Strain specificity in antimicrobial activity of silver and copper nanoparticles

Jayesh P. Ruparelia [a], Arup Kumar Chatterjee [c], Siddhartha P. Duttagupta [b], Suparna Mukherji [a,*]

[a] *Centre for Environmental Science and Engineering, Indian Institute of Technology Bombay, Powai, Mumbai 400 076, India*
[b] *Department of Electrical Engineering, Indian Institute of Technology Bombay, Powai, Mumbai 400 076, India*
[c] *Innovation Centre for Applied Nanotechnology, Kolkata, India*

Abstract

The antimicrobial properties of silver and copper nanoparticles were investigated using *Escherichia coli* (four strains), *Bacillus subtilis* and *Staphylococcus aureus* (three strains). The average sizes of the silver and copper nanoparticles were 3 nm and 9 nm, respectively, as determined through transmission electron microscopy. Energy-dispersive X-ray spectra of silver and copper nanoparticles revealed that while silver was in its pure form, an oxide layer existed on the copper nanoparticles. The bactericidal effect of silver and copper nanoparticles were compared based on diameter of inhibition zone in disk diffusion tests and minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of nanoparticles dispersed in batch cultures. Bacterial sensitivity to nanoparticles was found to vary depending on the microbial species. Disk diffusion studies with *E. coli* and *S. aureus* revealed greater effectiveness of the silver nanoparticles compared to the copper nanoparticles. *B. subtilis* depicted the highest sensitivity to nanoparticles compared to the other strains and was more adversely affected by the copper nanoparticles. Good correlation was observed between MIC and MBC ($r^2 = 0.98$) measured in liquid cultures. For copper nanoparticles a good negative correlation was observed between the inhibition zone observed in disk diffusion test and MIC/MBC determined based on liquid cultures with the various strains ($r^2 = -0.75$). Although strain-specific variation in MIC/MBC was negligible for *S. aureus*, some strain-specific variation was observed for *E. coli*.

Keywords: Silver; Copper; Nanoparticles; Antimicrobial activity

1. Introduction

Microbial contamination of water poses a major threat to public health. With the emergence of microorganisms resistant to multiple antimicrobial agents [1] there is increased demand for improved disinfection methods. The antimicrobial properties of silver ions were known since ancient times and silver ions are widely used as bactericide in catheters, burn wounds and dental work [2]. Researchers have also recommended the use of silver and copper ions as superior disinfectants for wastewater generated from hospitals containing infectious microorganisms [3,4]. However, residual copper and silver ions in the treated water may adversely affect human health [5]. The emergence of nanoscience and nanotechnology in the last decade presents opportunities for exploring the bactericidal effect of metal nanoparticles. The bactericidal effect of metal nanoparticles has been attributed to their small size and high surface to volume ratio, which allows them to interact closely with microbial membranes and is not merely due to the release of metal ions in solution [6]. Metal nanoparticles with bactericidal activity can be immobilized and coated on to surfaces, which may find application in various fields, i.e., medical instruments and devices,

water treatment and food processing. Metal nanoparticles may be combined with polymers to form composites for better utilization of their antimicrobial activity. Metal nanoparticles are also finding application in various other fields, i.e., catalysis and sensors [7–9]. However, it is also recognized that nanoparticles may have many undesirable and unforeseen effects on the environment and in the ecosystem [10,11].

The antimicrobial properties of silver nanoparticles are well-established [12–15] and several mechanisms for their bactericidal effects have been proposed. Although only a few studies have reported the antibacterial properties of copper nanoparticles, they show copper nanoparticles have a significant promise as bactericidal agent [16]. However, other nanoparticles, such as platinum, gold, iron oxide, silica and its oxides, and nickel have not shown bactericidal effects in studies with *Escherichia coli* [15,17,18]. Yoon et al. [19] reported the antibacterial effects of silver and copper nanoparticles using single representative strains of *E. coli* and *Bacillus subtilis*, where the copper nanoparticles demonstrated superior antibacterial activity compared to the silver nanoparticles. Silver and copper nanoparticles supported on various suitable materials, such as carbon, polyurethane foam, polymers and sepiolite have also been effectively used for bactericidal applications [13,14,20–22]. While various hypotheses have been proposed to explain the mechanism of antimicrobial activity of silver nanoparticles, it is widely believed that silver nanoparticles are incorporated in the cell membrane, which causes leakage of intracellular substances and eventually causes cell death [12,15]. Some of the silver nanoparticles also penetrate into the cells. It is reported that the bactericidal effect of silver nanoparticles decreases as the size increases and is also affected by the shape of the particles [23,24]. Although most studies have utilized spherical particles, truncated triangular shaped particles are reported to have greater bactericidal effect compared to that of spherical and rod-shaped particles [24]. It is also reported that bactericidal efficiency is affected by the type of microorganism. In studies with gram negative, *E. coli,* and gram positive, *Staphylococcus aureus*, Kim et al. [2] reported greater biocidal efficiency of silver nanoparticles for *E. coli*, and attributed it to difference in cell wall structure between gram negative and gram positive microorganisms. However, currently there is insufficient evidence to support such conclusions since most research on bactericidal effect of nanoparticles has been conducted with one or a very limited number of microbial strains [12–15].

The objective of this study was to compare the bactericidal effect of silver and copper nanoparticles using various microbial strains. Such a comparative study would reveal strain specificities and would eventually lead to better utilization of nanoparticles for specific application. Three representative bacteria typically recommended for use in antimicrobial assays, i.e., *E. coli*, *B. subtilis* and *S. aureus* were used and studies were conducted with eight strains, i.e. four *E. coli* strains, one *B. subtilis* strain and three *S. aureus* strains. The antimicrobial effect was quantified based on the inhibition zone measured in the disk diffusion tests conducted in plates and by determining the minimum growth inhibitory concentrations (MIC) and minimum bactericidal concentration (MBC) of nanoparticles in liquid batch cultures.

