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ORIGINAL ARTICLE

Investigations into the antibacterial behavior of copper nanoparticles against *Escherichia coli*

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Abstract Zerovalent copper nanoparticles (Cu^0) of 12 nm size were synthesized using an inert gas condensation method in which bulk copper metal was evaporated into an inert environment of argon with subsequent cooling for nucleation and growth of nanoparticles. Crystalline structure, morphology and estimation of size of nanoparticles were carried out by X-ray diffraction and transmission electron microscopy. The antibacterial activity of these nanoparticles against the Gram-negative bacterium *Escherichia coli* was assessed in liquid as well as solid growth media. It was observed from scanning electron microscopic analysis that the interaction of copper nanoparticles with *E. coli* resulted in the formation of cavities/pits in the bacterial cell wall. The antibacterial property of copper nanoparticles was attributed mainly to adhesion with bacteria because of their opposite electrical charges, resulting in a reduction reaction at the bacterial cell wall. Nanoparticles with a larger surface-to-volume ratio provide more efficient means for antibacterial activity.

Keywords Copper nanoparticle · Antibacterial activity · *Escherichia coli* · X-ray diffraction · Electron microscopy

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Introduction

Microbial contamination of air, water and soil due to different types of microorganisms creates problems in living conditions, and in the public health and industrial fields. As a result, an increased occurrence of antibiotic resistant genes in many bacterial species is found in human beings and animals. The over-use of antibiotics against pathogenic bacteria causes harmful side effects that has resulted in the emergence of resistance to antibiotics in bacteria (Levy 1991; Tong et al. 2005; Hu and Xia 2006). Some fungi have become a major health threat, particularly to patients with compromised immune systems, especially those suffering from acquired immune deficiency syndrome (AIDS) or receiving chemotherapy (Cioffi et al. 2005). To seek solutions to such problems a great deal of research work is being performed to develop materials with novel properties that have specific antimicrobial activities for use in fighting infections to create sterile conditions. A great deal of research work has focused on the synthesis of novel materials to design properties for innovative biotechnological applications in medical implants, drug delivery systems, sterile coatings for biomedical devices, adhesives and packaging (Cioffi et al. 2005).

Given their catalytic, optical, electrical and magnetic properties, metal nanoparticles and nanomaterials are considered a source of great importance for a wide range of biological and pharmaceutical applications (Mamunya et

al. 2004). The enhanced biological activity of metal nanoparticles against microorganisms needs to be thoroughly understood before they can be used in commercial products. There are some encouraging results showing the bioactivity of different drugs and antimicrobial formulations in the form of nanoparticles.

The antibacterial and antifungal properties of copper, silver, titanium and zinc are effective in reducing the growth of various microorganisms (Lee et al. 2003; Cioffi et al. 2005; Raffi et al. 2008). The antimicrobial efficacy of technologically appealing materials containing copper-based active powders or pigments in fabrics, paints or as coatings (Cioffi et al. 2004), aqueous copper solutions (Avery et al. 1996), complex copper species (Zoroddu et al. 1996) or copper-containing polymers has led to their use as antifungal compounds. Copper oxide (CuO)-containing phosphate-based glass fibers (PGF) have been developed for potential use in wound healing applications (Neela and Ahmed 2005).

The antimicrobial mechanism of nanomaterials can be understood by studying their specific binding to the surfaces of microorganisms and the consequent metabolism of such materials inside microorganisms. However, the development of effective antibacterial materials with tailored properties that are capable of delivering a controlled release of copper ions is still an open research area. Very little information is yet known about the antimicrobial properties of copper-based nanoparticles and nanocomposites (Chen and Chiang 2008). The purpose of the present study was to investigate the antibacterial behaviour of copper nanoparticles against *Escherichia coli* ATCC-15224 in liquid as well as in solid growth media. The ultimate goal of this study was to provide experimental evidence to help understand the mechanism of interaction of copper nanoparticles with *E. coli* resulting in bacterial growth inhibition.

