

BIOCIDAL PROPERTIES OF COPPER NANOPARTICLES

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Abstract

Metal nanoparticles (NPs) with antibacterial properties represent a promising alternative approach to antibiotics, whose overuse has led to the appearance of drug-resistant bacteria. This article addresses particularly copper (Cu) nanoparticles since Cu is a structural constituent of many enzymes in living microorganisms. In addition, Cu has a better antibacterial effect and minimal cost compared to silver. The properties of Cu nanoparticles are described here: antibactericide, toxicity mechanisms, oxidation, and copper oxide biocompatibility for medical applications. Along with the advantages of Cu nanoparticles, the nanotoxicity still remains to take into consideration such as in targeting different bacteria strains, bacteria's resistance, the effect of size, the effect of NP chemical composition, the effect of oxidation, and the corona phenomenon effect. The methodology of Cu nanoparticles synthesis, related to the biocidal effect, is illustrated by some limitations and some breakthrough such as chitosan stabilizer (CS), laser ablation, plasma induction, and flow-levitation method (FL). Although Cu nanoparticles are beneficial for bacterial elimination, these nanoparticles are graded harmful to the human body and the environment because of their toxic effects. Thus, it requires further improvement and further investigation to create super antibacterial Cu nanoparticles, to develop some interesting research work around this subject, and to reveal some promising medical findings.

Keywords: antibacterial properties, nanotoxicity, biocompatibility, copper nanoparticles

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Introduction

In this day and age, nanoparticles (NPs) applied to biology and medicine are found in various applications such as fluorescent biological labels, drug and gene delivery, biodetection of pathogens and proteins, probing of DNA structure, tissue engineering, tumour destruction via heating (hyperthermia), separation and purification of biological molecules and cells, MRI contrast enhancement and on a variety of surfaces to make them water repellent, anti-glare, self-cleaning, ultraviolet light resistant, scratch resistant, and even anti-microbial, antiseptic or disinfectant [1-4]. Several classes of antimicrobial NPs and nanosized carriers for antibiotics delivery have proven their effectiveness for treating infectious diseases, including antibiotic-resistant ones, *in vitro* as well as in animal models [5]. The application of nanomaterials as new antimicrobials should provide new modes of action and/or different cellular targets, compared with existing antibiotics which are somehow promoting multiple drug resistance microbes [6].

To date, the impact of NPs on various microbes (bacteria, biofilm, fungi, and viruses) has not yet been fully studied and their mechanisms of action are not thoroughly known [7-9]. In short, a biofilm is composed of bacterial cells attached to a surface, mixed in extracellular polymeric substances (EPS) produced by the bacteria [10]. It was reported that biofilms are more resistant than planktonic bacterial cells to various stress factors, including heavy metals, toxins, and bactericidal agents. However, the properties of materials at the nanoscale are different from those of the same materials at the micro and the macro scale [11-13]. Since some of these properties are not yet fully understood, researchers are occupied with investigating the possible toxic impact of NPs on microorganisms and endeavouring to pinpoint the toxicity mechanisms involved [14-17].

In this paper, first, we will review some socio-economic data on nosocomial infections and the importance of preventing biofilm formation in the current problematic of bacterial resistance against antibiotics. Second, we will examine how copper nanoparticles can be a promising alternative approach in terms of toxicity for microbes and biocompatibility for human cells. Finally, we will go over the main synthesis methods available nowadays and some strategies to overcome the oxidation layer in order to maintain the integrity of the NPs' properties.

Nosocomial infections (NIs): A Hospital-Acquired Infection

Nosocomial infections (NIs) are an important focus of infection prevention in all countries, but in developing countries, they are a major cause of preventable disease and death [18]. For example, NIs rates range from as low as 1-7% in several European countries and the Americas, to more than 40% in parts of Asia, Latin America, and sub-Saharan Africa [19]. In 1987, a prevalence survey, involving 55 hospitals in 14 developing countries in 4 World Health Organization Regions (Europe, Eastern Mediterranean, South-East Asia and Western Pacific), found an average of 8.7% of all hospital patients had NIs [20]. Thus, at any time, over 1.4 million patients worldwide will have infectious complications acquired in the hospital. According to the European Centre for Disease Prevention and Control, about 4.1 million patients are affected by healthcare-associated infections in the EU every year, including approximately 37 000 lethal cases. The most frequent are related to urinary tract infections (UTIs) (28%), followed by respiratory tract infections (RTIs) (25%) and bloodstream infections (BSIs) (10%) [21].

Many infections are associated with medical devices, such as catheters (urinary, vascular), surgical implants (e.g., vascular grafts, cardiac pacemakers), and mechanical ventilation devices [22,23]. The most significant of them (also shown in FIG. 1) are: i) infections following surgery or invasive medical procedures; ii) UTIs, pneumonia, diarrhoea, and skin lesions; iii) maternal and new-born infections. Overall, implant-associated infections contribute to increased patient morbidity and cost. The adhesion of serum proteins to the implant and the low vascularity in the area of the trauma, create an ideal environment for bacterial adherence [24].

The organisms causing most NIs usually come from the patient's own body (endogenous flora). They may also come from contact with staff (cross-contamination), contaminated instruments and needles, and the environment (exogenous flora) [25,26]. Virtually, every pathogen has the potential to cause infection in hospitalized patients, but only a limited number of both Gram-positive (Gr+) and Gram-negative (Gr-) bacteria are responsible for the majority of NIs. The most common pathogens that cause NIs are *Staphylococcus aureus* (*S. aureus*), *Pseudomonas aeruginosa* (*P. aeruginosa*), and *Escherichia coli* (*E. coli*) [27]. The most prominent pathogens include methicillin-resistant *S. aureus* (MRSA) and methicillin-sensitive *S. aureus*, that are two of the main causes of pneumonia and surgical wound BSIs, [28,29] followed by *E. coli*, a remarkable food-borne pathogen. *E. coli* is one of the most frequent causes of many common bacterial infections, including cholecystitis, bacteraemia, cholangitis, UTIs, traveller's diarrhoea, and other clinical infections, such as neonatal meningitis and pneumonia. In the US, *E. coli* is the leading cause of both community acquired and nosocomial UTIs. As many as 50% of women have had at least one episode of UTIs in their lifetime. *E. coli* causes 12-50% of NIs, and 4% of diarrhoeal disease [30].

Additionally, both *Klebsiella pneumoniae* (*K. pneumoniae*) and *P. aeruginosa* are opportunistic pathogens of pronounced multidrug resistance in individuals with impaired immune systems. Other important candidates include penicillin non-susceptible *Streptococcus pneumoniae*, *Enterococcus faecalis*, and vancomycin resistant *Enterococci* [21].

The origin of implant-associated infections can be tracked back to the implantation site, where bacterial colonization and subsequent biofilm formation generate a surface layer highly inert to current medical treatment. Consequently, in many cases, implant removal and/or amputation represent the only alternatives [31]. Upon implantation into the body, a "race for the surface" is initiated between tissue cell integration and microbial colonization, which is generally decided within several hours. Since most of the patients are immunocompromised, the host defence usually fails to prevent colonization prior to tissue integration [32]. Most of the pathogens are found ubiquitously on the patient's skin or in the patient's body and, thus, can freely access the implantation site. In the first 2 h after implantation, bacterial colonization is governed by reversible physical adsorption. After 2-3 h, chemical bridging reactions ensure a much stronger adhesion between bacteria and substrate. Subsequently, within a period of 24 h or longer, the excretion of extracellular substances ensures both microbial multiplication and resistance to mechanical, immunological and medical removal attempts [33]. This biofilm formation is highly dependent on the bacterial species and some colonized devices may not become clinically infected at all, or the infection can emerge only after several months after implantation [22].