2. Materials and methods

2.1. Materials and bacterial strains

The bactericidal experiments were carried out with gram negative bacteria *E. coli* and gram positive bacteria *B. subtilis* and *S. aureus* in nutrient media, composed of peptone (Loba Chemie Ltd., Mumbai) and NaCl (Merck Ltd., Mumbai) 5 g l^{-1} each, and yeast extract (Central Drug House, New Delhi) and beef extract (S.D. Fine Chem Ltd., Mumbai) 1.5 g l^{-1} each. Throughout this study, the same nutrient media was used for all strains, unless otherwise specified. For preparing solid media, the nutrient media was supplemented with 2% bacteriological agar (Himedia Laboratories Ltd., Mumbai) as solidifying agent. The silver and copper nanoparticles were prepared by wet chemical synthesis involving stoichiometric reaction between sodium borohydride and silver/copper ions [25]. The nitrate salts of silver and copper (S.D. Fine Chem Ltd., Mumbai) were used as precursors and reaction with sodium borohydride was conducted by vigorously stirring the reaction mixture. After synthesis, the nanoparticles were washed twice with DI (deionised) water to ensure removal of residual boron. The synthesis and drying of nanoparticles were conducted in a reducing environment provided using pure hydrogen gas. The nanoparticles were subsequently stored in air-tight containers.

E. coli strains MTCC 443 (ATCC 25922), MTCC 739 (ATCC 10536), MTCC 1302 (wild type), MTCC 1687 (ATCC 8739), and *B. subtilis* strain MTCC 441 (ATCC 6633) were procured from the Institute of Microbial Technology (Chandigarh, India). *S. aureus* strains NCIM 2079 (ATCC 6538P), NCIM 5021 (ATCC 25923) and NCIM 5022 (ATCC 29213) were procured from the National Chemical Laboratory (Pune, India).

2.2. Sample preparation and characterization

Semi-quantitative analysis of nanoparticles was carried out by energy-dispersive X-ray spectroscopy (EDS, FEI Quanta 200, Holland). The crystallinity of the nanoparticles was further characterized using a X-ray diffractometer (XRD, Philips PW3040/60 X'pert PRO, The Netherlands) employing Cu K_α radiation. The X-ray diffraction data was collected over the range 20–120° in increments of 0.017 per 15.18 s. This low scan speed was selected to obtain greater sensitivity for detection of impurities in the sample. The nanoparticles were digested with conc. HNO_3 (Suprapur, Merck, Germany) and analyzed by inductively coupled plasma-atomic emission spectroscopy (ICP-AES, GBC

Scientific 8440 Plazmalab, Australia) for determining the presence of residual boron in the nanoparticles. The size and morphology of silver and copper nanoparticles were examined using a transmission electron microscope (TEM, Philips CM200, The Netherlands). A suspension of the nanoparticles (0.1 mg) in methanol (10 ml) was sonicated for 15 min and a drop was placed onto a copper grid. After drying overnight for evaporation of the solvent, the nanoparticles on the copper grid were examined using a TEM.

Particle size analysis was performed to determine the size distribution of synthesized silver and copper nanoparticles in DI water/nutrient media. Silver and copper nanoparticles were suspended in DI water and nutrient media contained in conical flasks. After sonication for 10 min the flasks were incubated in an orbital shaker set at 200 rpm. The particle size of suspended nanoparticles was measured with time up to 24 h and the particle size distribution was obtained by dynamic light scattering (DLS, Brookhaven BI-200SM Goniometer equipped with BI-9000AT Digital Autocorrelation Version 2.0 software, USA). DLS analysis was performed using laser light (Coherent INNOVA 70C, USA) set at 488 nm and 15 mW. The analysis was carried out at a fixed scatter angle of 90° and at 25 °C. With appropriate dilution, the polydispersity index (PDI) was maintained at 0.2–0.3 in all the experiments to ensure proper dispersion.

2.3. Disk diffusion test

Bacterial sensitivity to antibiotics is commonly tested using a disk diffusion test, employing antibiotic impregnated disks [26]. A similar test with nanoparticle laden disks was used in this study. A 5 ml suspension of nanoparticles (5 mg ml^{-1}) was sonicated and subsequently filtered through a membrane filter (0.2 μm, 47 mm diameter Pall Gelman Laboratory). The nanoparticle laden filter paper was dried in an oven for 1 h and small disks of uniform size (6 mm diameter) containing 100 ± 15 μg nanoparticles were punched out and stored in a desiccator at room temperature. The bacterial suspension (100 μl of 10^4–10^5 CFU ml^{-1}) was applied uniformly on the surface of a nutrient agar plate before placing the disks on the plate (4 per plate). The plates were incubated at 35 °C for 24 h, after which the average diameter of the inhibition zone surrounding the disk was measured with a ruler with up to 1 mm resolution. The mean and standard deviation (SD) reported for each type of nanoparticle and with each microbial strain were based on six replicates.

