiss reaction. The lethal hydroxyl radicals, superoxide and hydrogen peroxide generated can react with proteins and other components present in the cell containing thiols and carbon radicals to form their respective radicals and eventually disrupt their function rendering the cell dead.

Source: [1]

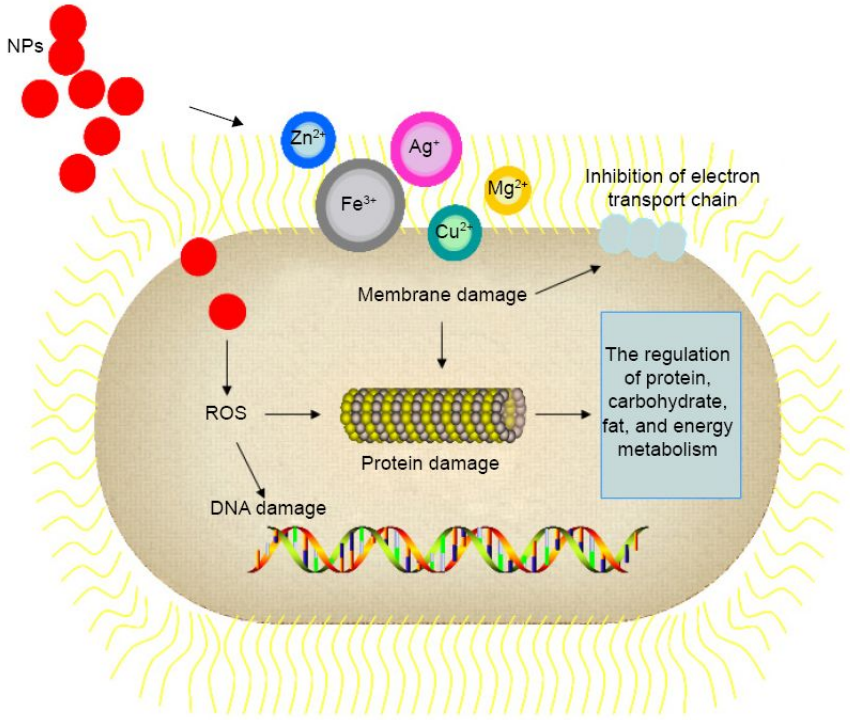


Figure 4: Metal nanoparticles (NPs) made from antimicrobial metals release ions into bacterial cells and arrest cell activity by damaging the membrane and entering the cell to produce reactive oxygen species (ROS) which damages the DNA and proteins. Metal ions can also affect the electron transport chain in cell respiration. Source: [21]

4.2 Disruption of proteins and loss of enzyme activity: Toxic doses of metals like Fe, Cu, Cd, lead (Pb), zinc (Zn), mercury (Hg) and nickel (Ni) have the capacity to destabilize proteins and enzymes in a cell. Amino acids with sulphur groups like methionine or cysteine are at the risk of getting oxidised by metals. Oxidation of the side chains of amino acids creates the loss of catalytic activity triggering degradation of proteins and cytosolic enzymes responsible for glycolysis or catabolic reactions. Cysteine residues of proteins in cells are oxidised to sulphonic and phonic products upon exposure to toxic doses of metals producing ROS like H_2O_2 with the release of iron as shown in **Figure 5**. Mononuclear iron metalloenzymes like peptide deformylase, threonine dehydrogenase and cysteine deaminase are disrupted in *Escherichia coli* are now susceptible to iron catalysed Fenton chemistry proving to be lethal for the cell. [22, 23]. Bacterial hydratases with [Fe-S] clusters are site specifically vulnerable to toxic metals. Metal toxicity also inactivates isopropylmalate isomerase affecting branched chain amino acid synthesis by decreasing levels of fumarase A and 6-phosphoglyceraldehyde in *Escherichia coli*. A copper catalysed aerobic reaction:

$[4Fe\text{-}4S]^{2+}$-------> [3Fe-3S] degrades the iron-sulphur clusters as shown in **Figure 5** causing Cu poisoning of the cell and is independent of ROS production. The active site of an enzyme aminolevulinic acid dehydratase undergoes site specific inhibition when Zn is replaced by Pb as shown in **Figure 5**. Similarly Ag replaces Cu in *Saccharomyces cerevisiae* leading to the loss of Cu-Zn superoxide dismutase (SOD) activity elevating the chances of superoxide toxicity in the cell. Metal substitution at non catalytic metal binding sites also inhibit enzyme activity [1, 24].

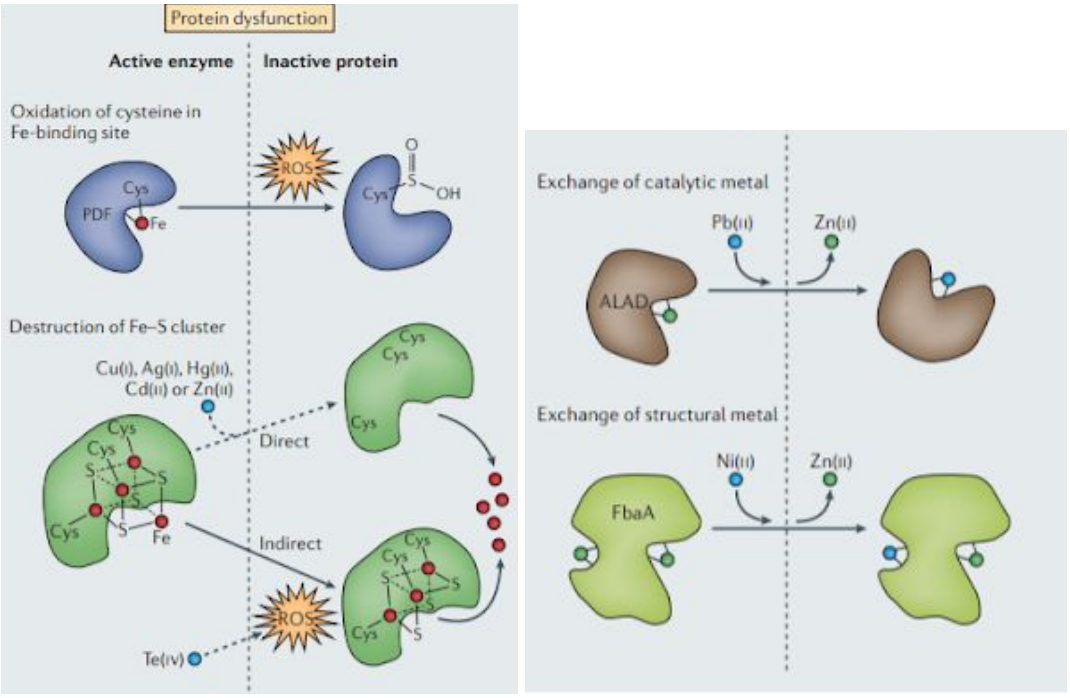


Figure 5: Proteins with cysteine side groups undergo inactivation upon exposure to iron which replaces the sulphur atom along with the production of ROS. Toxic doses of copper, silver or mercury disrupt the [Fe-S] clusters in certain enzymes. Metals such as lead and zinc can also transition with functional groups in several proteins to damage them.