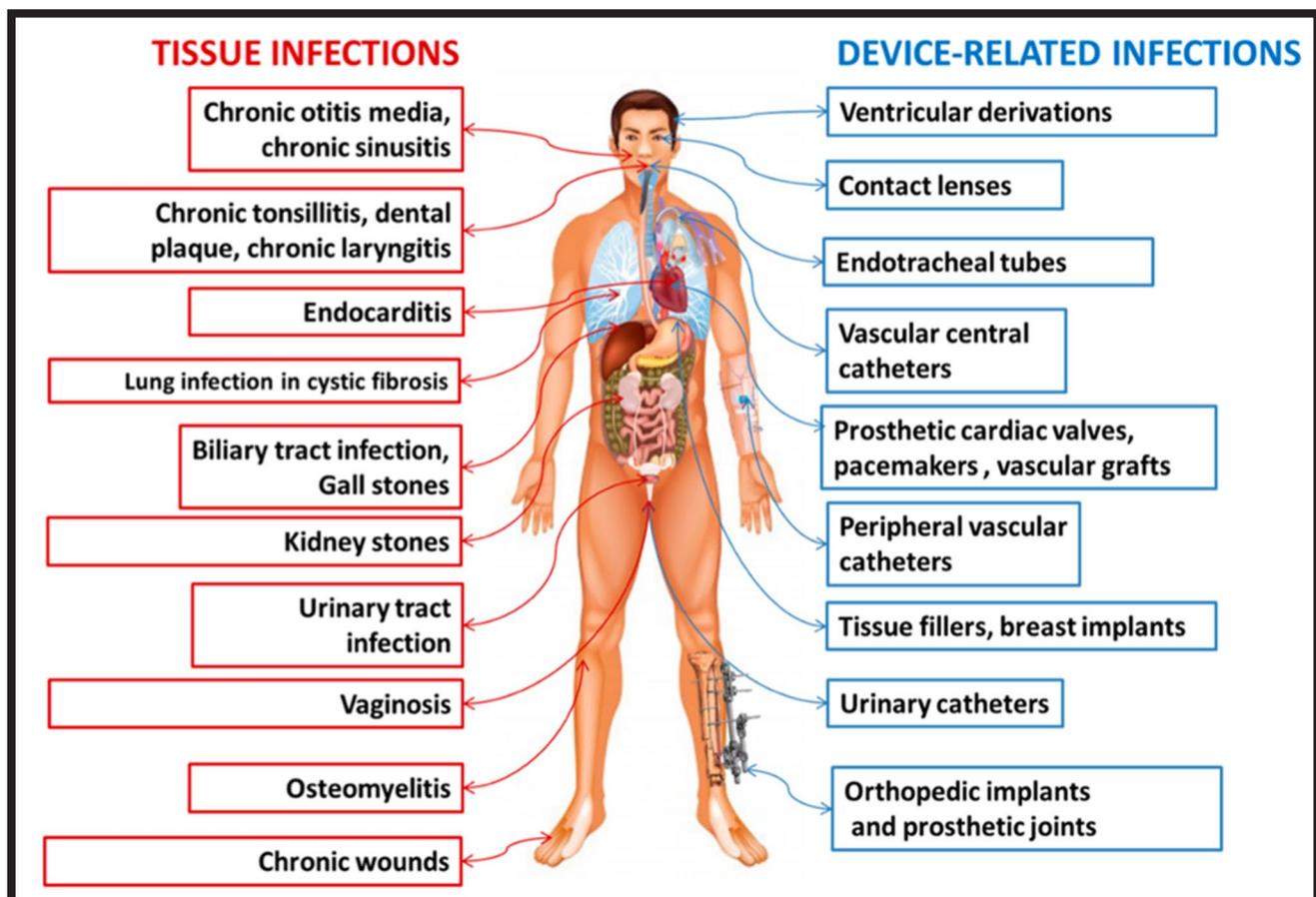


FIG. 1. Typical bacterial infections and their respective biomedical devices [25].

Biofilms are More Resistant than Planktonic Bacteria [34]

A biofilm is composed of bacterial cells attached to a surface, mixed in Extracellular Polymeric Substances (EPS) produced by the bacteria [10]. It is a natural tendency of microorganisms to attach to wet surfaces, to multiply and to embed themselves in a slimy matrix composed of EPS that they produce, forming a biofilm. The National Institutes of Health revealed that among all microbial and chronic infections, 65% and 80%, respectively, are associated with biofilm formation [35]. There is good evidence indicating that the biofilm mode of life leads to increased resistance to antimicrobial products [36,37]. Biofilm-associated bacteria are more resistant to antimicrobials, compared to planktonic cells, and this makes their elimination a great challenge [38,36]. Moreover, the emergence of bacteria resistant to conventional antimicrobials clearly shows that new biofilm control strategies are required [38,37]. As biofilm formation mechanisms will only be discussed briefly, the reader is directed to several excellent comprehensive reviews in this area [10,39,40].

The attachment of microorganisms to surfaces and subsequent biofilm development are complex processes, affected by numerous variables including heavy metals, toxins, and bactericidal agents. Several recent studies [41–45] have shown a role of cell-to-cell chemical signaling (quorum sensing) in biofilm formation [41], where this system determines extracellular matrix fine-structure by controlling its secretion through the bacterial cell wall [42]. Biofilms form a gel phase, within which microorganisms live [43]. The EPS matrix acts as a barrier, in which diffusive transport prevails over convective transport [43]. A function frequently attributed to EPS is their general protective effect on biofilm microorganisms against adverse conditions. As an example, it has frequently been observed that biofilm cells can tolerate high concentrations of biocides [36,44,45]. The EPS matrix delays or prevents antimicrobials from reaching target microorganisms within the biofilm by diffusion limitation and/or chemical interaction with the extracellular proteins and polysaccharides [44]. Moreover, within the EPS matrix, the molecules required for cell–cell communication and community behaviour may accumulate at concentrations high enough to be effective [43]. Ideally, preventing biofilm formation would be a more logical option than treating it. However, there is presently no known technique that can successfully prevent or control the formation of unwanted biofilms without causing adverse side effects.

The main strategy in preventing biofilm formation is to clean and disinfect regularly, before bacteria attach firmly to surfaces [46,47]. Several attempts have been made to avoid biofilm formation by the incorporation of antimicrobial products into surface materials, by coating surfaces with antimicrobials [48] or by modifying the surface physicochemical properties [49]. Other authors reported biofilm formation inhibition by coating surfaces with silver [50]. It was observed that planktonic organisms are more susceptible to environmental stress and bactericidal agents, compared to biofilms [51].

In the environment, bacteria can attach to various surfaces and live as an organized biological system, in a secreted glycocalyx matrix [52,53]. This biofilm provides additional protection for bacteria from environmental stress, toxins, and bactericidal agents. Costerton et al. (1999) [54] hypothesized that the ionic bonds between the bactericidal agent and the glycocalyx matrix caused the depletion of bactericidal agents with a depth of the biofilm matrix. It was reported that bacteria in biofilm produce more EPS under environmental stress or exposure to toxic compounds [55]. Interestingly, the chemical and physical properties of EPS may vary among bacterial types and strains [56].

Bacterial Resistance to Antibiotics is an Increasing Threat

Another major limitation of antimicrobial therapy is the development of bacterial resistance to antibiotics. The resistance of bacterial pathogens to traditional antibiotic therapy is an increasing threat. The resistance of microorganisms to antibiotics is steadily rising, with reports showing that quite a number of the presently recognized antimicrobial agents are less effective and resisted by one species of microorganisms or another, so it appears to be no single antimicrobial agent, available for human and animal use, which is not resisted by some microorganisms. Gram-negative bacteria include pathogenic strains of *E. coli*, *Acinetobacter baumannii* (*A. baumannii*), *K. pneumonia* and *P. aeruginosa*, all of which, are increasingly resistant to most available antibiotics. More than 70% of bacteria causing infections are now resistant to at least one of the drugs most commonly used for treatment. A global medical challenge in the 21st century is the treatment of vancomycin-resistant microbes, because vancomycin is the latest generation of antibiotics and is assumed most effective against *S. aureus* infection [42]. One of the most recent new wave of “super super bugs” came with the emergence of mutant New Delhi metallo beta-lactamase NMD-1, which is an enzyme that confers bacterial multiple drug resistance [57]. It first emerged in New Delhi and has now spread worldwide from Britain to New Zealand. In 2009, *K. pneumoniae* was the first bacterium identified to produce NMD-1 in a patient with an infection that did not respond to many antibiotics [58]. Two classes of antibiotic-resistant pathogens are emerging as major threats to public health. First, MRSA is estimated to cause ~19,000 deaths per year in the U.S. [59]. Apart from their high mortality rate, MRSA infections lead to an estimated \$3-4 billion US dollars of additional health care costs per year. Further, the rising prevalence of MRSA increases the likelihood that vancomycin resistant *S. aureus* [60]—just as deadly as MRSA, but more challenging to treat—will become a new scourge in hospitals. Pathogens from the second class, multidrug-resistant (MDR) and pan-drug-resistant (PDR) Gr- bacteria, are less prevalent than MRSA, but they pose the grave threat of infections that are truly untreatable [61]. These strains of *A. baumannii*, *E. Coli*, *K. pneumoniae*, and *P. aeruginosa* are resistant to some MDR or all PDR of the antibiotic classes commonly used to treat Gr- bacteria such as penicillin, cephalosporins, carbapenems, monobactams, quinolones, aminoglycosides, tetracyclines, and polymyxins [61]. Prospects for finding new antibiotics for Gr- pathogens are especially poor: their outer membranes block the entry of some antibiotics, and efflux pumps expel many of the remainder. Despite the rise of resistant pathogens, the rate of new antibiotic approvals is dropping. Where will new antibiotics come from? In the past, this question was generally answered through the synthetic tailoring of a small group of “scaffolds.” Novel therapeutic strategies to tackle bacterial infection are imperative to maintain the ability to rapidly evolve antibiotic treatment regimens.