2.4. Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

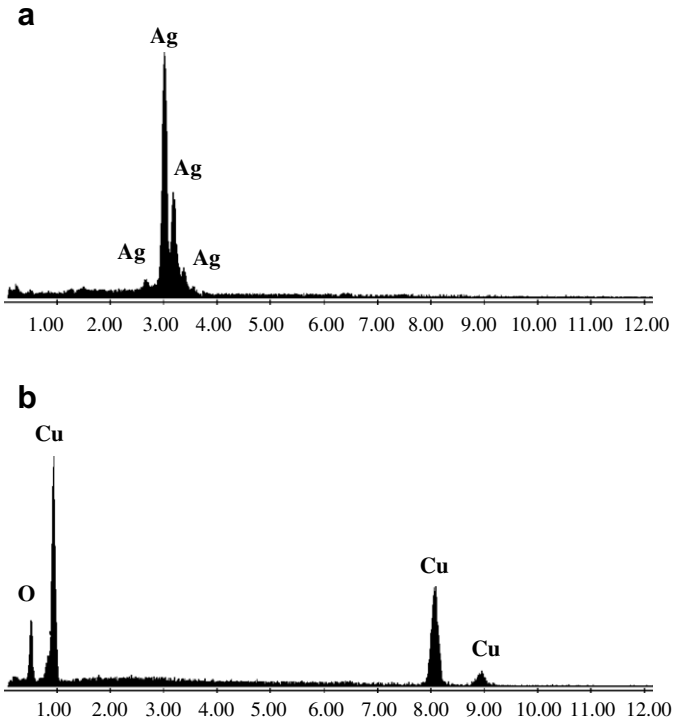
The minimum inhibitory concentration (MIC), defined as the lowest concentration of material that inhibits the growth of an organism [27], was determined based on batch cultures containing varying concentration of silver/copper nanoparticles in suspension (20–300 mg l^{-1}). Sterile Erlenmeyer flasks (500 ml), each containing 100 ml nutrient broth were sonicated for 10 min after adding the nanoparticles to prevent aggregation of the nanoparticles. Subsequently, the flasks were inoculated with 1 ml of the freshly prepared bacterial suspension in order to maintain initial bacterial concentration 10^3–10^4 CFU ml^{-1}, and then incubated in an orbital shaker at 200 rpm and 30 °C. The high rotary shaking speed was selected to minimize aggregation and settlement of the nanoparticles over the incubation period. Lower rpm setting during incubation may cause underestimation of the antimicrobial activity of the nanoparticles. Bacterial growth was measured as increase in absorbance at 600 nm determined using a spectrophotometer (Thermo Spectronic, Helios Epsilon, USA). The experiments also included a positive control (flask containing nanoparticles and nutrient media, devoid of inoculum) and a negative control (flask containing inoculum and nutrient media, devoid of nanoparticles). The negative controls indicated the microbial growth profile in the absence of nanoparticles. The absorbance values for positive controls were subtracted from the experimental values (flasks containing nutrient media, inoculum and nanoparticles) [17]. All the experiments were carried out in triplicate. Both silver and copper nanoparticles were tested for bactericidal effect using all the microbial cultures selected for the study.

The minimum bactericidal concentration (MBC), i.e., the lowest concentration of nanoparticles that kills 99.9% of the bacteria was also determined from the batch culture studies. For growth inhibitory concentration ($\geqslant$MIC) the presence of viable microorganisms was tested and the lowest concentration causing bactericidal effect was reported as MBC as suggested by Avadi et al. [28]. To test for bactericidal effect, a loopful from each flask was inoculated on nutrient agar and incubated at 35 °C for 24 h. The nanoparticle concentration causing bactericidal effect was selected based on absence of colonies on the agar plate.

The release of Ag^+ and Cu^{2+} ions from the nanoparticles into DI water and nutrient media was studied by suspending 10 mg of nanoparticles in 100 ml DI water/media and sonicating for 10 min. The suspension was kept in a rotary shaker under the same conditions as in the above studies and residual Ag^+ and Cu^{2+} concentration in the aqueous phase was determined by ICP-AES after 24 h.

3. Results and discussion

The EDS profile of silver nanoparticles (Fig. 1a) indicates that the sample contains pure silver, with no oxide layer. In contrast, an oxygen peak is observed in the EDS profile of the copper nanoparticles (Fig. 1b), suggesting the presence of an oxide layer. The XRD pattern of silver and copper nanoparticles (Fig. 2a and b) were compared and interpreted with standard data of International Centre of Diffraction Data (ICDD). The eight characteristic peaks for silver nanoparticles appeared at 38.1°, 44.3°, 64.4°, 77.4°, 81.5°, 98.7°, 110.9° and 114.9°, which



Element	Wt%
Ag	100
Total	100

Element	Wt%
O	12.48
Cu	87.52
Total	100

Fig. 1. EDS profile of (a) silver and (b) copper nanoparticles and quantitative analysis.

correspond to crystal facets of (111), (200), (220), (311), (222), (400), (331) and (420) of silver. The eight characteristic peaks for cuprite appeared in the sample of copper nanoparticles at 29.6°, 36.3°, 42.2°, 61.3°, 73.5°, 77.5°, 104.1°, 108.8°, which correspond to crystal facets of (110), (111), (200), (220), (311), (222), (331) and (420). Each crystallographic facet contains energetically distinct sites based on atom density. The silver and copper nanoparticles both contain high atom density facets such as (111) that are known to be highly reactive [6,29]. The XRD pattern of silver and copper nanoparticles confirm the presence of silver in pure form whereas copper is present as cuprite. No additional impurities were detected either in the EDS or in the XRD profile. While EDS only depicts elements present in excess of 5% (w/w), XRD conducted at low scan speed is more sensitive [30] and assumed to detect elements at low concentration. Estimation of residual boron by ICP-AES analysis after digestion of nanoparticles in conc. HNO_3 indicated boron levels of 0.1% and 0.4%, respectively, in the silver and copper nanoparticles. Thus, the possible presence of other trace level impurities cannot be excluded.