Materials and methods

Synthesis of copper nanoparticles

Copper nanoparticles were synthesized from pure copper metal wire using an inert gas condensation (IGC) method. A process vacuum glass chamber was cleaned by achieving a base pressure of 10^{-6} Torr. Small cuttings of polycrystalline copper wire (99.9% purity, Sigma-Aldrich, St. Louis, MO) were loaded in a molybdenum evaporation boat. Argon gas (99.99% purity) was introduced into the process vacuum chamber through a moisture trap at a pressure of 100 mTorr. Copper atoms were evaporated by supplying electric current to the evaporation boat. A disc shutter over the evaporation boat was opened when super-saturation conditions were achieved. The particles formed in gas phase were allowed to deposit on a stainless steel flat surface cooled by flowing liquid nitrogen over it. The synthesized Cu^0-nanoparticles were collected by brushing the stainless steel plate with a Teflon scraper.

X-ray diffraction analysis

Copper nanoparticles were characterized by X-ray diffraction (XRD) using CuK_α radiation (λ=0.154051 nm) and a vertical wide-range goniometer equipped with a diffracted beam monochromator. Samples were scanned from 30° to 80° of 2θ in increments of 0.04° 2θ with a 4-s counting time.

Transmission electron microscopy analysis

For transmission electron microscopy (TEM) analysis, the copper nanoparticles were suspended/ dispersed in pure acetone by ultra-sonication and a few drops of particle suspension were placed onto carbon coated copper-grids and dried in a desiccator. TEM analyses were carried out in a JEOL-fx-2000 microscope. TEM was used for estimation of crystalline structure, morphology and mean size of nanoparticles.

Antibacterial activity tests

To study the bactericidal effect of copper nanoparticles against *Escherichia coli* ATCC-15224, the nanoparticles were dispersed in pre-sterilized deionized water by ultra sonication (model 934, Yamato, Tokyo, Japan). A freshly grown axenic culture of *E. coli* (10^4 cells/mL) was inoculated into flasks containing liquid nutrient growth medium (CM-01, Oxoid, Basingstoke, UK) supplemented with various concentrations of copper nanoparticles (20, 40, 60, 80 and 100 μg Cu^0/mL). These flasks were incubated at 37°C and 150 rpm on orbital shaking incubator. The total volume of liquid medium in each flask was kept at 50 mL. All experiments were performed in triplicate under sterile conditions.

Samples were taken periodically from the flasks to measure optical density at a wavelength of 625 nm using UV-Vis spectrophotometer (model 9453, Agilent, Wilmington, DE) to index growth of bacterium. After 48 h of incubation, liquid samples treated with nanoparticles in flasks were spread onto nutrient agar plates to observe the growth of *E. coli* as colony forming units (CFU).

Scanning electron microscopy analysis

Bacterial cells treated with nanoparticles were collected by centrifugation (Microfuge-18, Beckman-Coulter, Fullerton,

CA) of liquid medium at Relative Centrifugal Force (RCF) 10700 (average g- force) for 10 min. The bacterial cell biomass was treated with gradients of ethanol for fixing on aluminium stubs which were air-dried in desiccators before coating with a thin layer of gold. Samples were analyzed by scanning electron microscopy (SEM: LEO-440*i* Oxford Microscopy, Oxford, UK).

Atomic absorption spectroscopic analysis

The supernatants obtained after centrifugation of copper nanoparticles treated with the bacterium were analyzed using the standard atomic absorption spectroscopy (AAS) technique (Z-8000, Hitachi, Tokyo, Japan) to determine copper ion concentrations released into the growth medium.

Results and discussion

Synthesis and structural characterization of Cu^0-nanoparticles

Inert gas condensation is one of the most widely used methods for synthesis of metal nanoparticles. In this process, a metallic or inorganic material is vaporized using thermal evaporation sources, electron beam evaporation devices or sputtering sources in an inert atmosphere of argon or helium gas to synthesize nanoparticles with better control over size, distribution and purity (Birringer et al. 1984). An XRD diffractogram of copper nanoparticles is shown in Fig. 1. The diffraction peaks of samples correspond to the characteristic face centered cubic (FCC) copper lines indexed as (111), (200) and (220) that were observed in these samples at diffraction angles of 43.2°, 50.3° and 73.9°, respectively. A broad diffraction peak of cuprite (111) was observed at a diffraction angle of 36.2°. These diffraction peaks were similar in terms of angular positions to that of FCC pure bulk copper crystalline peaks but were relatively broad, as the mean size of the particles was of the order of nanometers.