Nanotechnology approach

In comparison to conventional antibiotics, nanostructured antimicrobial agents help in reducing toxicity, overcoming resistance, and lowering the cost. Metal and metal oxide NPs offer a new line of research in combating infectious diseases due to resistance developed by several pathogenic bacteria against antibiotics. An advantage of these nano-antibiotics is that naturally occurring microbes have so far not developed a known resistance against them.

Moreover, they use multiple biological pathways to exert their antimicrobial mechanisms such as disruption of the cell wall, inhibition of DNA, protein, or enzyme synthesis, generating photocatalytic reactive oxygen species (ROS), and some unexpected activities which are absent in previously used antibiotics and disinfectants. In addition, the preparations of these NPs are more cost-effective than antibiotic syntheses, they are also more stable during long-term storage and, unlike antibiotics, can withstand harsh processing conditions, such as high pH and temperature, without being inactivated [62].

Copper (Cu) and Copper Oxide (CuO) as Antibacterial Candidates

There are two main reasons for inspecting the biological activity of copper NPs (Cu-NPs). Firstly, as Cu-NPs are released into a natural ecosystem and human environment when applied as catalyst components, anticorrosive coatings, and conducting materials, it is of utmost importance to consider the risks caused by them [63,64]. Cu is a structural constituent of many enzymes in living organisms and it can generate toxic effects at high concentration, when in free ionic form, by generating ROS that disrupt the DNA and amino acid synthesis [65]. Indeed, Cu-NPs could then present some risks, since an excess of Cu in the human body leads to the generation of the most damaging free radicals, such as the hydroxyl radical ($\bullet\text{OH}$) [66]. Secondly, Cu-NPs could play important roles in developing a new generation of drugs for medicine and agriculture thanks to their known antibacterial potential [67,68]. Therefore, it becomes necessary and of interest to identify the underlying mechanism(s) of its biological activity.

The antimicrobial activity of Cu-NPs against several microorganisms has already been reported [69–73]. One of us (Deryabin et al., 2016) [74], for instance, showed in a comparative study with 8 metal and metal oxide NPs, and 10 carbon-based NPs, that the Cu- and CuO-NPs had the highest antibacterial activity against *B. subtilis* and *E. coli* but were less effective against the marine bacterium *Photobacterium phosphoreum*. A study investigating the toxicity of Cu-NPs on microorganisms by Yoon et al. [71] reported their preliminary studies on the toxicity of Cu-NPs on *E. coli* and *B. subtilis* using agar plate assay. Their assay showed the antimicrobial characteristics of the particles on both microbes tested. Moreover, Cu-NPs are very reactive because of their high surface-to-volume ratio and can easily interact with other particles and increase their antimicrobial efficiency. For instance, Cu monodispersed NPs (2–5 nm) have revealed a strong antibacterial activity and were able to decrease the microorganism concentration by 99.9% [69].

Ren et al. (2009) [75] investigated the antimicrobial potential of CuO-NPs generated by a thermal plasma technology that contained traces of pure Cu- and CuO-NPs, against a range of bacterial pathogens, including MRSA and *E. coli*. Their study revealed that the ability of CuO-NPs to reduce the bacterial populations to zero was enhanced in the presence of a subminimal inhibitory concentration of silver or copper NPs. However, Cu is a component in many vital processes in cells including microorganisms. Therefore, bacteria will want to find an adequate balance in Cu quantity as a cofactor to prevent toxicity from a high amount [76,77]. The minimum inhibitory concentration being the lowest concentration of NPs that completely inhibits the initiation of growth of a bacterium. Ruparelia et al. (2008) [78] showed that Cu-NPs have a greater affinity toward the carboxyl and amine groups at high density on the surface of *B. subtilis*, than that of silver NPs, showing superior antibacterial activity. CuO, being less costly than silver, and easily miscible with polymers, can be an alternative to silver NPs.

Toxicity Mechanisms of Copper-NPs Compared to Copper Microparticles and Bulk Forms

Cu is an essential micronutrient, being part of the structure of several enzymes and other proteins. It is also a redox-active metal, existing in two major oxidation states, Cu^{1+} and Cu^{2+} . The redox cycling of Cu between Cu^{1+} and Cu^{2+} catalyses the intracellular production of hydroxyl radicals, $\bullet\text{OH}$ [79,80]. Cu is an important cofactor for many enzymes, although some *in vitro* analysis, using comet assays and Trypan blue staining, suggested that Cu-NPs cause DNA damage and cytotoxicity [81].

Since cytotoxic effects reflect the holistic damage induced by the particles, the cytotoxicity of Cu-NPs, as well as Cu-Microparticles (MPs) and soluble Cu^{2+} were tested against the host strain, *E. coli* RFM443 [81]. It was found that Cu-NPs possess higher biotoxicity to the ecosystem than Cu-MPs and Cu^{2+} , due to their multiple and severe damage effects at much lower toxic concentrations. Moreover, Cu-NPs induce not only oxidative stress in *E. coli*, but also protein, DNA, and cell membrane damage, and ultimately cause cell growth inhibition. This was demonstrated in a study, in which small Cu-NPs (4–5 nm) caused similar dose-dependent degradations of isolated DNA molecules, via the generation of singlet oxygen $^1\text{O}_2$ in both U937 (from human lung lymphoblast) and HeLa (from cervical cancer cells) cells [82].

Li et al. (2013) [83] used spherical, homogeneous, crystalline Cu-NPs and Cu-MPs, with primary particle sizes of about 90 nm and 10 μm , respectively; when exposed to the ambient atmospheric conditions, both surfaces became partly oxidized into CuO. Compared with Cu-NPs, Cu-MPs showed totally different effects on cells and no obvious responses of any strains were observed, although these two kinds of particles had the same chemical composition. These results imply that the major toxic effects of Cu^{2+} ions are membrane damage and protein damage, which occur only at high concentrations (>140 mg/L), whereas Cu-NPs cause all four kinds of damaging effects (i.e., oxidative stress, and DNA, membrane, and protein damage) at low concentrations (maximum at 80 mg/L). Moreover, the different concentration profiles and damage effects between Cu-NPs and Cu^{2+} ions suggest that the toxic mechanism of Cu-NPs probably does not depend on Cu^{2+} ions.

Cu is an essential nutrient in many organisms, and enzyme-associated Cu is a requirement for aerobic metabolism. On the other hand, excess accumulation of Cu, or intracellular release of free Cu, leads to severe toxicity. Aerobically, Cu readily catalyses reactions that result in the production of hydroxyl radicals $\bullet\text{OH}$ through the Fenton and Haber-Weiss reactions ($\text{Cu}^{1+} + \text{H}_2\text{O}_2 \rightarrow \bullet\text{OH} + \text{HO} + \text{Cu}^{2+}$; $\bullet\text{O}_2 + \text{Cu}^{2+} \rightarrow \text{Cu}^{1+} + \text{O}_2$) [84,85]. The highly reactive oxygen intermediates are responsible for lipid peroxidation, oxidation of proteins, and cleavage of nucleic acids [85–87]. Additionally, free Cu-ions are able to oxidize sulfhydryl-groups, such as cysteine in proteins or the cellular redox-buffer glutathione [87,88]. Finally, Cu might cause inactivated proteins by replacing related metal cations such as those of zinc or iron from active sites.

Therefore, intracellular free Cu-concentrations are low and tightly regulated [89]. Thus, tight regulation of uptake, distribution and excretion of Cu is necessary for maintaining optimal cellular Cu levels. Defining the molecular mechanisms of Cu metabolism, including Cu uptake, intracellular trafficking, incorporation into Cu-requiring proteins, excretion, and regulation of these processes, is an important emerging research area. While high affinity Cu transporters play a critical role in Cu acquisition, excess Cu can enter cells by non-specific mechanisms. Once Cu is transported across the cell membrane, it must efficiently reach its appropriate destinations to be incorporated into Cu-requiring proteins without participating in harmful reactions. It has been demonstrated that target-specific cytosolic Cu carriers distribute Cu in eukaryotes ranging from yeast to humans [78,90,91].

Toxicity Mechanisms of Copper Oxide-NPs Compared to Copper Oxide-Microparticles and Bulk Forms

Since there is a published review paper available regarding the effects of DNA damage induced by CuO-NPs in *in vitro* studies [92], only a few studies are mentioned here. DNA damage, as a result of oxidative stress, identified by increased levels of 8-isoprostane and the ratio of glutathione disulphide to total glutathione (GSSG/GSH) in human airway epithelial (Hep-2) cells, has been reported [93]. Oxidative stress increased the expression of plasminogen activator inhibitor-1 (PAI-1), by mediating p38 phosphorylation in endothelial cells treated with CuO-NPs (42 and 200 nm) [93]. Elevated oxidative stress may lead to DNA damage, which, in turn, has the potential for carcinogenesis. In another study by Karlsson et al. (2008) on A549 cells (from adenocarcinomic human alveolar basal epithelial cells), CuO-NPs were the most potent, regarding cytotoxicity and DNA damage [94].