TEM images (Fig. 3a and b) confirm that the metal particles are in the nano range and that they are approximately spherical in shape. Subsequent image analysis revealed that the silver nanoparticles are relatively smaller (mean $\pm$ SD: 3.32 ± 1.129 nm; size range: 2.26–10.34 nm for a scan of $n = 2582$ particles) than the copper nanoparticles (9.25 ± 1.79 nm; range: 6.86–16.53 nm; $n = 683$). Considerable asymmetry was observed in the particle size distribution profile.

The antibacterial activity of silver and copper nanoparticles was compared for various microorganisms using the diameter of inhibition zone in disk diffusion test. The diameter of inhibition zone (DIZ) reflects magnitude of susceptibility of the microorganism. The strains susceptible to disinfectants exhibit larger DIZ, whereas resistant strains exhibit smaller DIZ. The disks with silver nanoparticles were surrounded by a larger DIZ compared to the copper nanoparticles for all *E. coli* and *S. aureus* strains selected for this study (Figs. 4 and 5a–c). While the DIZ was affected by the type of microorganisms, it was essentially invariant across the various strains of *E. coli* and *S. aureus*. The DIZ for silver nanoparticle impregnated disks was almost 40–50% greater than that observed with the copper nanoparticle impregnated disks for all the *E. coli* strains selected for this study. Similarly, for *S. aureus* the silver nanoparticle impregnated disks were found to be more effective compared to copper nanoparticle impregnated disks, however the difference in the DIZ was merely 10–15%. In contrast, for *B. subtilis*, the disks impregnated with copper nanoparticles showed a significantly larger DIZ, almost 90% greater compared to that observed with silver nanoparticles. Since DIZ was measured on agar plates using a ruler with 1 mm resolution, the possibility of measurement errors exist; however, the method illustrates the potential biocidal effect of nanoparticles to different microbial strains.

The MIC and MBC representing the antimicrobial activity of nanoparticles dispersed in batch cultures is summarized in Table 1. Representative growth profile of microbial strains in the presence of varying concentration of

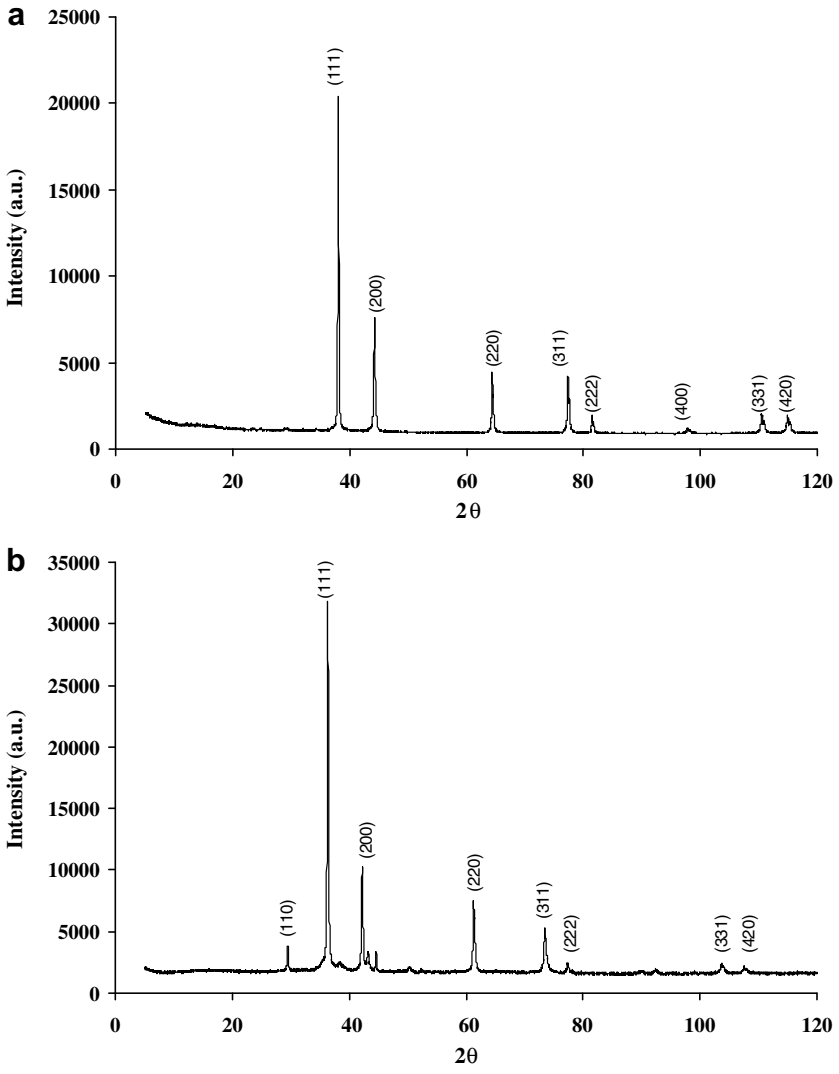


Fig. 2. XRD pattern of (a) silver and (b) copper nanoparticles.

silver and copper nanoparticles are depicted in Fig. 6a–c. In batch studies, a greater lag phase and lower maximum absorbance (at 600 nm) were observed as the concentration of nanoparticles increased. Similar observation was reported by Sondi and Salopek-Sondi [12] in their studies on effect of silver nanoparticles on a single strain of *E. coli*. As concentration of nanoparticles increased to MIC of the respective strains, no growth was observed in the flask. The bactericidal effect of nanoparticles is dependent on the concentration of nanoparticles and the initial bacterial concentration [24]. In this study, the initial bacterial concentration was almost constant at 10^3–10^4 CFU ml^{-1} irrespective of nanoparticle concentration and microbial strain.