Source: [1]

4.3 Impaired membrane function: Cell membranes consist of phospholipids with electronegative chemical metal cation adsorption sites. Exposure to toxic doses of metals deeply damages the cell membrane and is seen through electron microscopy. Metals also disrupt the electron transport chain. Ag inhibits the activity of NADH-ubiquinone oxidoreductase (NQR) in *Vibrio harveyi* by hampering the chemiosmotic potential of the membrane which leads to proton leakage. Copper and cadmium toxicity leads to lipid

peroxidation, the details of which is not yet studied. Metals also increase thiobarbituric acid reactive substances (TBARS) which have a similar toxicity to cells as ROS [25, 26].

4.4 Effect of metals on nutrient uptake: Few metals interfere with the utilization of specific nutrients in a cell, for example chromium doses prevent sulfur uptake by the cell causing sulfur starvation and works better than competitive uptake of metals. Gallium (Ga) causes iron starvation in *P.aeruginosa* and the mechanism is not just competitive uptake but Ga represses the iron responsive transcriptional regulator PudS which downregulates the genes responsible for uptake of iron. Metals therefore can inhibit cell growth by preventing the assimilation of essential nutrients in the cell [27, 28].

4.5 Genotoxicity: Genotoxicity assays such as the Ames test show that metals such as Cr, Cd, As, manganese (Mn), cobalt (Co), molybdenum (Mo) and antimony (Sb) are mutagenic to the cell. Lethal DNA damage is brought about by iron Fenton chemistry reactions in a cell. Toxic metal doses displace the essential iron in the cell releasing more iron available for Fenton reaction [1].

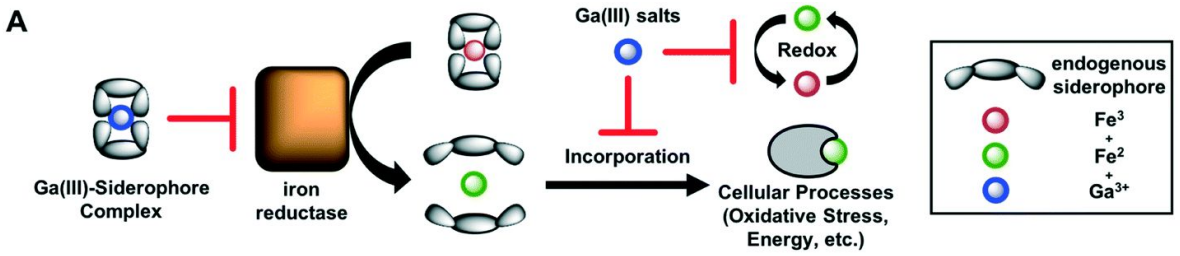
5.0 Mode of delivery of metal antimicrobials:

Metals, metalloids and metal NPs have the ability to enter and act inside mammalian and bacterial cells and hence are currently being researched with their applications as drugs especially to tackle antibiotic resistant pathogens [30]. Tricking the bacteria to take up lethal metals instead of essential metals is one of the properties that can be used to deliver metal based drugs [5]. NPs are usually combined with antibiotics to tackle antibiotic resistant strains of bacteria. Antibiotic resistance mechanism is often due to the reduction of the drug permeability through the cell envelope. This is overcome when NPs are attached to the hydroxyl and amino groups of antibiotics. This complex has increased synergistic effects and easily passes through the cell envelope and is ready to build up toxicity in the cell [43]. Siderophores such as desferrioxamine (DFO), enterochelin or protoporphyrin IX (PPIX) are used in combination with metals for administration in humans. Among scandium (Sc), indium (In), manganese (Mn) and gallium (Ga), Ga is the best for attachment with siderophore as shown in **Figure 7A**. Ga(NO_3) Ga-DFO, Ga-PPIX are used in the form of anti

biofilms and antibiotics which are effective against multi drug resistant *P.aeruginosa* and several gram positive and gram negative bacterial cells. These are tested in vitro and in animal models [31]. The most electronegative metals such as the ones in **Figure 6** are better effective as antimicrobials as they get attracted to the negatively charged cell wall of bacteria. Metals like silver works best synergistically, meaning when combined with antibiotics, other metals or siderophores in a stable manner. Silver ions are said to bind to proteins and enzymes in the cell wall and membrane, disrupting the cell wall increasing permeability. Individual and combined metals must be studied in greater detail elucidating their synergistic effects also with antibiotics. Antimicrobials in combination when used are much more effective against pathogens [3, 32]. Ga-DFO with amino-glycoside antibiotic gentamicin is one such example. Bismuth has a history of curing gastrointestinal ailments. Combining Bi citrate and salicylate for triple and quadruple therapy is effective against *Helicobater pylori* and treating stomach ulcers. Alkaline copper quaternary is an important fungicide. Cu-Ag ionisation is particularly used in water disinfection killing the main pathogen *Legionella spp* [33, 34, 35].

Figure 6: Antimicrobial metal surfaces such as gold, silver and copper.

Source: [36]



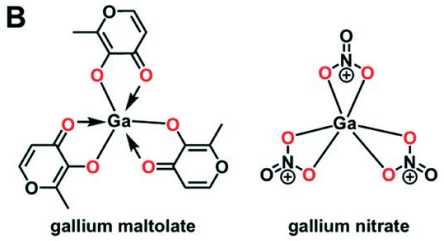


Figure 7A: Gallium siderophore complexes are said to inhibit iron and iron reductase activity in the cell along with the formation of gallium salts which blocks cellular redox reactions in turn rendering the cell dead. Ga mimics the properties of iron and forms complexes with cellular proteins to inhibit their functions.
7B: This shows the structures of complexes formed by Ga and Fe with chelating atoms in red. Gallium nitrate is said to stop the growth of *P.aeruginosa, S.aureus* and *A.baumannii*, whereas gallium maltolate inhibits *S.aureus* infections inside the host organism. Source: [37]

6.0 Metal Nanoparticles (NPs)/ Nanomaterials:

Metal nanomaterials are made up of metals (such as Cu or Ag) which are of the size 1-100nm with organic and inorganic moieties. These nanoparticles directly participate in physical interaction with bacterial cell surfaces. The main characteristics that affect the efficacy of these metal nanomaterials in releasing ions are the size, shape and surface charge [1]. The types of metals most likely to be used to create nanoparticles are heavy metals with a density more than $5g/cm^3$ or transition elements that can undergo redox reactions. NPs are manufactured using a bottom-up approach where the salt of a metal is reacted with a strong reducing agent such as sodium borohydrate. Metal cations are reduced to neutral atoms where metal atoms aggregate at the nucleation site to form NPs. Silver is the most popular metal used to make NPs but other metals such as Al, Au, Bi, Ce, Cu, Fe, Mg, Ti and Zn are also used. Other forms of NPs such as mixed metal oxides, antibiotic and enzyme conjugated NPs are made in order to use them as metal antimicrobials [38]. Adding a capping agent to NPs increases the stability and dispersion of the NP in turn reducing its aggregation [39]. NPs are commonly stabilized with citrate, chitosan or polyvinyl acetate and this facilitates an accelerated generation of ions [40]. Using plant extracts, bacteria, fungi and yeasts as solvents and stabilizing agents during the production of NPs as shown in **Figure 8** reduces the amount of toxic byproducts released as compared to conventional NP synthesis processes [41]. One of the factors to be considered while formulating a metal NP is the type of target bacterial cell wall. Gram positive bacteria have just a single thick peptidoglycan layer as the