In a study conducted in 2009 by K. Kasemets et al. [95], toxic effects of ZnO-, CuO- and titanium oxide (TiO₂)-NPs on the single-cell eukaryotic organisms *Saccharomyces cerevisiae* (yeast), were evaluated. The effect of metal oxide NPs, as well as bulk oxide and ion formation, were compared. Both forms of ZnO showed the same toxicity, while CuO-NPs were 60 times more toxic than bulk CuO. There was increased toxicity for both CuO-NPs and bulk CuO after 24-hour exposure when compared to an 8-hour exposure, due to an increased Cu ion dissolution. CuO-NPs were found about 60-fold more toxic than bulk CuO. In fact, at 8 h, the maximal effective concentrations needed to induce 50% response after exposure called EC50, for NPs and bulk, were, respectively, 20.7 and 1297 mg CuO/L and, 13.4 and 873 mg/L at 24 h. The increase in toxicity of both CuO forms after 24 h was due to the increased dissolution of Cu ions from CuO over time. A comparison of EC50 values for CuO-NPs, bulk CuO and Cu²⁺, with bioavailable Cu concentrations in the growth medium, showed that the solubilized Cu ions explained only about 50% of the toxicity of both NPs and bulk CuO.

Three studies on CuO-NP-induced DNA damage in *in vitro* cellular models reported detection of significant single-strand break (SSB) lesions, using the alkaline comet assay [81,77,94], and two of the studies specifically applied the Fpg-modified comet assay (formamidopyrimidine-DNA glycosylase as a biomarker for the detection of oxidative DNA damage) to detect significant SSBs, due to the accumulation of oxidatively modified purine lesions [77,94]. Interestingly, all three studies noted significant oxidative damage to DNA at NPs concentrations of 80 µg/mL and, in each case, the DNA damaging effects of CuO-NPs were stronger than those of CuO-MPs or than added Cu²⁺ ions, or Cu²⁺ ions released from the CuO-NPs themselves. The mechanisms behind the induction of DNA damage are not clear, but it is apparent that released Cu²⁺ ions are not the causative factor for *in vitro* oxidative stress and the resulting DNA damage.

Cells treated with CuO-NPs presented reduced catalase and glutathione reductase enzyme activities and elevated glutathione peroxidase activities. The increasing ratio of glutathione disulphide (GSSG) to glutathione (GSH) indicated that CuO-NPs produce ROS and obstruct cellular antioxidant defences [96]. It is valuable information that CuO-NPs had higher toxicity than other metal oxides *in vitro* [97].

Moreover, it was shown that both CuO-NPs and bulk CuO exhibit higher toxicities on biofilms, compared to the low toxicity of 35% observed for Cu ions on planktonic cells. The concentration of Cu ions had to be 250 ppm to suppress bacteria growth completely; however, 90% of biofilm growth could be inhibited by 0.02 ppb [76]. Nonetheless, the detailed mechanism needs to be further investigated.

Cu/CuO-NPs caused a range of effects, including oxidative stress, cytotoxicity, neurotoxicity, DNA damage and DNA lesions, in a variety of cell lines. For Cu ions, the mechanism may involve oxidative stress [67]. The redox cycling of Cu ions results in the depletion of glutathione and affects the sulfhydryl groups of proteins, causing DNA damage and lipid oxidation [79]. Likewise, other *in vitro* studies using cultured lung epithelial cells have shown that exposure to Cu containing NPs led to increased intracellular ROS formation, oxidative DNA damage, and cell death [98,99]. Rushton et al. [100] showed that the native oxidant potential of metallic Cu-NP powders – as determined via oxidation of dichlorodihydrofluorescein and other methods – correlated well with oxidant stress related inflammatory mediator production in cultured cells and with their acute *in vivo* inflammatory potency.

Copper NPs Biocompatibility for Medical Applications

In 2008, Williams et al. [101] redefined biocompatibility as follows: “biocompatibility refers to the ability of a biomaterial to perform its desired function with respect to a medical therapy, without eliciting any undesirable local or systemic effects in the recipient or beneficiary of that therapy but generating the most appropriate beneficial cellular or tissue response in that specific situation and optimising the clinically relevant performance of that therapy”. In this context, NP toxicity refers to the ability of the particles to adversely affect the normal physiology, as well as to directly interrupt the normal structures of organs and tissues in humans and animals. It is widely accepted that toxicity depends on physicochemical parameters, such as particle size, shape, surface charge, chemistry, composition, and NP stability.

Cu is considered as an essential micronutrient, necessary for the proper growth, development and maintenance of bone, connective tissue, brain, heart and numerous other organs [102–104]. It is also involved in the stimulation of the immune system to fight infections, repair injured tissues and promote healing [105], and it aids neutralization of free radicals, which cause severe cell injury [106]. The average level of stored Cu in the body (mostly in the liver) is approximately 120–150 mg. Cu is an essential element for maintaining homeostasis in organisms [107]. Cu ions will become toxic once their concentration is not tolerated in the physiological range [108,109].

Toxicity assessment studies have primarily focused on investigating the effects of different exposure routes, such as the respiratory or gastrointestinal tracts. Yokohira et al. [110] analysed the carcinogen test from lung biopsy after intratracheal instillation of CuO-NPs. Histopathological assessment showed that they induced severe acute inflammatory changes in the rat lung at high doses, and chronically at low doses or with frequent instillations. Karlsson et al. [94] illustrated that CuO-NPs could lead to cell cytotoxicity and DNA damage in the human lung epithelial cell line A549. Oxidative lesions were verified by measuring the intracellular production of ROS with the oxidation-sensitive fluorescent probe, 2',7'-dichlorofluorescein diacetate. Associating the relationship between ROS generation and DNA damage, Chen et al. [111] reported that oxidative stress caused by CuO-NPs was the prior toxic effect. França et al. (2013) [112] investigated the effects of three chitosan differing only in their degrees of deacetylation and of carboxyethyl chitosan, as candidates for Fe₃O₄ NPs coatings. All the samples were found to be biocompatible and nontoxic before sterilization and remained so subsequently.

Cu ions may cause toxicity once they exceed the physiological tolerance range *in vivo* [113]. Therefore, the possible health effects and toxicology of CuO-NPs have caused great concern to both the public and scientific researchers.

Generally, metallic NPs show antibacterial and antifungal activity, even though there are environmental and human safety concerns regarding the release and consumption of metal NPs which are yet to be explored. Excessive release of silver, for example, causes environmental pollution which in turn makes silver harmful to humans and animals. And Cu is no exception, because an excess of Cu in the human body leads to generation of the most damaging radicals, such as the $\cdot\text{OH}$ [66]. However, there are Cu-transporting adenosine triphosphatases; including ATP7A and ATP7B, which play an important role in Cu homeostasis and export excess Cu through the intestine (ATP7A) as feces, the liver (ATP7B) as a bile product, and the mammary gland (ATP7B) as milk [66,114].

Copper NPs Nanotoxicity: Concerns and Challenges

Strain Differences: *E. coli* (Gr-) and *S. aureus* (Gr+)

It was previously hypothesized that a thick peptidoglycan layer of bacteria is a significant barrier to the NPs but in fact Gr+ bacteria often are more sensitive than Gr-. Data from previous studies suggest that NPs toxicity may vary significantly between Gr+ and Gr- bacteria, and in some cases, vary by organism [17]. For example, a range of studies showed higher antibacterial action of silver NPs against Gr- rods than against Gr+ cocci. The results indicated higher sensitivity of *E. coli* than *S. aureus*: the minimum inhibitory concentration against *E. coli* was in the range of 3.3-6.6 nM while against *S. aureus* – more than 33 nM. These differences in susceptibility were explained by the different structure of the cell wall, that is, by the higher concentration of peptidoglycan in the cell wall of *S. aureus*. Also, because peptidoglycan is a unique structure of bacteria absent in mammal cells Cu-NPs are promising agents with directed action on microorganisms by the facilitated influx of smaller-sized NPs into the cell wall of Gr- bacteria which consists of a unique outer membrane layer and a single peptidoglycan layer as compared to the cell wall of Gr+ bacteria with several peptidoglycan layers [115,116]. Thus, the cell wall is more exposed to NPs through the outer bacterial membrane. The unique high surface to volume ratio of Cu-NPs enables them to interact with the bacterial cell membrane through its surface [117], which leads to the death of the bacterium [118]. These findings underline that the size of the NPs plays an important role in their antimicrobial activity.