As for the disk diffusion tests, the batch studies also reveal differences in sensitivity to silver and copper nanoparticles for the various microbial strains. Among all the *E. coli* strains selected for this study, the strain most sensitive to silver and copper nanoparticles was MTCC 443. The *E. coli* strains least sensitive to silver and copper nanoparticles were MTCC 739 and MTCC 1687, respectively. The MIC observed in this study for silver nanoparticles are 40 μg ml^{-1} for MTCC 443, 120 μg ml^{-1} for MTCC 1302, 140 μg ml^{-1} for MTCC 1687 and 180 μg ml^{-1} for MTCC 739. Our results are in contrast with some studies reporting negligible inhibitory effect of silver nanoparticles on *E. coli* up to 100 μg ml^{-1} [12,23]. However, these studies employed silver nanoparticles of larger size (12–40 nm) and higher initial concentration of bacteria in the batch cultures (10^5–10^8 CFU ml^{-1}). For *E. coli* at initial concentration of 10^6 CFU ml^{-1} suspended in distilled water, Li et al. [31] reported the MIC of silver nanoparticles (~20 nm) as 40 μg ml^{-1}. The relatively low MIC is possibly due to suspension of the cells in distilled water compared to suspension in nutrient media as employed in our study. For studies conducted on agar plates, the MIC of silver nanoparticles for *E. coli* was reported as 75 μg ml^{-1} [6]. In batch studies with *E. coli* and colloidal silver nanoparticle (size range 2–25 nm), MIC was reported to be in the range of 3–25 μg ml^{-1} for initial bacterial concentration 10^5–

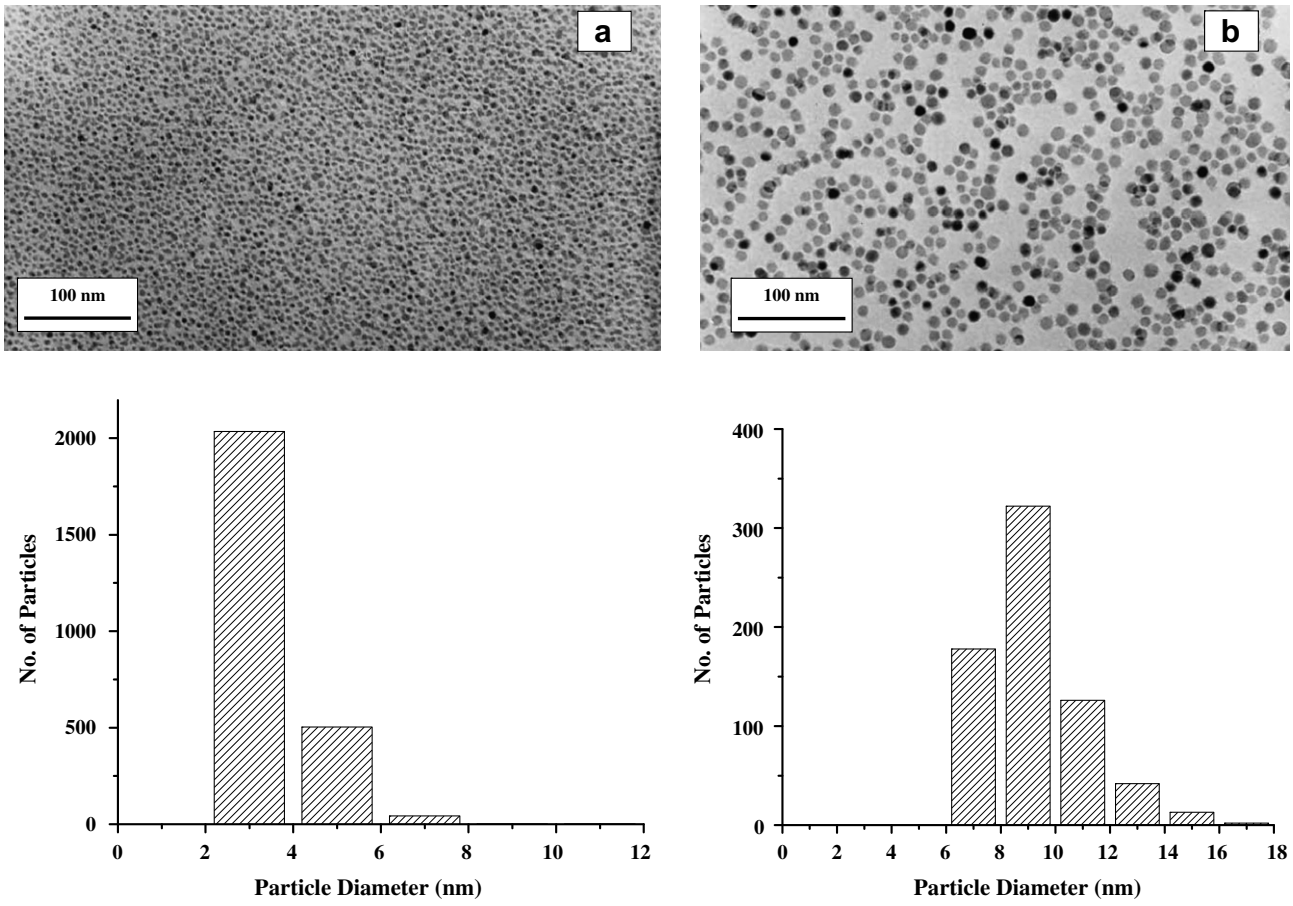


Fig. 3. (a) TEM image of silver nanoparticles and particle size distribution obtained by image analysis. (b) TEM image of copper nanoparticles and particle size distribution obtained by image analysis.