cell wall and gram negative bacteria have a thin peptidoglycan layer with an additional lipopolysaccharide outer membrane which is negatively charged making them more vulnerable for attack by positively charged metal ions which can infiltrate the double wall. Gram positive bacteria have an 80nm thick protective peptidoglycan layer with teichoic acids and have a certain resistance to toxic metals [52]. However, the negative charge on either of the cell walls allows attack by metal ions. The positively charged detergent cetyl trimethylammonium bromide (CTAB) boosts the NP toxicity by paving way for absorption on specific crystal planes and negatively charged cell walls of bacteria [42]. Adding halogens to NPs help aerogel formation of chlorine or bromine powders. The toxic effectiveness and oxidising power of the halogen is highly effective against many gram negative and positive bacteria including their endospores.

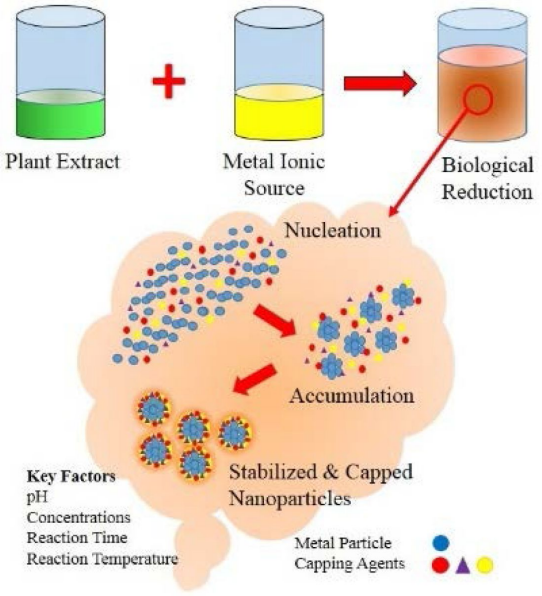


Figure 8: An ionic metal solution is reduced with chemicals or plant extracts (as shown above) followed by nucleation, i.e., crystallization of the metal ions which undergo capping and stabilization as mentioned above. The final product includes agglomerated metal NPs ready for commercial application. The main factors affecting the synthesis of metal NPs are the pH of the reaction, concentrations of the reducing agent and the metal, reaction time and temperature. Source: [44]

6.1 Characteristics of NPs: Features such as formulation process, environmental conditions, bacterial defence mechanisms and physical characteristics of the NPs affect the antimicrobial activities of the NPs.

6.1.1 Size and shape: Smaller NPs have higher antimicrobial activity with the ability to increase ROS production causing cell damage and death. NPs can be of a variety of shapes such as spherical, sheets, plates, tubes, cubes, rods or triangles. Nanocubes and rods made with CeO_2 are said to be more effective amongst others. The diameter of the NP influences the toxicity as well [45, 46].

6.1.2 Surface area: A larger area provides better adsorption and binding of compounds. Introducing surface defects increases the surface to volume ratio leading to higher ROS production. Exposed surfaces and oxidation levels of the metal also contribute to its toxicity [47].

6.1.3 Charge: Positively charged NPs with amino functionalized polystyrene particles are easily attracted and passed through the negatively charged cell membrane of any cell. They alter the electron transport chain of the cell [48].

6.1.4 pH of the environment also matters while using NPs as antimicrobials. Acidic conditions promote NP binding with the cell wall [49].

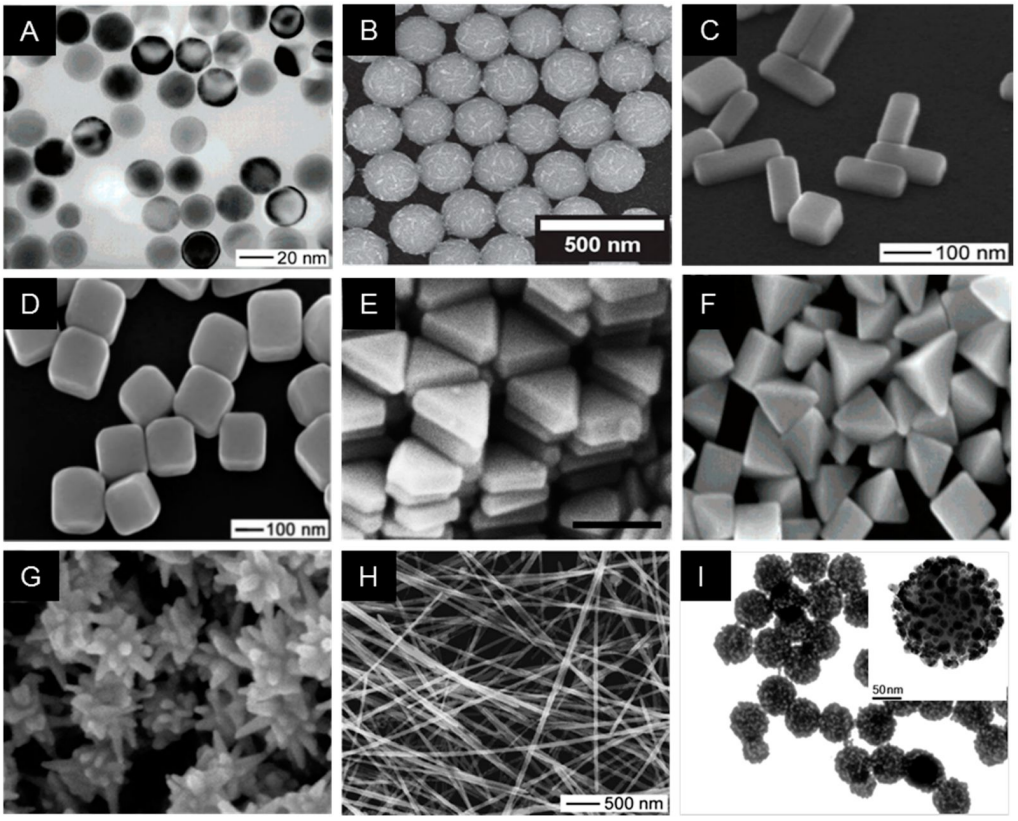


Figure 9: Various shapes of silver NPs as seen on an electron microscope. (A) Silver nanosphere, (B) Silver necklaces, (C) Silver nanobars, (D) Silver nanocubes, (E) Silver nanoprism, (F) Silver bipyramids, (G) Silver nanostar, (H) Silver nanowire, (I) Silver nanoparticle embedded silica particle.
Source: [50]

6.2 Mechanism of action of antimicrobial metal NPs:

Metal NPs release ions into the cell disrupting the cell membrane, generating ROS and associating with R-SH groups to cause toxicity to proteins in the cell as shown in **figure 10** [51]. The exact mode of action of ROS is not cell wall damage but has a complex toxicity to the cell. ROS producing hydrogen peroxide causes catalytic oxidation and oxidative stress in the cell. This depletes GSH (glutathione) which is an antioxidant. The intracellular concentrations of ROS could be measured by calculating the ratio of GSH that gets oxidised to glutathione (GSSG). GSH is a thiol which reduces disulphide bonds in cysteine and gets oxidised to GSSG. The measurement is done by scavenging ROS in the cell [67].