Bacterial Resistance to NPs

The scientists hope that the microbes are less likely to develop resistance to NPs, which means it could be used to combat the emerging problem of antibiotic resistance. Some evidence suggests that NPs accelerate horizontal gene transfer between bacteria, helping them to become resistant against multiple antibiotics. As Zhigang Qiu et al. [119] described in the Proceedings of the National Academy of Sciences, the transfer of genes can increase by up to 200 times, helping diseases to acquire resistance against antibiotics from harmless gut bacteria in wastewater. Nanoalumina in water increased the horizontal transfer of multidrug-resistance genes across genera with an increase in the concentration of nanoalumina, density of parent bacteria, conjugation time, and temperature. In contrast, aquatic factors had no or little effect on the transfer of antibiotic resistance genes. The mechanisms by which nanoalumina promotes the transfer of drug-resistance genes may involve the damage of bacterial membranes by oxidative stresses, an enhancement of the expression of conjugative genes, and the repression of global regulatory factor genes for RP4 plasmid conjugation.

Qiu et al. (2012) [119] suggest that the application of nanoalumina in water and waste treatment should be evaluated carefully so as not to cause public health and environmental and ecological hazards. Further investigations are needed regarding other types of NP's contribution in bacterial resistance.

Bacterial Resistance to Metals

Several resistance mechanisms to metals have been described, the most common, which is enhanced efflux of metal ions from the cell, is a high-level, single-step and target-based mutation. This mutation enhances the efflux of metal ions from cell and makes metal resistance less probable owing to its multifaceted mode of action [120]. Bacteria have been shown to develop resistance to various heavy metals, including ionic Cu [76] and CuO [121]. Resistance mechanisms to heavy metals include sequestration of metals into complexes, reduction of a metal to a less toxic species and direct efflux of metal out of the cell [122]. However, these mechanisms are obviously ineffective for resistance to NPs, and may play a role in the removal of ions dissociating from the NPs surface only. Nonetheless, little is known about the effectiveness of CuO-NPs on biofilms and the changes in biological activity as a function of the size and shape of the NPs. Interactions between NPs and different bacterial structures are studied better for silver NPs; the mechanism of action of other NPs is still not well understood, especially the effect and mechanism of action of NPs combinations on bacterial cells.

Effect of NPs Size: Ions, NPs, MPs, and Bulk Material

A prevalent number of reports stated that smaller NPs demonstrated a higher toxicity rate in comparison to bigger NPs or bulk (micro scale) materials [123-125]. However, it was also observed that materials in NPs were more toxic than in MPs [94]. Other studies credited observed toxicity to soluble metal ions [126,127]. Moreover, it was shown that the toxicity of nanosized oxides (CuO-NPs) was much higher than their bulk counterparts [128]. The decline in the biotoxicity level of the Cu compounds in a series ions \rightarrow NPs \rightarrow MPs was observed in good agreement with the results of animal studies [111] estimating Cu-NPs toxicity (LD50 = 413 mg/kg) as being below the one of CuCl₂ but exceeding the one of MPs (LD50 = 110 mg/kg and LD50 > 5000 mg/kg, respectively). At the same time, the results indicate the similarity of this mechanism for various Cu compounds with NPs being less effective than Cu²⁺ ions but superior to MPs of this metal. Thus, nanosized particles are more toxic. This is also a possible reason to explain the toxicity of CuO-NPs, which exceeded that of bulk CuO by about 45- or 50-fold [64]. Midander et al. [81] also showed that Cu-NPs were much more toxic than Cu-MPs in terms of DNA damage and cytotoxicity *in vitro*. Moreover, this study [81] demonstrated that Cu can be released from CuO-NPs, and the soluble quantity was different in various media. Though NPs released more Cu ions than the MPs, the released fraction did not contribute significantly towards cytotoxicity when compared to particles themselves [92].

Effect of NPs Chemical Composition

Chemical composition of NPs is the base factor associated with antibacterial properties. Hence, the choice of an appropriate chemical element is extremely important and is linked to biocompatibility as well as toxicity properties [129]. For instance, Heinlaan et al. [130] pointed out that the toxicity of three oxides (both nano and bulk) to the Gr- bacteria *Vibrio fischeri* decreased as follows: TiO₂ < CuO < ZnO.

Babushkina et al. (2010) [131] compared the antibacterial properties of Cu and iron NPs of 30–40 nm and 30–70 nm, respectively, on 10 clinical multidrug-resistant isolates of *S. aureus*. The authors revealed more noticeable antibacterial action of Cu-NPs on *S. aureus* compared with iron NPs: 30 min exposure to Cu-NPs even at the concentration of 0.001 mg/mL significantly reduced the number of living cells compared with a control without NPs, while iron NPs reduced bacterial growth only starting from 0.1 mg/mL concentration after 30 min exposure. Moreover, besides chemical composition, it was demonstrated in the study of Raffi et al. (2010) [132] that the antibacterial activity of Cu-NPs against *E. coli* behaved in a concentration-dependent way in liquid and solid media where 100 µg/mL of Cu-NPs of 12 nm totally inhibited bacterial growth, while concentrations of 20 and 40 µg/mL were less effective [64,95].

Oxidation of Copper based-NPs Results in Particle Aggregation and Reduces ROS Production Capabilities

Copper-based-NPs on the other hand face a major limitation which is rapid oxidation upon exposure to air, which can result in particle aggregation [133]. As the oxide layer is formed, the ROS generating capacity of the Cu-NPs is reduced. Since Cu oxidizes to CuO and cuprous oxide (Cu₂O), and it converts to Cu²⁺ during preparation and storage, it is difficult to synthesize pure Cu-NPs in an ambient environment. Therefore, alternative pathways exist to preserve metal NPs antibacterial properties. In fact, the idea is to form a coating on the surfaces of NPs, generally in the presence of polymers (e.g., polyvinyl pyrrolidone, polyethylene glycol, and chitosan) and surfactants (cetyl trimethyl ammonium bromide) as stabilizers. In fact, even though the Cu nanopowder can be completely reduced by a strong reducing agent, such as hydrazine or sodium borohydride, it can also be instantly oxidized by the dissolved oxygen (O₂(aq)) that exists in the aqueous reduction system [134,135]. Therefore, the development of an efficient approach for preventing the oxidation of Cu nanopowders during synthesis in an aqueous solution is required urgently.

Corona Phenomenon Effect

The organic rich environment present in nature is simulated by the presence of organic media ingredients in the nutrition broth. Kim et al. (2012) [136] demonstrated that the uncontaminated and surfactant-free NPs themselves possess moderate cytotoxicity to human cells in a cell-dependent manner. They used MTT assays using HeLa human cervical cancer cells, PC3 human prostate cancer cells, and MCF-7 human breast cancer cells, to address the inherent toxicity of the prepared ultra-pure NPs with nascent surfaces. Cells were maintained in tissue culture plates at 37°C in an atmosphere of 5% CO₂ in MEM/EBSS (Minimum Essential Medium with Earle's Balanced Salts) media for HeLa cells and in RPMI-1640 media for PC3 and MCF-7 cells, all supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 100 IU/mL penicillin and 0.1 mg/mL streptomycin. In order to keep cells in log phase, the cultures were re-fed with fresh media two or three times/week. However, the MEM/EBSS or RPMI-1640 media contains inorganic salts, amino acids, vitamins and other components which could «contaminate» these supposed «ultra-pure» NPs by the corona phenomenon. The corona phenomenon which depends on nanoparticle properties such as surface charge, hydrophobicity, presence of ligands, size, and morphology; medium composition such as protein source; medium condition such as pH; and exposure time; is defined as the phenomenon where NPs or any synthetic material will become quickly covered by resident proteins when they come in contact with any biological fluid.

The corona layer composition depends on all previously mentioned NPs' properties and its architecture is made of two distinct layers: the hard corona, in direct contact and strongly interacting with the NP; and the soft corona, more external and unsteady.

Eco-Toxicity Depends on NPs Concentration Accumulation and Should Be Addressed Early

Since modern environment contains enhancing amounts of metal NPs, including NPs of Cu, in electronics, in biomedicine and in other technical processes, an evaluation of potential risks against human and animal health is of great importance. While the bactericidal effect for deliberate disinfection of pathogenic bacteria via application of antimicrobial coatings to various surfaces [137,138] is a desirable feature, the uncontrolled and unmanageable potential bactericidal effect from NPs released into the environment could damage the ecosystems of creeks, rivers, wetlands and other ecological systems that harbour bacterial communities. The accumulation of NPs in the environment might shift ecological niches and damage ecological systems.