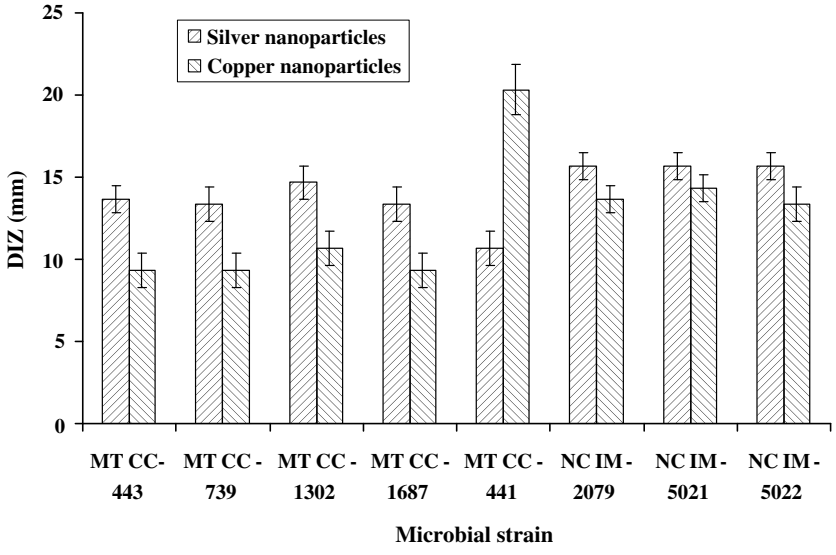


Fig. 4. The diameter of inhibition zone (DIZ) surrounding silver/copper nanoparticle impregnated disks (6 mm diameter) in presence of various microorganisms.

10^8 CFU ml^{-1} [15,23,32]. Due to variation in the *E. coli* strain employed, variation in the size of silver nanoparticles and initial bacterial concentration, direct comparison between the studies is not feasible. Several studies on antimicrobial activity of silver nanoparticles were carried out with colloidal nanoparticles [2,23,32,33], while dry nano-

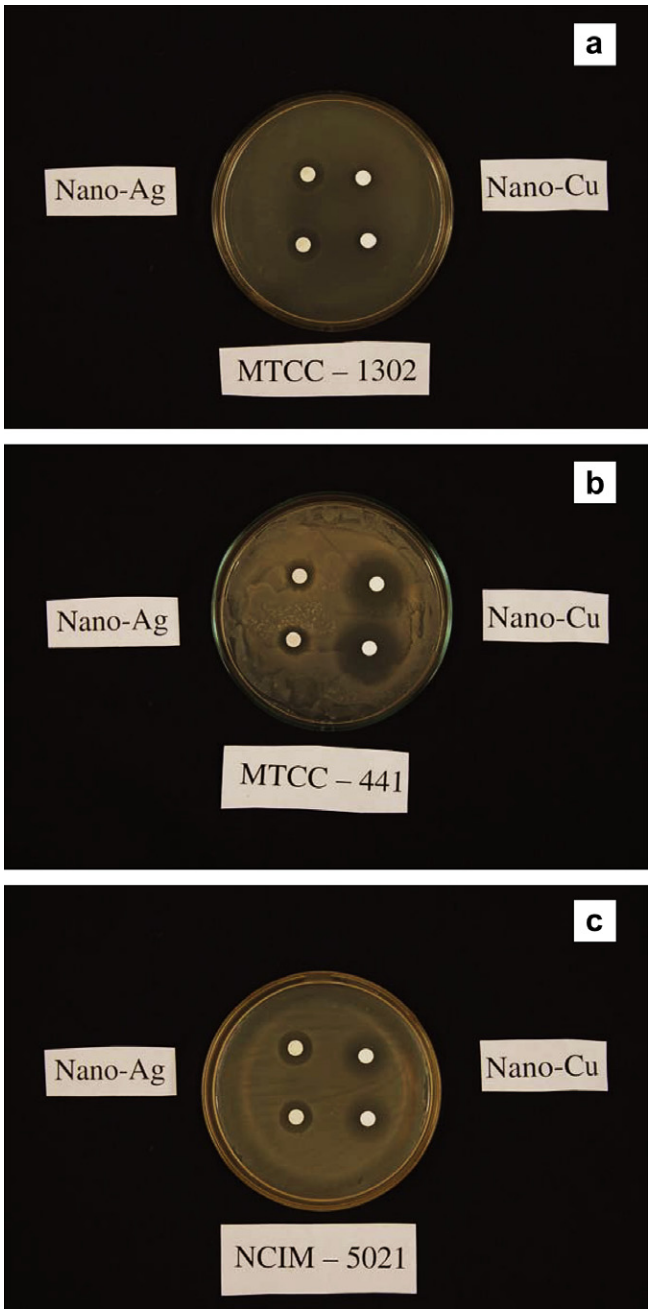


Fig. 5. Representative images of agar plates containing silver and copper nanoparticle impregnated disks and DIZ for (a) *E. coli* (MTCC 1302), (b) *B. subtilis* (MTCC 441) and (c) *S. aureus* (NCIM 5021).