Salmonella typhimurium when observed for the charge distribution of its cell envelope, by allowing the attachment of cationized compounds, a mosaic of anionic surface which is discontinuous, was seen. This structure facilitates metal NPs to accumulate in the gaps and increase focal toxicity [53]. The electrochemical potential between the NP and the cell helps release of ions from the NP. The NP concentration is directly proportional to the toxicity and the NP is viable for short periods of time [54, 55]. Upon exposure to metal and metal oxide NPs such as silver, copper oxide and cerium the respective ions react with the cell wall and affect the proteins responsible for the cell wall synthesis and increase the damage to the cell [56, 57, 58]. Metal ions can further enter the cytosol and cation selective porins affecting the porin channels [59, 68]. *Shewanella oneidensis* has a mosaic structured cell wall which is non uniform having spatial heterogeneity. NPs thus get attached to the sub polar area of the cell aggregating itself to form a toxic environment [60]. Few NPs like MgO or $Mg(OH)_2$-NP enter the cell without the cell wall disruption. This is confirmed by observing no damage to the cell wall through microscopy [61]. Ag-NPs neutralises the cell wall surface charge to weaken the wall increasing permeability of an *Escherichia coli* cell causing electrostatic imbalance, proton motive force collapse and leakage of intracellular potassium all within 5 minutes of exposure. Leakage can be measured by using assays to detect intracellular contents [62, 63, 64]. NPs can also affect the cellular respiration process in cells by damaging b-cytochrome

and cyt-α2 [65, 66]. A high resolution image of a bacterial cell exposed to metal ions shows a dense central area indicating the defence mechanism of the cell where the contents of the cell becomes condensed. NPs cause nuclear fragmentation by attachment to DNA [69, 70]. When metal ions enter *E.coli*, they affect proteins and enzymes responsible for the production of ATP. 30s ribosome is denatured inactivating succinyl-coA synthetase. This in turn inactivates the citric acid cycle and is confirmed by measuring ATP levels extracellularly which was found to be negligible indicating that there was no leakage of ATP and it was indeed the inhibition of ATP production [71].

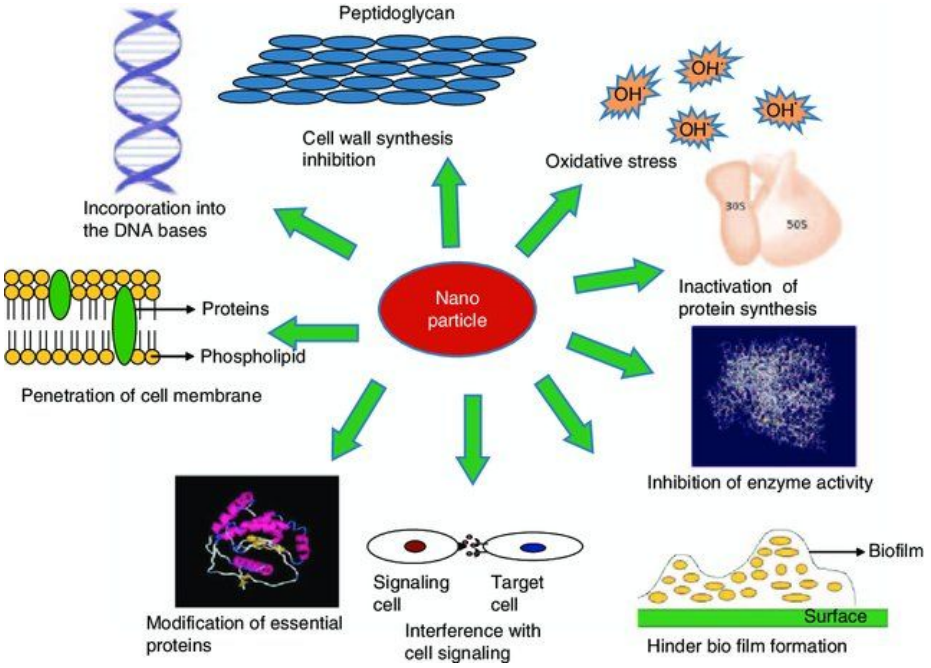


Figure 10: Metal NPs are lethal to cells through its various mechanisms of actions as mentioned in the figure and the above text.

Source: [72]

7.0 Metal resistance mechanisms in cells:

Excess concentrations of metals when present in cells trigger efflux pumps which induce removal of metal ions through pores in the cell envelope. Cells also form complexes with toxic metals and sequester them extracellularly or inactivate them by enzymatically converting them to less or non toxic forms as shown in **Figure 11**. Certain gram negative bacteria like *Cupriavidus metallidurans* are resistant to heavy metals and metal NPs like TiO_2, Al_2O_3 and carbon nanotube NPs. This was confirmed by exposing the same to

Escherichia coli which was killed immediately. A transcriptome analysis of these resistant bacteria revealed that there are 2 plasmids pMol-28 and pMol-30 which upregulated 83 and 143 genes respectively related to metal resistance and protection. pMol-28 holds resistance against Co, Cr, Hg and Ni whereas pMol-30 has resistance factors against Ag, Cd, Co, Cu, Hg, Pb and Zn. Further, transcriptome analysis of a cell exposed to metal NPs showed that genes responsible for biofilm formation (bolA), citric acid cycle (sdhC), electron transport chain (sdhC), cellular transport (mdfA), protein efflux (fsr, yajR, cmrE) and DNA repair (recN, uvrA, ybfE) were upregulated [74]. CeO_2-NP is shown to disrupt respiration or iron homeostasis and the respective genes were seen to be downregulated [75]. Similarly in *Escherichia coli* with the presence of MgO-NP, 83 out of 109 genes mainly involved in central metabolism, genetic transcription and cellular function were seen to be downregulated [76]. *Pseudomonas spp* when exposed to Ag-NPs show upregulation of antioxidant genes that protect against oxidative stress. Stress also causes envelope proteins and heat shock proteins to be upregulated [77]. The bacteria shows resistance by modifying the peptidoglycan layer, activating genes for cell wall repair and removal of ions by metabolites and proteins [73]. Bacteria also have certain protective mechanisms that flush out toxic ROS produced during metal toxicity in cells and mutants without ROS scavenging enzymes have varied sensitivity to metals. When bacteria and yeast are exposed to toxic doses of metals, genes related to elimination of ROS are upregulated. Redox sensing transcription factors regulate ROS detox enzymes and modify metal homeostasis [17]. Bacterial cells have proteins such as IscS and SufA which restore the activity of metal induced damage of enzymes as shown in **Figure 11**. Evidence suggests that presence of copper in the cell acts as a protective mechanism against genotoxicity since Cu catalysed Fenton reactions occur only at the periplasmic space in a cell, away from the DNA [29]. These resistance mechanisms must be studied carefully while designing antimicrobial metal drugs.