Environmental research into CuO-NPs' toxicity has mostly focused on the effects on organisms, especially those in aqueous environments. The most common experiment models are algae and zebrafish, whose growth and toxicity are treated as environmental relevance indicators. Aruoja et al. [126] studied the toxicity of CuO-NPs on the algae *Pseudokirchneriella subcapitata* using bulk formulation of metal oxide as a control. At low concentrations, CuO-NPs (EC50 = 0.71 mg Cu/L) were more soluble and more toxic than the control (EC50 = 11.55 mg Cu/L). The results showed that the toxicities of bulk and nanosized CuO were largely influenced by soluble Cu ions. These findings were similar to the conclusions drawn by Grosell et al. [139] and Griffith et al. [140]; whose publications both proved that the soluble Cu forms were highly toxic to fish. Some studies also reported that CuO-NPs' suspensions might damage gill lamellae and inhibit epithelial cell proliferation by altering plasma metal levels [140], as well as chloride cell number and diameter [141]. Therefore, Gomes et al. [142] considered that mussel digestive gland could aggregate CuO-NPs and result in toxicity. The results of Shi et al. [143] indicated that CuO-NPs decrease chlorophyll content of the duckweed, and that CuO-NPs toxicity is three to four times higher than that of ionic Cu, because of the larger uptake of NPs-released Cu. Griffith et al. [140] compared the responses of fish exposed to Cu-NPs solution and soluble Cu and reported that the effects of gill morphology and transcription were not solely due to the dissolution of Cu-NPs.

Cu-NPs Synthesis Methods Reviewed

The synthesis procedures affect to a great extent the properties of Cu-NPs. Chemical, physical and biological techniques, considering bottom-up or chemical or biological methods and top-down or physical methods have been studied, FIG. 2 shows the different synthesis methods for Copper Oxides NPs [144].

The main techniques for NPs synthesis through the chemical approach are: Chemical reduction [145], microemulsion (colloidal) techniques [146], sonochemical reduction [144], electrochemical [147], microwave-assisted [148], and hydrothermal [149] synthesis. Biological or biosynthesis [150], techniques are also considered as bottom-up or chemical processes.

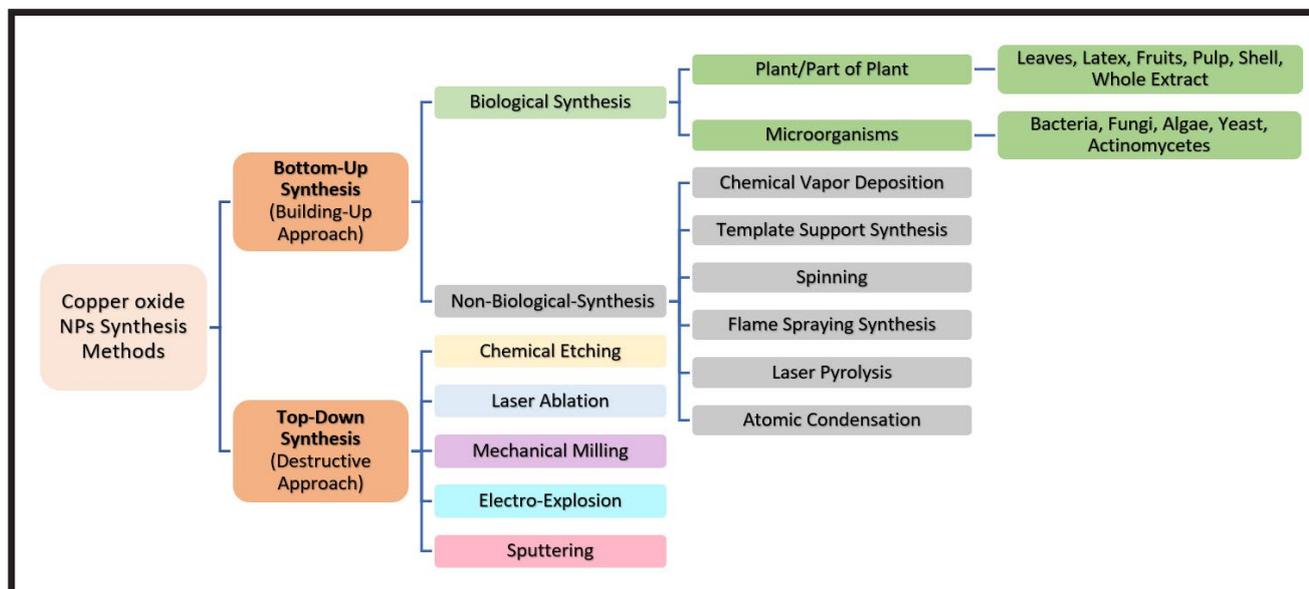


FIG. 2. Copper Oxide Nanoparticles synthesis methods [155].

Physical methods for nanoparticles synthesis are laser (pulse) ablation [151], vacuum vapour deposition [152], pulsed wire discharge [153], and mechanical milling [154]. The main disadvantages of these physical methods are the quality of the product, which is less as compared to nanoparticles produced by chemical methods. Additionally, these methods require costly vacuum systems or costly equipment to prepare nanoparticles such as plasmas.

Why Chemical Reduction is Often Preferred?

Among all methods, the chemical reduction is the most preferred because it has the advantages of being simple and economical, and it is also easy to control the particle size and morphology of the generated NPs. The following TABLE 1 summarizes the variety of NPs sizes produced and the concentration and incubation time used to assess a bactericidal effect against *E. coli* and *S. aureus*. Most reactions are carried out in water given the considerations of mass production, cost, and environmental consistency. However, using water as the solvent for the chemical reduction of Cu is a great challenge in contrast to its use for the synthesis of Au or Ag nanopowders because Cu is less chemically stable than Cu^{2+} and Cu^{1+} oxides; thus, a major problem is the usual occurrence of surface oxidation during its synthesis.

A detailed comparative study published by Umer et al. (2012) [156] revealed that chemical reduction methods are most suitable for the synthesis of Cu-NPs. Chemical reduction of Cu salts using ascorbic acid (Vitamin C) is a new and green approach in which Vitamin C is used both as the reduction and the capping agent. This approach is the most effective and is also economical.

Besides, another aqueous reduction method for the efficient synthesis of nonoxidative Cu nanopowders without using a protecting agent or introducing an inert gas is presented by Chang et al. (2013) [157]. In this approach, a capping layer composed of a hydrophobic solvent with a high boiling point is applied to and covered on the top of the reduction system. The proposed approach based on the use of a capping solvent is highly effective for preventing the oxidation of the produced Cu nanopowder. By this method, high purity Cu nanopowders can be produced that exhibit good electrical conductivity with an electrical resistivity on the order of $10^{-4} \Omega\cdot\text{cm}$.

Physical Method Using Laser Ablation

A laser-assisted method called laser ablation has proven itself as an alternative physical nanofabrication method [158–163]. It consists in the ablation of a target (mostly solid) by intense laser radiation and leads to an ejection of its constituents and the formation of NPs. [158–163]. Laser radiation is used to ablate a solid target (Ag, Au, Co and Cu) placed on the bottom of a glass vial filled with a biocompatible aqueous solution (7 mM NaCl solution or neat water). In other words, size selection of CuO-NPs is obtainable using laser ablation. Laser ablation in liquid has several advantages over the traditional chemical reduction methods for NPs, such as technical simplicity and chemical purity, because this method when performed in a clean and well-controlled environment enables us to avoid the use of toxic chemical reduction agents to control the growth of the colloidal NPs, resulting in production of ultrapure NPs with nascent surfaces [158–163]. Laser ablation provides a solution for the study on the biological effects of NPs since conventional NPs lead to cytotoxicity possibly caused by contamination with chemical precursors or additives during their synthesis and/or purification procedures. Thus, laser ablation is expected to offer novel opportunities to provide insight into the origin of NP toxicity.

Kim et al. (2012) [136] introduced this novel laser-assisted method called laser ablation to generate Ag-, Au-, Co-, and Cu-NPs in biocompatible aqueous solution and they obtained supposedly ultra-pure NPs. They evaluated the intrinsic/inherent toxicity of ultra-pure Ag-, Au-, Co-, and Cu-NPs generated by laser ablation in a liquid ambient, revealing that the chemical nature of metallic NPs strongly influences the toxicity and cellular uptake into the model cells. For instance, a research group investigated the dependence of the particle properties on the *in situ* annealing temperatures and selection sizes. CuO- and Cu_2O -NPs with narrow size distributions were well dispersed in the samples [164]. Nath et al. (2011) [165] revealed that high pressure (60 GPa) increases the material covalency at Cu-water interface. The growth of CuO phase was thermodynamically stable under tightly focused conditions. Reduction in size incited lattice distortion transformation into highly symmetric Cu_2O containing insufficiently oxidized Cu-NPs [165]. In another study, this synthesis procedure, which was used for the ROS generation by Cu-NPs, seems to be an appropriate procedure for the enhancement of bactericidal effect of these NPs [166].

TABLE 1. Copper and Copper Oxide Nanoparticles synthesis method, size and concentration used against bacteria in several references from the literature.