particles in powder form was re-suspended in the nutrient media in our studies. In aqueous suspension, the mean hydrodynamic diameter of the nanoparticles is expected to be in the same range as that based on TEM [34]. However, in this study, the DLS results depicted that maximum number of silver and copper nanoparticles were in the size range of 12–21 nm and 41–82 nm, respectively both in DI water and in nutrient media. No systematic variation in size was observed with increase in time up to 24 h. The measured particle size using DLS is higher compared to the size measured by TEM possibly due to agglomeration

Table 1
MIC ($\mu g\ ml^{-1}$) and MBC ($\mu g\ ml^{-1}$) of silver and copper nanoparticles for various microorganisms

Culture	Strain no.	MIC		MBC	
		Ag	Cu	Ag	Cu
Escherichia coli	MTCC 443	40	140	60	160
Escherichia coli	MTCC 739	180	220	220	260
Escherichia coli	MTCC 1302	120	200	160	220
Escherichia coli	MTCC 1687	140	280	180	300
Bacillus subtilis	MTCC 441	40	20	60	40
Staphylococcus aureus	NCIM 2079	120	140	160	160
Staphylococcus aureus	NCIM 5021	120	140	160	160
Staphylococcus aureus	NCIM 5022	120	140	160	160

of nanoparticles in water. However, the agglomeration effect was not enhanced by presence of salts in the nutrient media and increasing agglomeration over time was not observed. The agglomeration effect may have affected the bactericidal efficiency and MIC/MBC values as also suggested by Gan et al. [35].

While both *E. coli* and *S. aureus* depict higher sensitivity to the silver nanoparticles compared to the copper nanoparticles, the difference is less for *S. aureus* compared to *E. coli*. Moreover, all the *S. aureus* strains exhibited identical sensitivity to silver and copper nanoparticles and no strain specificity was observed. Similar results indicating no strain specificity was reported by Panacek et al. [23] for effect of silver nanoparticles on two strains of *S. aureus.* Kim et al. [2] reported that gram positive *S. aureus* is more resistant to silver nanoparticles compared to gram negative *E. coli*, based on studies with single strains of each culture. Here, we demonstrate that some *E. coli* strains such as MTCC 739 and MTCC 1687 are more resistant than the *S. aureus* strains. Hence, the bactericidal efficiency of nanoparticles is not solely dependent on the structure of the bacterial membrane.

The gram positive *B. subtilis* strain MTCC 441 was found to be more sensitive to the copper nanoparticles compared to the silver nanoparticles as also observed in the disk diffusion studies. The bactericidal effect of silver nanoparticles on *B. subtilis* appears to be significantly greater in the studies with dispersed nanoparticles compared to the disk diffusion studies. Yoon et al. [19] carried out studies with 200 CFU ml^{-1} of specific strains of *E. coli* and *B. subtilis* inoculated in plates with silver (size ~40 nm) and copper (size ~100 nm) nanoparticles and reported that *B. subtilis* is more sensitive to both copper and silver nanoparticles compared to *E. coli*. Moreover, Yoon et al. [19] reported that copper nanoparticles have greater bactericidal effect compared to silver nanoparticles for the single strains of *E. coli* and *B. subtilis* studied. Our study conducted in flasks with initial bacterial concentration of 10^3–10^4 CFU ml^{-1}, also revealed greater sensitivity of *B. subtilis* compared to *E. coli* or *S. aureus*, although the size of silver (size ~3 nm) and copper (size ~10 nm) nanoparticles employed were different. However, in the disk diffusion tests conducted on plates, the four strains of *E. coli* demon-