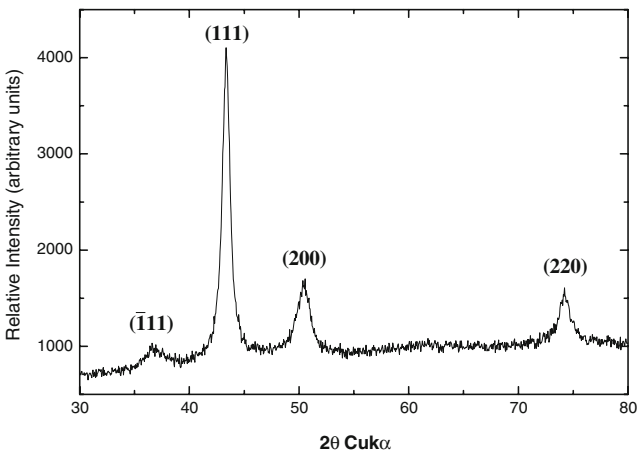

Fig. 1 X-ray diffraction (XRD) diffractogram of copper nanoparticles

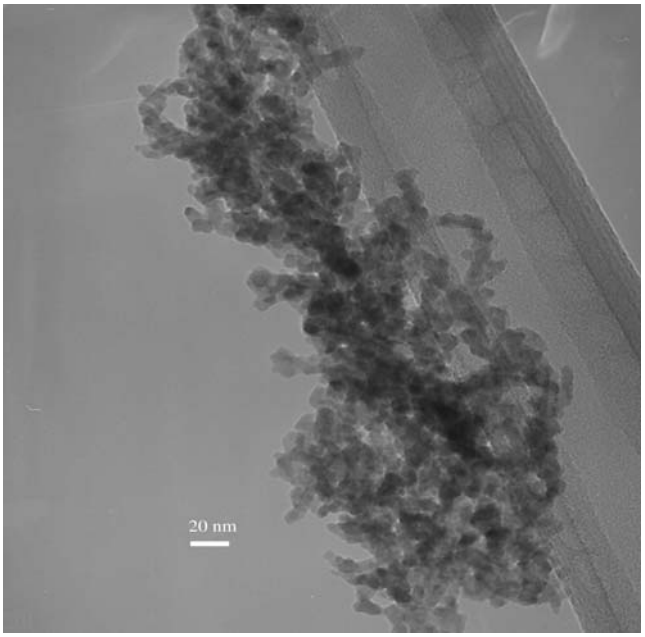

Fig. 2 Transmission electron microscopy (TEM) micrograph of copper nanoparticles showing shape and particle size estimation

Scherer's equation was used to estimate mean size of nanoparticles.

$$d = \frac{0.9\lambda}{\beta \cos\theta}$$

where d is the mean diameter of nanoparticles, λ is the wavelength of X-ray radiation source, β is the angular full width at half maximum (FWHM) of the X-ray diffraction peak at the diffraction angle (Cullity 1978). The mean size of copper nanoparticles estimated by XRD data was 12 nm. A thin copper oxide passivation layer is believed to have formed on the surface atoms of copper nanoparticles, which prevented further oxidation of copper atoms inside. Mean particle size of the copper nanoparticles estimated by TEM was about 15 nm, which compared well with that estimated by XRD data. The TEM micrograph of copper nanoparticles shown in Fig. 2 shows agglomerated clusters of copper nanoparticles. Metal nanoparticles have a higher surface free energy, resulting into their agglomeration. It is an established fact that metal nanoparticles formed in a gas phase synthesis process follow the lognormal distribution function (LNDF; Granqvist and Buharman 1976).