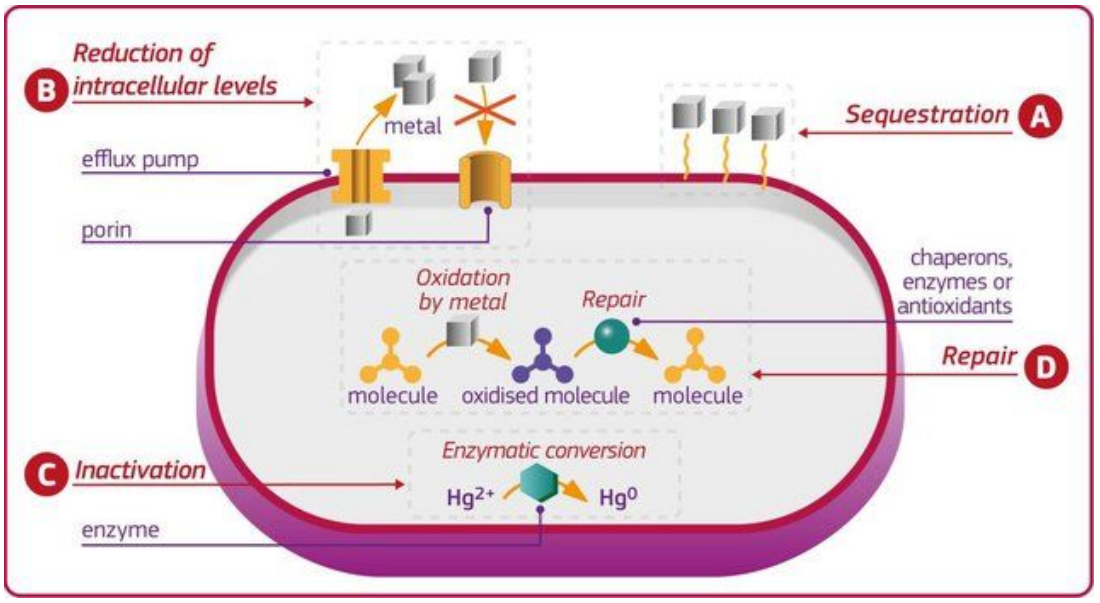


Figure 11: Various metal resistance mechanisms in bacteria. (A) Sequestration or chemically altering metal composition, (B) Reduction of metal uptake and increase of efflux outside the cell, (C) Inactivation of metals by an enzymatic conversion to a less harmful form (e.g. from Hg 2 + to Hg 0), (D) Repair of molecules vulnerable to oxidation by metals which is mediated by cellular chaperones, enzymes or antioxidants.

Source: [78]

8.0 Toxicity of metal based antimicrobials to human cells:

Silver NPs can cause cytotoxic effects in the cardiovascular and respiratory systems, osteoblasts and osteoclasts. It can cause the damage of DNA synthesis and mitochondria triggering apoptotic pathways and malformations in embryo development. The toxicity depends on the size and types of the NPs. They can affect red blood cells if the size of NPs are 50nm. Gold NPs have been tested on mice through injection and were lethal to them. It also blocks uptake pathways in maize plants. Titanium NPs with its application in cosmetics must be tested for their toxicity on the skin before commercial production. Similarly, various transition metals, rare earth metals and their NPs have a certain level of toxicity depending on the amount of NPs and exposure levels to human cells such as hepatocytes and Kupffer cells as shown in **Figure 12 which** must be studied carefully before creating the antimicrobial. The cytotoxicity properties can be managed by changing the size or shape of the NP or by using conjugating agents when used for drug delivery in humans [79].

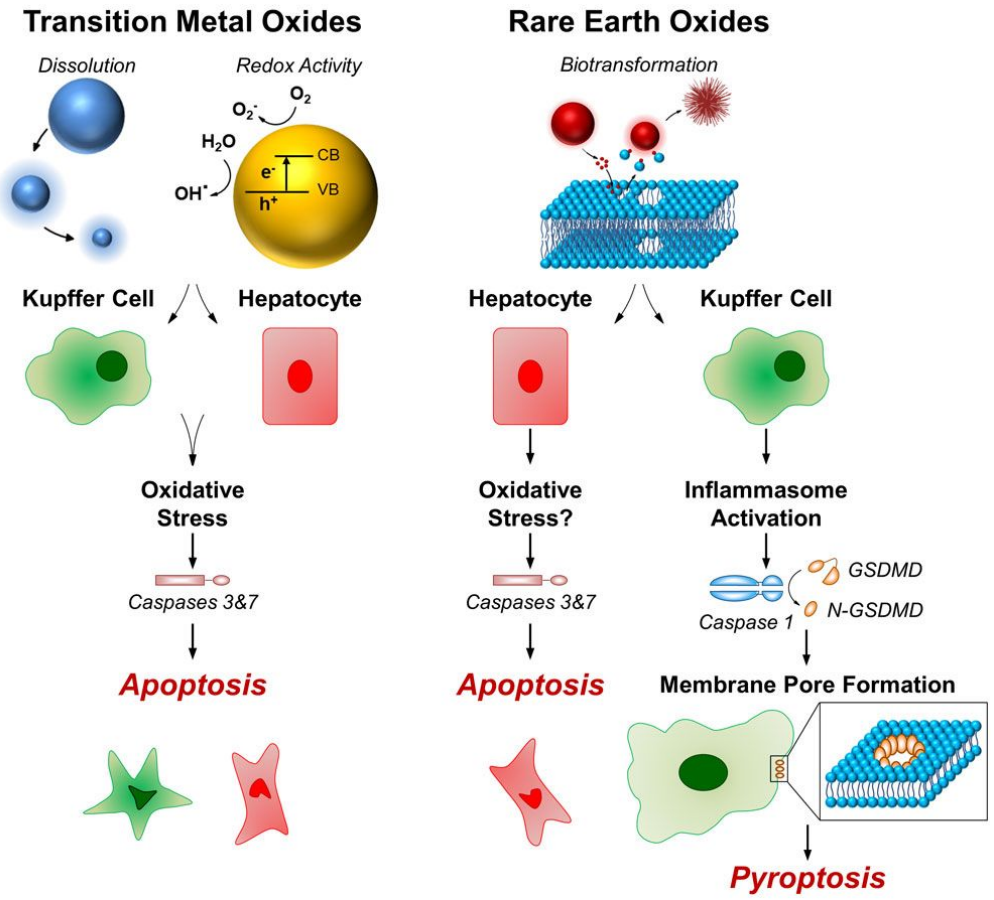


Figure 12: Certain transition metal oxides are cytotoxic to human cells like Hepatocytes and Kupffer cells and induce apoptosis through oxidative stress catalysed by the action of caspases 3 and 7. Similarly rare earth metal oxide NPs cause the death of Hepatocytes and Kupffer cells due to the action of the GSDMD protein and cell wall rupture.