Nanoparticle Type	Bacteria Strain	Authors	NPs size	Concentration	Incubation time	Synthesis Method
Cu	<i>E. coli</i>	Raffi, 2010	12-15 nm	0.06 mg/mL	48 h	Thermal plasma
		Chatterjee, 2012	80 nm	12 µg/mL	18 h	
		Cubillo, 2006	2-5 nm	1 wt%	24 h	Reduction in gelatin medium
		Yoon, 2007	90 nm	33.49 µg/mL	24 h	
		Valodkar, 2011	32 nm	0.63 mg/L	24 h	
		Ruparelia, 2008	9.25 nm	160-300 µg/mL	24 h	
		Deryabin, 2013	24 nm			
		Giannousi, 2014	44 nm	71.31 µg/mL	24 h	Chemical synthesis
	<i>S. aureus</i>	Bagchi, 2014	10-20 nm	63.5 µg/mL		
		Betancourt-Galindo, 2014	<20 nm	1.6 mg/mL	16 h	Chemical synthesis, 99% inhibition
		Valodkar, 2011	2-5 nm	1 wt%	2 4	
		Ruparelia, 2008	32 nm	1.11 mg/L	24 h	
		Ruparelia, 2008	9.25 nm	160 µg/mL	24 h	NCIM 2079
CuO	<i>E. coli</i>	Ramyadevi, 2012	9.25 nm	160 µg/mL	24 h	NCIM 5022
		Bagchi, 2012	44 nm	>100 µg/mL		Hydrothermal method
		Das, 2013	15-30 nm	1 mg/mL	9 h	Reduction
		Ren, 2009	22.4-94.8 nm	0.25 mg/mL	24 h	
		Azam, 2012	29.11 nm	25 µg/mL	24 h	Gel combustion method
		Azam (2) 2012	20-28 nm	30-95 mg/mL	24 h	Hydrothermal method
		Baek, 2011	20-30 nm	28.6 mg/mL	24 h	Hydrothermal method
		Giannousi, 2014	16 nm	>100 µg/mL	24 h	
	<i>S. aureus</i>	Giannousi, 2014	12 nm	80.93 µg/mL	24 h	
		Ren, 2009	22.4-94.8 nm	250 µg/mL	24 h	Thermal plasma
		Das, 2013	15-30 nm	1 mg/mL	6 h	
		Ren, 2009	22.4-94.8 nm	2.5 mg/mL	24 h	
		Baek, 2011	28 nm	100 mg/mL	24 h	Hydrothermal method
<i>S. aureus</i>	Giannousi, 2014	20-30 nm	65.9 mg/mL	24 h	Hydrothermal method	
	Giannousi, 2014	16 nm	>100 µg/mL	24 h		
	Ren, 2009	12 nm	96.13 µg/mL	24 h		
	Ren, 2009	22.4-94.8 nm	250-2500 µg/mL	24 h	Thermal plasma	

Physical Method Using Plasma Induction Process

Plasma induction method is generated through an inductive coupling mechanism. When an alternative current of radio frequency and a high voltage is imposed on a spiral coil, the conductor placed in the center of the coil will be heated up under the alternative electromagnetic field. Introducing a continuum gas flow into such a coil, the gas could be ionized and heated into plasma. The plasma so generated is called inductively coupled plasma or induction plasma for short.

The plasma induction method used for nanopowder synthesis has many advantages over alternative techniques such as high purity, high flexibility, ease of scale-up and ease of operation and control. For example, the plasma induction system developed by Tekna [167] has been successfully used in the synthesis and preparation of advanced materials such as new ceramics, nanometric metallic powders, biomaterials, and superconductors. The typical size range of the nanoparticles produced is from 20 nm to 100 nm, depending on the quench conditions employed. Materials at such a small-scale display unusual properties, as chemical, physical, electrical, optical, mechanical, magnetic, etc., different from their bulk state.

Recently, our team synthesized CuO powders from the reaction of copper with oxygen in plasma using HF-75 torch of Tekna [167]. Since copper has two states of oxidation, i.e., tenorite (CuO) and cuprite (Cu₂O), a preliminary effort has been made in the study to convert pure copper into tenorite. The extent of the conversion is mainly determined by two factors: the oxygen concentration in plasma and the temperature in the reactor. The antibacterial activities of these CuO powders are published in a separate paper [168].

Flow-Levitation (FL) Method

Flow-levitation (FL) method is based on the levitation melting technology [169] and has been used as a successful method for the preparation of some metals [170–172] and ZnO-NPs [173]. Moreover, this method has been used for the preparation of alloy NPs. Sivaprahasam et al. [174] used this method for the synthesis of FeCu nanopowders and other groups used it to produce intermetallic Ag₂Al, FeAl, and FeNi₃-NPs. [175,176].

In comparison to the conventional evaporation-condensation, the main advantages of the FL method are high purity of the product (due to the container less nature of the process) and high production rate (due to the rapid heating and continuous manner of the method) [177]. FL method is a novel method capable of producing high purity intermetallic NPs with a relatively high production rate. Cu- and CuO-NPs of various average sizes and chemical compositions were synthesized via FL condensation method [170].

However, main restrictions of FL method are as follows: relatively low productivity (1-100 g/hour), impossibility to work with refractory metals (with melting point over 2500°C) and relatively wide particle size distribution (that causes different chemical composition of particles with different sizes) [178].

Among main advantages one should emphasize an opportunity of fast and effective variation of particle production regimes to provide a wide range of particle sizes and chemical reaction conditions, and an opportunity to fulfill some steps of successive chemical transformations of the particles *in situ* within the one experimental apparatus in controlled atmosphere and temperature.

Addition of Stabilizing Agent to Counteract NPs Oxidation and Aggregation

We have established now that NPs' properties can be controlled depending on the synthesis method. One of the main effects, which are enhanced by controlling particle size, is their antimicrobial action [78,75]. Nonetheless, one main drawback in most of the synthesis technique is the rapid oxidation of copper-based-NPs upon exposure to the air.

For protecting Cu-NPs from oxidation during synthesis, the sufficient addition of surfactants such as hexadecyltrimethylammonium bromide [135,179], poly(N-vinyl-2-pyrrolidone) [134], oleic acid [180], gelatin [181], diethanolamine [182] and sodium dodecylbenzenesulfonate [137] is necessary for full coverage of the particle surface. However, the electrical properties of the Cu-NPs can be hindered because of the presence of the insulating surfactants. In fact, a mixture of metallic Cu and Cu₂O can be seen. It has been largely reported that Cu₂O or CuO generally accompanies the production of Cu nanopowders, even though the added amount of reductant is sufficient [134,179].

One approach is to add a capping layer composed of a hydrophobic solvent with a high boiling point and apply it to cover NPs. Without using this layer, O₂(aq) can be easily supplemented from air [134,179]. That is, when O₂(aq) is consumed by the reductant or the less-oxidized Cu⁰ or Cu^I colloids, oxygen is immediately supplemented from air based on dynamic equilibrium. The additional O₂(aq) from air maintains the oxidation of the Cu⁰ and Cu^I colloids. However, with the hydrophobic capping layer, the dissolution of oxygen is unfavourable, and thus the reduction system can be secluded from air and the system can remain deficient in O₂(aq). Without the addition of supplemental O₂(aq), oxidation of the produced Cu⁰ colloids will not be easy. In addition to efficiently reducing the concentration of O₂(aq) in the reduction system, this method has other advantages. Unlike an inert gas, the capping solvent can be easily recycled and reused. Moreover, the use of surfactants as protecting agents is not a must, and thus good electrical conductivity of the as-synthesized Cu⁰ can be anticipated.

Therefore, the Cu nanopowder is generally generated along with a mixture of its oxides (Cu₂O or CuO). Contrarily, when the capping solvent is applied, only the product of metallic Cu NPs is present, generally obtained in a face-centred cubic crystal structure. It becomes then obvious that the proposed approach based on the use of a capping solvent is highly effective for preventing the oxidation of the produced Cu nanopowder. This result may be attributed to the less favourable dissolution of oxygen in hydrophobic n-octane, which prevents the penetration and dissolution of oxygen in the aqueous solution [179,183].

Chemical Methods using Chitosan Stabilizer

It is known that Cu-NPs tend to agglomerate on synthesis due to the high tendency of Cu nuclei to bond. The aggregation may also be due to the high surface area of the Cu-NPs [133]. Therefore, the use of a polymer coating is essential to avoid NPs clusters.

Usman et al. (2013) [184] investigated the antimicrobial properties of metallic Cu-NPs synthesized in chitosan polymer medium through chemical means. The surfaces of chitosan-Cu-NPs are covered by fragments of chitosan (CS) which protect against aggregation and oxidation [185]. CS is a natural copolymer of D-glucosamine and N-acetyl-D-glucosamine and is produced by alkaline deacetylation of chitin. The nuclei of the individual nanocrystals were attracted to each other by weak van der Waals forces, and the stabilizer (CS) provided insulation between the particles by overcoming these forces, a phenomenon seen with both polymers and surfactants [154,185].