Bacterial growth inhibition mechanism of Cu^0-nanoparticles

Optical density at a fixed wavelength of 625 nm of solutions containing different concentrations of copper

nanoparticles was measured as a function of time (Fig. 3). The number of bacterial colonies observed on solid nutrient agar plates was a function of copper nanoparticle concentration (Fig. 4); CFUs were reduced significantly with increasing copper nanoparticle concentration in the growth medium. Virtually no growth of bacterial colonies was observed in samples containing copper nanoparticles at 60 μg Cu^0/mL and above. The growth inhibition trend of *E. coli* observed by CFU values was in good agreement with the results of optical density measurement in liquid medium. The total soluble copper concentrations estimated by AAS in liquid medium are shown in Fig. 5. This data clearly shows that the soluble copper concentration increased in liquid medium as the nanoparticle concentration in the growth medium increased, which inhibited bacterial growth. SEM of damaged bacterial cells upon incubation with copper nanoparticles revealed the formation of cavities and pits (Fig. 6). The outer cell membrane of *E. coli* is constructed predominantly of tightly packed lipopolysaccharide (LPS) molecules, which provides an effective permeability barrier (Thiel et al. 2007).

Copper-containing compounds such as $CuSO_4$ and $Cu(OH)_2$ are the traditional inorganic antibacterial materials (Hughes and Poole 1989). The overall charge of bacterial cells at biological pH-values is negative due to the excess of carboxylic groups present in the lipoproteins at the bacterial surface, which, upon dissociation, makes the cell surface negative (Stoimenov et al. 2002). The opposite charges of bacteria and copper ions released from nanoparticles are thought to cause adhesion and bioactivity due to electrostatic forces. Since peptidoglycans are negatively charged molecules, they bind Cu^{2+} ions released from copper nanoparticles in liquid growth medium. Being

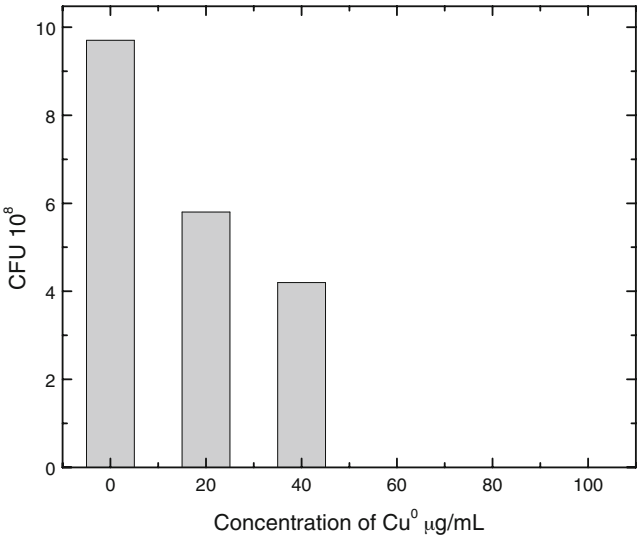

Fig. 4 Antibacterial characterization of copper nanoparticles by colony forming unit (CFU) as a function of concentration on agar plates

Gram-negative, the bacterium *E. coli* may allow more Cu^{2+} ions to reach the plasma membrane but is generally considered less susceptible to antibiotics and antibacterial agents than Gram-positive bacteria (Koch 1990). Bacterial cell growth enhances the turbidity of liquid nutrient medium because microbes have a higher refractive index than water, thus scattering incident light. The optical density of the growth medium was found to decrease compared to control solution with gradually increasing concentration of copper nanoparticles. Increasing concentrations of copper nanoparticles caused a delay in bacterial growth, and higher concentrations of copper nanoparticles (100 μg Cu^0/mL) were found to have an effective