Source: www.nanowerk.com

9.0 Scope for commercial applications of antimicrobial metals and metal NPs:

Metals and metal NPs have various applications in the food, textile, biofilm and pharmaceutical industries as shown in **Figure 13**. Silver and copper surfaces are commonly used as disinfectants in hospitals and laboratories as a way to contain the spread of pathogenic microorganisms [80]. Silver NPs are used in water treatment, medical devices, burn dressings and also in food preservation. Palladium alloys have a history of being used as temporary implants to prevent cardiovascular disease infections [79]. Platinum has also been used as antimicrobial in medical implants in cases of cardiovascular defibrillators, hip and

knee implants and catheters. In a study conducted by *Vaidya et al*., it was shown that palladium, gold and platinum have very high antimicrobial properties, greater than copper and silver. These metals were tested as antimicrobials with three species of bacteria including *Enterococcus faecium, Klebsiella pneumoniae* and *Acinetobacter baumannii*. Zone of inhibition tests conducted showed that the combination of metals Au/Pt, Au/Pd and Pt/Pd were most effective at higher concentrations. MIC tests represented that the best metal antimicrobial against *K.pneumoniae* was Pt at a concentration 3.9mgL^{-1}, following with gold and silver against *A.baumannii* at the same concentration and Pt and gold against *E.faecium* at a concentration 11.71mgL^{-1}. A biofilm accumulation assay showed that all metals kill all the bacteria at 500mgL^{-1} implicating that concentration plays a vital role in enhancing antimicrobial activity [81]. An experiment conducted by *Miyano et al*., showed that various metals such as Co, Ni, Cu, Zn, Zr, Mo and Pb have a high rate of killing *S.aureus* organisms when subject to film contact method, i.e., when a bacterial culture was incubated on a metal surface with a covering, rather than exposing metals to the bacteria in a shaker incubator. A transmission electron microscope image of the cells after exposure by film contact method revealed that the cell wall was completely damaged [82]. Gold NPs are tested to have anticancer activity and is dependent on the size and shape of the NP with spherical ones having the best activity. They are used for targeted drug delivery and since gold nanoclusters have fluorescence activation properties, they form complexes with fluorophore compounds that can be detected by spectroscopy. Titanium NPs are used as antimicrobials in orthopaedic implants and stitches and also to store medical equipment. Zinc NPs are highly effective when in oxide form and is used in dental prosthetics to prevent fungal growth in cases of denture stomatitis. They are also used in anticancer treatments as they promote the production of tumor suppressor protein p53 [79]. Copper, nickel and cobalt combined with amino thiols or coumarin ligands pose a heavy threat to *K.pneumoniae* and are more lethal at higher concentrations [81].

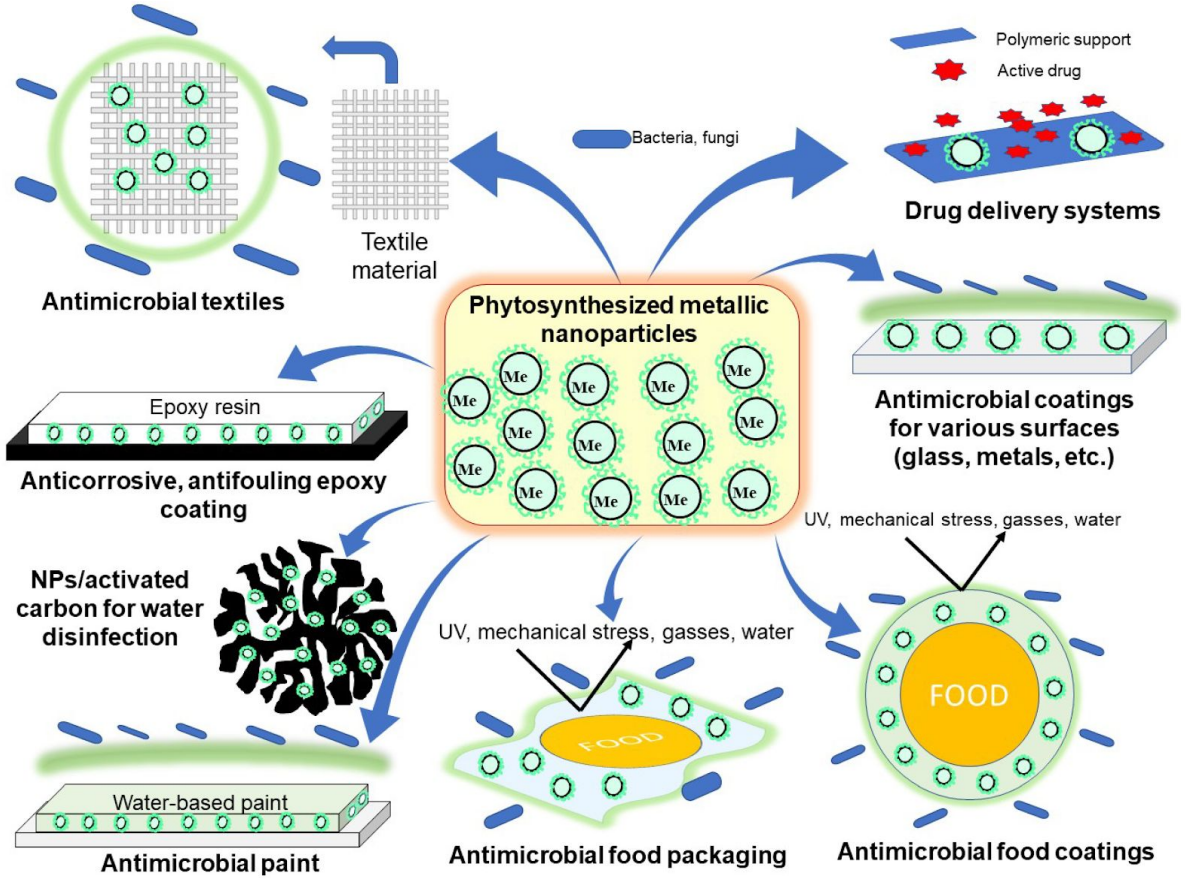


Figure 13: Metal NPs have several applications including production of antimicrobial textile materials, food packaging, water treatment, antimicrobial surface coating and making antimicrobial drug delivery complexes. Source: [83]