No other CuO or Cu₂O impurity peaks were observed in any of the spectra, suggesting that the synthesized particles were of high purity. The images obtained for the various concentrations of the stabilized crystals indicate that the Cu-NPs were embedded within the matrix of the polymer. These findings accentuate the important role of the polymer as a stabilizer. It is worth noting that the chitosan stabilized Cu-NPs exhibited both antibacterial and antifungal activity against Gr+ bacteria, Gr- bacteria, and yeast.

However, CS is deemed to also have antioxidant properties that may inhibit ROS generated by Cu-NPs. For instance, Xie et al. (2001) [186] studied the antioxidant activities of water-soluble chitosan derivatives which were considered to be hydroxyl radical scavengers. CS is also biodegradable, biocompatible, and non-toxic and, therefore, has been employed in biomedical applications such as drug delivery [187–190]. CS has many significant biological properties including bioactivity and biodegradability with a reactive chemical group including OH and NH₂. CS has shown good antioxidant activity, dependent on the molecular weight and the degree of deacetylation. The mechanism of action is still not exactly known, but it is postulated that it reacts with unstable free radicals to form more stable macromolecular radicals.

Other Examples of Polymeric Embedding of NPs

Esteban-Cubillo et al. [69] have shown that Cu/sepiolite NPs strongly inhibit the growth of *E. coli* and *S. aureus* by 99.9%. Ruparelia et al. [78] showed the specificity of Cu-NPs to selectively inhibit the growth of few strains of *E. coli*. The bactericidal and fungicidal properties of Cu-NPs are known [67,90,191]. Shamel et al. (2012) [192] reported that plant extracts could be used to stabilize NPs in green synthesis. Cu-NPs (about 6 nm) embedded in polyvinyl methyl ketone films exhibit a noticeable inhibitory effect on the growth of microorganisms [86]. Due to the stability of Cu-NPs supported on a matrix and their disinfecting properties, Cu-NPs can be used as a bactericide agent to coat hospital equipment. Christian et al. (2008) [12], revealed that Cu-NPs get more attention due to their excellent thermal properties after the introduction of nanofluids. Nanofluids of Cu-NPs are used in both heating and cooling applications [193].

Concluding remarks

It is noteworthy that in 2006 the US had about 720,000 hospital-acquired infections, causing \$125 billion in excess hospital charges and more than 74,000 fatalities [194], (numbers for the US were extrapolated from data acquired for Pennsylvania). A hospital-acquired infection raised a patient's percent mortality from 2.1 to 12.3%, the length of hospitalization from 4.4 to 19.3 days and the cost per visit from \$33,000 to \$176,000. These numbers emphasize the need for new antimicrobial approaches that tackle this severe public health problem.

Despite the many effective antimicrobial strategies against planktonic bacteria, most antimicrobials are rarely tested or effective against biofilms [195]. Novel approaches to treat established biofilms are thus urgently needed. Clearly, new strategies for battling biofilms are warranted.

The potential usefulness of metallic Cu in hospitals and other public places to control and reduce hospital acquired infections is very promising. Nevertheless, research is also required that investigates the possible emergence and spread of Cu surface resistant bacteria. CuO- and Cu-NPs have shown to have adverse effects on bacteria, and Cu²⁺ dissolving from the NPs induced also toxic effects by triggering ROS production and DNA damage in bacteria. Therefore, copper-based NPs can be used as a novel antifungal agent in agriculture to control the plant pathogenic fungi as well as a potent disinfectant in poultry and animal husbandry.

However, in any organism, the molecular mechanisms responsible for killing and the cellular target(s) of Cu toxicity have not been delineated yet and remain elusive.

Applications for Cu NPs include antimicrobial, antibiotic, and antifungal (fungicide) agent when incorporated in coatings, plastics, textiles, in Cu diet supplements, in the interconnect for micro integrated circuits, for its ability to absorb radioactive cesium, in super strong metals, in alloys, in nanowire, in nanofiber, in certain alloy and catalyst applications. Further research is being done at various levels for their potential electrical, dielectric, magnetic, optical, imaging, catalytic, biomedical and bioscience properties. It is interesting to note that Cu-NPs are generally immediately available in most volumes.

Synthesis and use of NPs are influenced by a variety of factors which affect the activity of the NPs, such as chemical composition, concentration, contact time, size, shape, target microorganism and photo-activation. Further investigations should be devoted to the assessment of influence of biological fluids, pH, oxygen pressure and other chemical and biological factors on antibacterial properties of NPs, biocompatibility of NPs, and interactions between different physico-chemical properties of NPs in their combined use. Special attention should be paid to the study of activity of NPs and their combinations with other classes of antimicrobial agents against multidrug-resistant microorganisms.

Researchers have also recommended the use of silver and Cu ions as superior disinfectants for wastewater generated from hospitals containing infectious microorganisms. Cu-NPs, due to their excellent physical and chemical properties and low cost of preparation, have been of great interest. Cu-NPs have wide applications as heat transfer systems [84], antimicrobial materials [85,86], sensors [86], super strong materials [87,88], and catalysts [89,196]. However, Cu-NPs can easily oxidize to form CuO. To protect Cu-NPs from oxidation, they are usually encapsulated in organic and inorganic coating such as carbon and silica [11,85,197,198].

Considerable interest has been focused on noble metal NPs in recent years because of their potential applications in a wide range of fields such as catalysis, optics, biology, microelectronics, and electrical conductors. Among the popularly used noble metals, Cu is non-toxic; non-magnetic and has good thermal and electrical conductivity at the least cost compared to Au, Pd and Ag. **Therefore, Cu nanopowders are believed to be the most competitive material for the replacement of Ag nanopowders, which have been widely used to date in the above applications.**

Abbreviations

NPs	Nanoparticles
EPS	Extracellular polymeric substances
E. coli	Escherichia coli
NIs	Nosocomial infections
UTIs	Urinary tract infections
S. aureus	Staphylococcus aureus
P. aeruginosa	Pseudomonas aeruginosa
K. pneumoniae	Klebsiella pneumonia
A. baumannii	Acinetobacter baumannii
MRSA	Methicillin-resistant S. aureus
BSIs	Bloodstream infections
VRE	Vancomycin resistant Enterococci
VRSA	Vancomycin resistant S. aureus
NDM-1	New Delhi metallo beta-lactamase
MDR	Multidrug-resistant
PDR	Pan-drug-resistant
Gr+	Gram-positive
Gr-	Gram-negative

Cu	Copper
CuO	Cupric Oxide
Cu ₂ O	Cuprous oxide
Cu-NPs	Copper NPs
Cu-MPs	Copper microparticles
ROS	Reactive oxygen species
EC10	Maximal effective concentration to induce 10% of response after exposure
EC30	Maximal effective concentration to induce 30% of response after exposure
EC50	Maximal effective concentration to induce 50% of response after exposure
A549	Adenocarcinomic human alveolar basal epithelial cells
937	Human lymphoblast lung cells
HeLa cells	Human epitheloid cervix carcinoma cells
Hep-2	Human airway epithelial cells
PAI-1	Plasminogen activator inhibitor-1
p38	Mitogen-activated protein kinases
E. coli RFM443	Escherichia coli strain RFM443
ppm	Part per million
ppb	Part per billion
SSB	Single-strand break
Fpg	Formamidopyrimidine-DNA glycosylase
H ₂ O ₂	Hydrogen peroxide
•OH	Hydroxyl radical
TiO ₂	Titanium dioxide
ZnO	Zinc oxide
O ₂ ^{•-}	Superoxide
GC/MS	Gas chromatography-mass spectrometry
DCFH-DA	Fluorescent probe, 2',7'-dichlorofluorescein diacetate
GR	Glutathione reductase
GPx	Glutathione peroxidase
DCFH	Dichlorodihydrofluorescein
CFU	Colony-forming units
ZVCN	Zero valent Cu-NPs Cu0
Vitamin C	Ascorbic acid
CS	Chitosan
CMCH	Carboxymethyl chitosan
PC3	Human prostate cancer cells
MCF-7 cells	Human breast cancer cells
LD50	Lethal Dose inducing 50% mortality
MEM/EBSS	Minimum Essential Medium with Earle's Balanced Salts
RPMI-1640	Roswell Park Memorial Institute medium N1640
LD100	Lethal Dose inducing 100% mortality
S. albus	Staphylococcus albus
E.coli AB1157	Escherichia coli strain AB1157
E.coli K12	Escherichia coli strain K12
AAS	Atomic absorption spectroscopy
CuCl ₂	Cupric chloride
O ₂ (aq)	Oxygen in aqueous medium GSH
	Glutathione

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