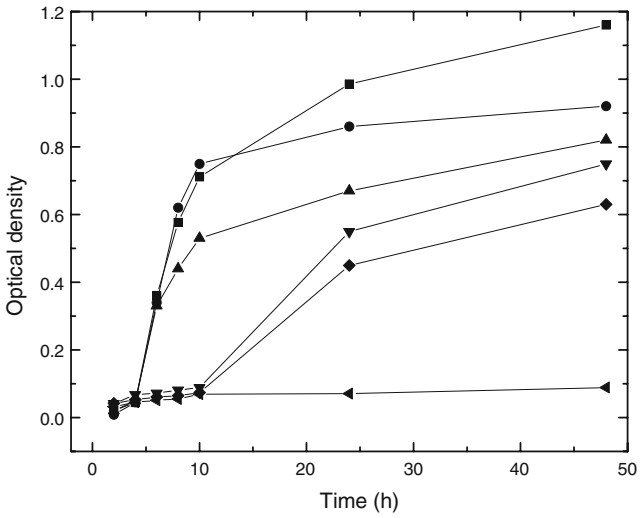

Fig. 3 Optical density as a function of time in the solution studies with varying concentrations of copper nanoparticles (μg Cu^0/mL): ■ 0, ● 20, ▲ 40, ▼ 60, ♦ 80, ◄ 100

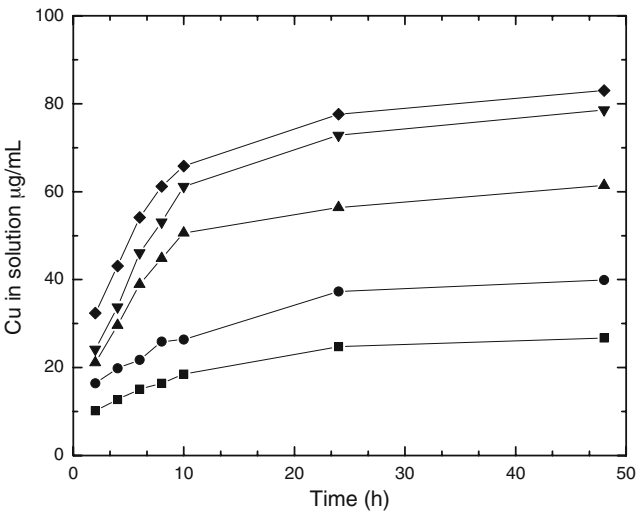

Fig. 5 Concentration of soluble copper released from copper nanoparticles in liquid growth medium. Copper nanoparticles (μg Cu^0/mL): ■ 20, ● 40, ▲ 60, ▼ 80, ♦ 100

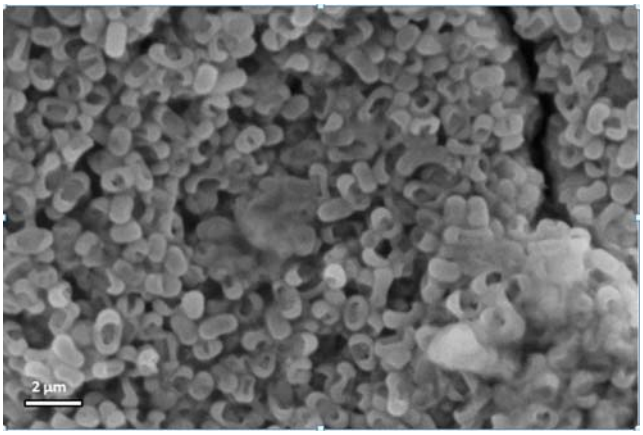

Fig. 6 Scanning electron microscopy (SEM) micrograph of *Escherichia coli* cells treated with copper nanoparticles showing pits/cavities in bacterial cell walls

bactericide effect on the growth of *E. coli*. At lower concentrations of nanoparticles, only a delay in the lag-phase was observed, which indicated that copper acted as a micronutrient for bacteria, whereas at higher concentrations bacterial growth ceased. The estimated concentration of Cu^{2+} ions released from the nanoparticles in liquid medium was highest in samples containing 100 µg Cu^0/mL nano-sized copper particles. These results support the conclusion that growth inhibition of *E. coli* depends on the release of an appropriate copper ion concentration in liquid medium.