10.0 COPPER AS AN ANTIMICROBIAL

In 2008, the environmental protection agency in the US declared copper and its 300 alloys as antimicrobial surfaces which kills 99.9% of bacteria within 2 hours [84]. There are various proteins in a cell that contain copper as an essential metal. For example, lysyl oxidase which is responsible for collagen cross linking, tyrosinase which catalyses melanin synthesis, dopamine β-hydroxylase which plays a role in the catecholamine pathway, cytochrome-c oxidase which is an electron acceptor in the respiratory chain and superoxide dismutase which protects against oxidative stress are all enzymes which require copper in minute quantities for their proper function [85]. Deficiency of copper in the body can cause anemia and abnormal fetal development [79]. The lethal dosage of copper to wild type *E.coli* is greater than 8uM of Cu(Ⅱ). When the resistance genes CopA, CueO and CusCFBA are knocked out, the lethal dose is just about 0.25um [86]. Copper amino acid chelates are said to

have the best antimicrobial efficacy. The amino acid chelates are strain specific and have 10 times more activity against *E.faecalis* than copper ions, Cu-EDTA chelates and Cu nanoparticles [87]. Copper in combination with algaecides, bactericides and fungicides is used in water purification methods. An example of this is the use of copper against *Microcystis aeruginosa*, the main producer of microcystins in water bodies which are a major health risk to humans. A study was done comparing various copper algaecides like Cu-ethanolamine (cutrine-plus), Cu citrate and gluconate (Algimycin-PWF) with copper ions and CuSO4. Chelation allows better copper transport over cell membranes and facilitates copper to be in aqueous form longer. The main organism producing toxic microcystins in water was identified to be *Microcystis aeruginosa* and these copper chelates were tested against this organism. The rate of degradation of microcystin was measured along with the concentration of *Microcystis aeruginosa*. Exposure to copper decreased the number of the organism and the toxin production was controlled [88].

10.1 MOA of copper toxicity on cells:

Copper induces the production of ROS and damages enzymes, proteins and amino acids as mentioned in the mechanism of action of metals [86]. The ROS and superoxide production can be monitored using a NBT (nitroblue tetrazolium) assay as suggested by *Choi et al* [89]. There was no observable external superoxide formation implying that the Cu-NPs or CuO-NPs enter the cell and induce intracellular ROS production [90]. Copper is said to disrupt iron from iron-sulphur clusters of dihydroxy-acid dehydratase and isopropylmalate isomerase and also displace Zn and other metals from important proteins as shown in **Figure 14**. Transcriptome analysis of cells exposed to toxic doses of copper has shown reduced activities of Fumarase A and 6-phosphoglyceraldehyde dehydratase. Copper damages Fumarase B, an anaerobic isoenzyme of Fumarase A, showing that copper toxicity is non-oxidative. Fumarase A was restored when copper was removed from the medium showing that the cell inactivation was due to damage of Fe-S cluster and not the polypeptide itself. Ferrous iron chelator dipyridyl helps measure iron loss from enzymes exposed to copper. 10uM of Cu(Ⅰ) is said to knock off more than one atom of Fe(Ⅱ) per enzyme. The remaining Fe-S cluster is stable until further exposed to copper. Non Fe-S cluster containing

proteins were seen to be unaffected by copper and hence one of the major MOA of copper toxicity is the targeting of proteins and enzymes especially with Fe-S clusters [86].

Copper also induces DNA fragmentation. *Deinococcus radiodurans* has an enhanced DNA repair mechanism but is still as susceptible to copper toxicity as *E.coli*. Copper utilization mechanisms in a bacterial cell is not yet well understood and might be the key to understanding copper toxicity. *Synechocystis* is an organism in which ATPases facilitates copper uptake to the photosynthetic thylakoid membrane and methanotrophic bacteria secrete siderophores which scavenge extracellular copper for its utilization [84].

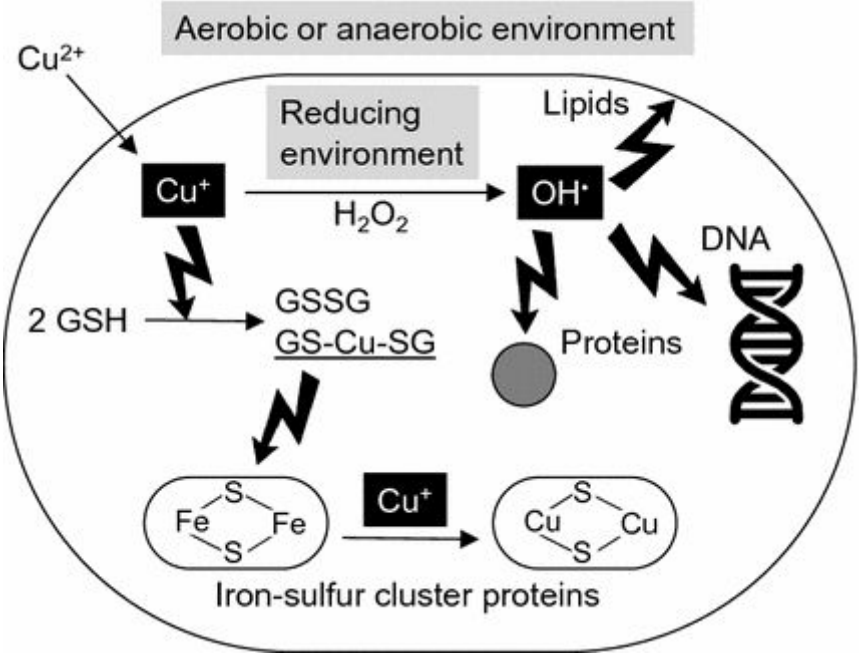


Figure 14: Copper has the ability to transition between its ionic states and in a reducing cell environment, it can cause severe damage to the cell through the production of ROS, damaging DNA and [Fe-S] clusters in proteins. Source: [91]

10.2 Copper toxicity resistance mechanisms in cells

Although copper is an essential metal in small quantities in the cell, if present in excess can be highly toxic to the cell and has been reported to cause Indian childhood cirrhosis and Tyrolian infantile liver cirrhosis in humans. Wilson's disease is a genetic disorder caused by a defective copper transporter in cells. Most cells have an active copper extrusion system as shown in **Figure 15**. *E.coli* has a distinct mechanism of copper extrusion starting with CopA which is a copper transporting ATPase in the cytoplasmic membrane and pumps excess

Cu(Ⅰ) to the periplasm. Lower levels of copper intracellularly activates the CueO enzyme which oxidises periplasmic Cu(Ⅰ) to Cu(Ⅱ) and prevents its flow to the cytoplasm. Excess levels of Cu in the cell activates the CusCFBA system which pumps copper from periplasm to extracellular environment. This was confirmed when mutants without Cus could not grow on a copper medium. *E.coli* also encodes plasmid related resistance systems which increases copper resistance in the cell. In gram positive organisms, since there is no outer membrane, only the CopA transporter extrudes excess copper out of the cell. Certain copper binding proteins and reductases support the resistance mechanism of the cell. Gene knockout studies involving deleting resistance genes allowed faster killing of organisms than the wild type organisms [84, 86].