It is logical to state that binding of copper nanoparticles to bacteria depends on the surface area available for interaction. As nanoparticles have a large surface area, their bactericidal efficacy is enhanced compared to large-sized particles; hence, they are believed to impart cytotoxicity to microorganisms (Stoimenov et al. 2002). The mechanism by which nanoparticles are able to penetrate into bacteria is not understood completely but studies have suggested that when *E. coli* is treated with copper nanoparticles, changes take place in its cell membrane morphology. An SEM micrograph of *E. coli* treated with copper nanoparticles (Fig. 6) showed that bacterial cells changed from normal rod-shaped to having an irregular appearance after treatment with nanoparticles. Some bacterial cells were discovered even to have shrunk, the cell wall was found destroyed and uneven, and bacterial inner vacuoles were found to appear. An efflux of nutrients is believed to occur between the cell wall and the cell membrane, which widened, and the cytoplasm tends to concentrate. Copper ions have the capacity to kill bacteria by destroying their cell walls and membranes because they have a strong reduction ability, which can extract electrons from the bacteria, causing their cytoplasm to escape and oxidizing the cell nucleus. It is suggested that when copper nanoparticles penetrate inside bacteria, they impart damage by interacting with phosphorus- and sulfur-containing compounds such as deoxyribonucleic acid (DNA) because they have a high affinity to react with such compounds. Cu^{2+} ions can combine with the plasma membrane by electrostatic attraction and then penetrate the cell membrane through opening or closing of membrane channels. This alters the permeability of cellular membranes, which causes leakage of intracellular ions and low molecular weight metabolites. At the same time, Cu^{2+} ions entering cells combine strongly with intracellular amino acids and proteases, resulting in degeneration that leads ultimately to denaturation of proteins (Tong et al. 2005). It is also considered that nanoparticles accumulating on the envelope proteins destabilize the outer cell wall, resulting in collapse of the plasma membrane potential and depletion of the level of intracellular ATP (Lok et al. 2006).

The antibacterial mechanism of copper nanoparticles has been attributed to the fact that Cu^{2+} ions eluted from nanoparticles are absorbed by bacteria when the nanoparticles concentration is high enough. Copper ions are absorbed onto the bacterial cell surface, imparting damage to the cell membrane by solidifying protein structure or altering enzyme function (Ohsumi et al. 1988; Dan et al. 2005). Bacterial cells are immobilized and become inactivated by the presence of copper nanoparticles in the growth medium, which results in hampering of their replication process, with subsequent cell death (Hu and Xia 2006). Reportedly, recycling redox reactions between Cu^{2+} and Cu^{1+} are possible at the surface of *E. coli* cells, generating hydrogen peroxide, causing damage to the cytoplasmic membrane (Hoshino et al. 1999). Under physiological conditions, the intracellular enzyme activity of bacteria treated with copper nanoparticles is believed to increase, suggesting that permeability of the cell membrane also increased, with bacteria suffering injury as a result. From these results, it is believed that binding of copper ions to the bacterial cell surface plays an important role in bactericidal activity (Hoshino et al. 2000). Copper has the potential to disrupt cell function in multiple ways, since several mechanisms acting simultaneously may reduce the ability of microorganisms to develop resistance against copper (Michels et al. 2005). These investigated mechanisms provide insight into the complicated antimicrobial action of copper nanoparticles.

In conclusion, high concentrations of copper nanoparticles demonstrate complete cytotoxicity against *E. coli*. These nanoparticles adhere to the bacterial cell wall and penetrate through the cell membrane. Copper ions destroy the bacterial cell wall, which becomes thick and coarse, the cytoplasm is then degraded and disappears, leading finally to cell death. The antibacterial mechanism is attributed mainly to the strong adsorption of copper ions to bacterial cells, which imparts antibacterial efficacy in a concentration-dependent manner. Nanoparticles have a large surface-to-volume ratio,

which enhances their bioactivity and makes them effective bactericidal agents. It is hoped that, in future, copper nanoparticles could replace some antibiotic medicines used to combat pathogenic bacteria in the gastrointestinal tract of animals.

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