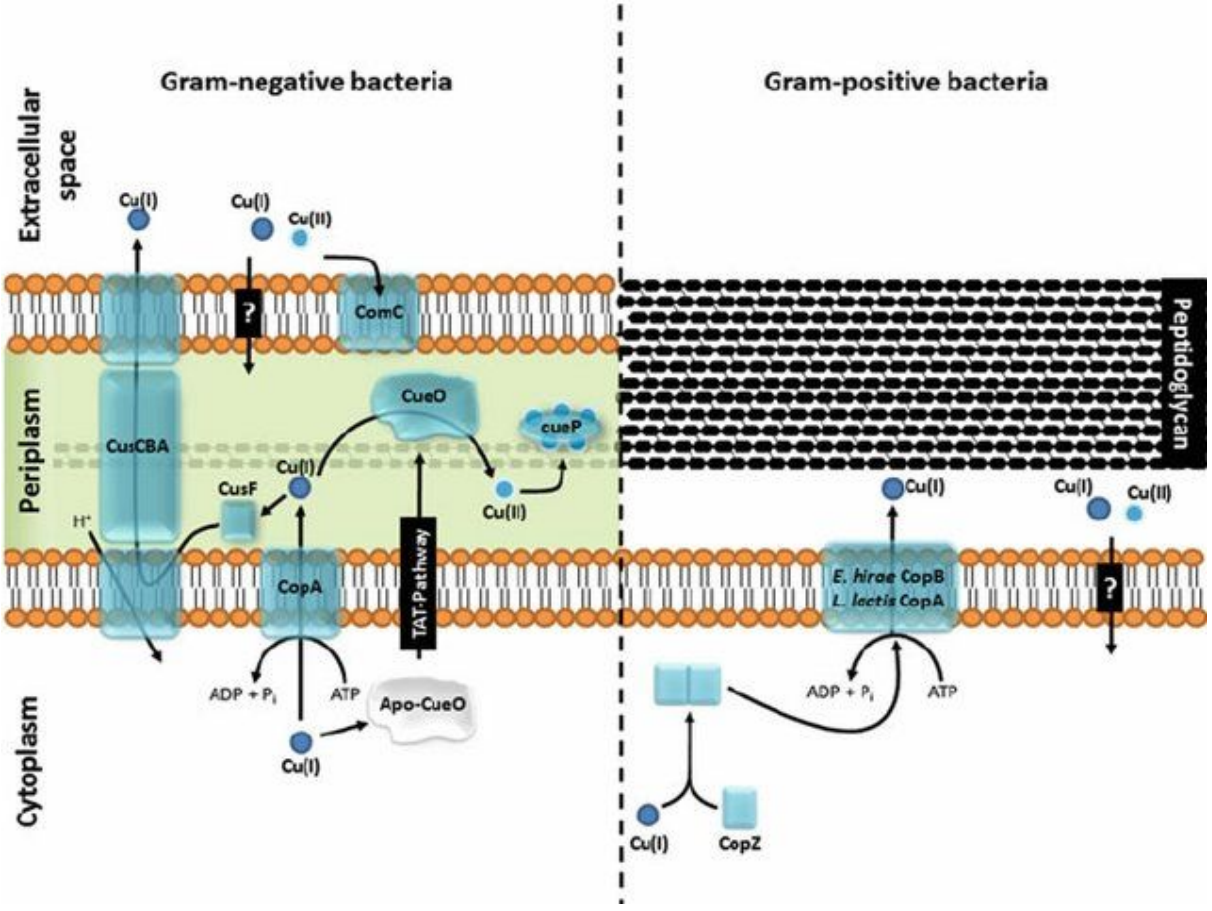


Figure15: This shows how both gram negative and positive bacteria maintain copper homeostasis. CusCBA is an efflux for copper from the periplasm, CusF is a copper chaperone that directs the copper to the CusCBA efflux system. CopA and ComC are outer membrane transporters regulating the influx of copper ions. CopA is a P-type ATPase that expels excess Cu to the periplasm.

Source: [92]

10.3 Copper nanomaterials

Copper nanomaterials are in the form of pure Copper NPs or copper oxide NPs but the former is a better antimicrobial, whereas the latter has slight cytotoxic properties. An increased surface area to volume ratio allows copper NPs to penetrate the cell membranes of microorganisms. Larger the size of the NP, higher the toxicity they hold. Toxicity also depends on the hydrophilicity and surface charge of the NP. Copper NPs are formed by several techniques including chemical reduction, laser ablation, sol-gel processing and thermal reduction. Each technique used provides different antimicrobial properties to the NPs. Exposure to UV light during the formulation of these NPs enhances their antimicrobial efficacy. Copper NPs have applications in food packaging where antibiotic resistant *Pseudomonas spp* are prevalent and can be inhibited to grow. Bimetallic NPs like CuFe NPs have a much better efficacy than pure copper NPs [79, 90].

10.4 Applications of copper and copper NPs

Staphylococcus aureus and *Acinetobacter sp* are common surface pathogens in healthcare industries. MRSA and New Delhi metallo-**β**-lactamase (NDM) are an infectious threat in the healthcare sectors. Hospital acquired infections also increase costs of treatments. Due to these factors, stainless steel surfaces are being replaced with copper surfaces which act as a very effective antimicrobial preventing unnecessary infections [84]. Copper surfaces are prevailing in the veterinary field as well. Copper oxide NPs made biogenically with the *T.divaricata* leaves (Indian medicinal plant) are known to kill the pathogen responsible for urinary tract infections. Copper NPs have applications in drug delivery, cancer cell photothermolysis and majorly in water treatment [79]. In a study conducted in two hatcheries in Brazil, it was seen that early broiler death was caused due to bacterial and fungal infections. Hatching trays made of stainless steel were sampled. *Enterobacteriaceae, Staphylococcus spp, Pseudomonas spp, Aspergillus spp* and *Penicillium spp* were identified. C11000 (99.9% Cu) copper plates were used to test the growth of these organisms using stainless steel as control. It was observed that the organisms did not survive on the Cu plates which was toxic to them. Copper surfaces, if used in hatcheries, prevent the use of carcinogenic disinfectants such as formaldehyde to control the growth of harmful pathogens. Copper sheets are currently being

used in animal production equipment like feeders, cages and drinkers. Copper nanoparticles are used in fiberglass stainless steel plates and silicon surfaces as a disinfectant [93].

11.0 CONCLUSIONS AND FUTURE PERSPECTIVES

Metals have a property highly interesting to microbiologists due to their ability to inhibit the growth of certain pathogenic microorganisms and also the ease of formation of metal nanoparticles which have the potential to be used individually or synergistically with antibiotics to tackle drug resistant pathogens. The complex mechanisms of toxicity such as cell wall or membrane damage, superoxide or ROS production, DNA damage and protein disruption with exposure to metals or metal ions makes it highly recommended for application in the antimicrobial field. Copper specifically has been used since ancient times as an antimicrobial metal and with its potential to kill pathogens upon contact, has been put to use in the medicine industry as a surface disinfectant, in water purification to kill water borne pathogens and in agriculture to kill harmful soil microorganisms. Copper and its oxides along with other metals have been used to make nanoparticles which have the capacity to be used as an ingestable drug. Toxicity mechanisms to human cells must be studied carefully in order to use copper or any other metal NP as a drug to treat pathogen induced diseases. Although cells have resistance mechanisms to metals, as long as the DNA is completely damaged, there is no worry about resistance being transferred laterally. Evidence suggests that there are no mutants discovered so far which have resistance to metal based antimicrobials. Transcriptome analysis and observation of the regulation of genes in organisms being killed by metals would elucidate the exact working mechanisms of these antimicrobial metals. With the advent of modern molecular biology, bioinformatics and microbiology techniques, personalised medicines and pathogen specific medicines could be produced and would be the future of tackling diseases.

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