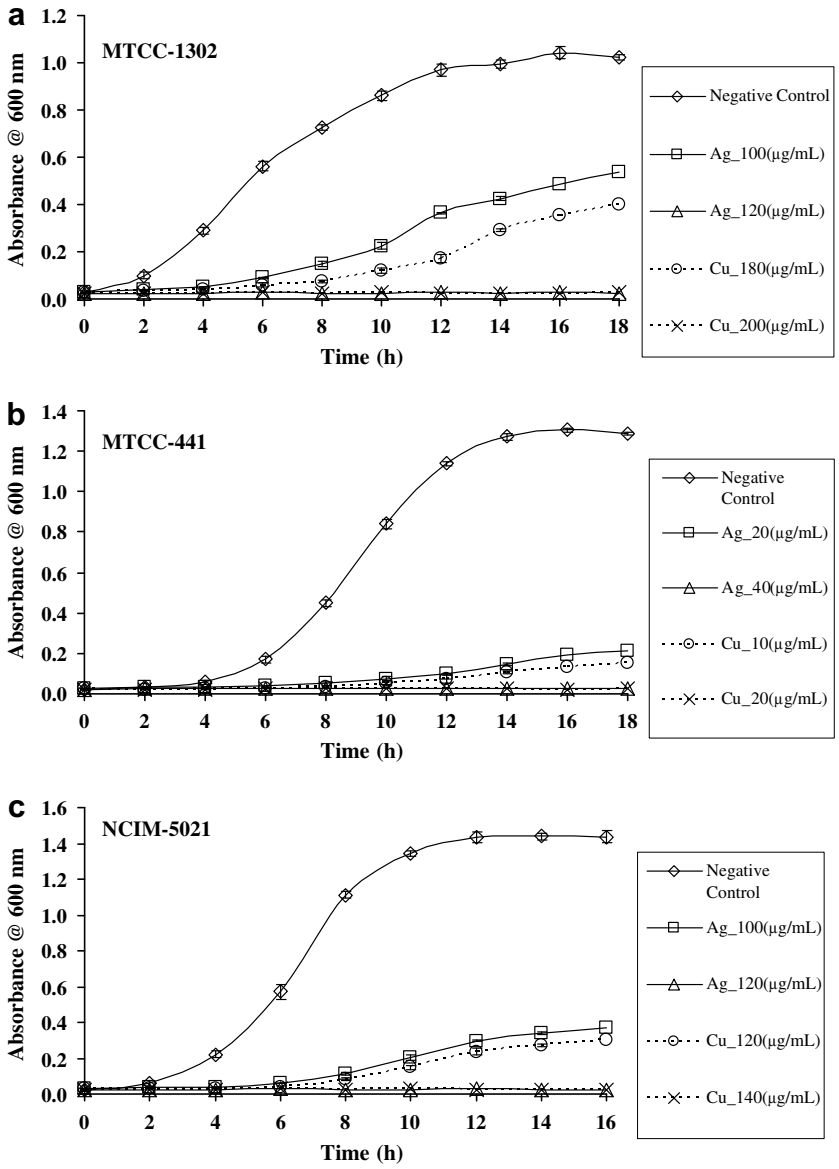


Fig. 6. Representative batch growth profile in presence of varying concentration of silver/copper nanoparticles for (a) *E. coli* (MTCC 1302), (b) *B. subtilis* (MTCC 441) and (c) *S. aureus* (NCIM 5021).

strated greater sensitivity to silver nanoparticles compared to copper nanoparticles, which is contradictory to the results demonstrated by Yoon et al. [19]. It is possible that results of the disk diffusion test reporting antimicrobial activity in terms of DIZ is less accurate compared to the tests conducted in batch culture with dispersed nanoparticles. This may also explain why strain specificity across the various *E. coli* strains observed in batch cultures with silver nanoparticles was not observed in the disk diffusion test. A good correlation is observed between the MIC and MBC values across all the cultures and the two types of nanoparticles studied (correlation coefficient, $r^2 = 0.98$). A negative correlation ($r^2 = -0.75$) was observed for values of MIC/MBC of copper nanoparticles and DIZ obtained with copper nanoparticles across all the cultures. No correlation could be observed for MIC/MBC of silver nanoparticles and DIZ obtained with silver nanoparticles across all the cultures.

A few studies have been performed to elucidate the mechanism of bactericidal action of nanoparticles. It is difficult to distinguish between the bactericidal activity of nanoparticles from that of the ions released by the nanoparticles [19]. Although this study was not designed to distinguish between the effect of nanoparticles as distinct from the effect of ions, the ions released into the aqueous phase were estimated for 10 mg of nanoparticles suspended in 100 ml nutrient media and DI water. The aqueous phase Ag^+ and Cu^{2+} concentration were 4 and 17 mg l^{-1}, respectively, after 24 h of incubation in a rotary shaker. The corresponding values for nanoparticles suspended in DI water under the same conditions over a period of 24 h were 0.3 and 0.5 mg l^{-1} for Ag^+ and Cu^{2+}, respectively. Thus, it

appears that the nutrient media facilitated the release of Ag^{+} and Cu^{2+} ions possibly due to reaction with the nutrient media constituents. The significantly greater release of Cu^{2+} ions in the nutrient media is possibly due to presence of the oxide layer on the copper nanoparticles and reaction with chloride ions in the media. For oxidized copper particles embedded in an inert, Teflon-like matrix, Cioffi et al. [36] demonstrated significant antimicrobial activity due to release of ions. In addition to the direct effect of the nanoparticles on bacterial membrane, the bactericidal effects observed in our study are also impacted by the release of Ag^{+}/Cu^{2+} ions in solution. The presence of nanoparticles in suspension would ensure continuous release of ions into the nutrient media. Silver or copper ions released by the nanoparticles may attach to the negatively charged bacterial cell wall and rupture it, thereby leading to protein denaturation and cell death [4]. Siva Kumar et al. [13] proposed that oxygen associates with silver and reacts with the sulfhydryl (–S–H) groups on cell wall to form R–S–S–R bonds thus, blocking respiration and causing death of cells. Cho et al. [15] reported that the surface of the cell walls of *E. coli* treated with silver nanoparticles were severely damaged compared to untreated *E. coli*. Cell wall rupture due to silver ions and silver nanoparticles was reported by Lok et al. [33]. The attachment of both silver ions or nanoparticles to the cell wall caused accumulation of envelope protein precursors, which resulted in dissipation of the proton motive force. Silver nanoparticles also exhibited destabilization of the outer membrane and rupture of the plasma membrane, thereby causing depletion of intracellular ATP [33]. The mode of action of both silver nanoparticles and silver ions were reported to be similar, although the nanoparticles were reported to be effective at significantly lower concentration than that of the ions. However, Morones et al. [6] proposed that the bactericidal mechanism of silver nanoparticles and silver ions are distinctly different. For treatment with silver nitrate, a low molecular weight central region was formed within the cells as a defense mechanism, whereas for treatment with nanoparticles, no such phenomenon was observed, although the nanoparticles were found to penetrate through the cell wall. With a detailed study of DNA/protein migration profiles Gogoi et al. [32] demonstrated that silver nanoparticles have no direct effect on either cellular DNA or protein, although the silver nanoparticles were more efficient bactericidal agent compared to the silver ions [33]. For *E. coli* (ATCC 10536) and *S. aureus* (ML 422), silver nanoparticles demonstrated greater bactericidal efficiency compared to penicillin [37]. Moreover, for bactericidal effects on *E. coli*, silver nanoparticles have also depicted synergistic effects with known antibiotics, such as amoxicillin [31].

No reports are available for mechanism of bactericidal effect of copper nanoparticles. We speculate a similar mechanism of action for copper nanoparticles, as for the silver nanoparticles. The reason for greater sensitivity of *B. subtilis* to the copper nanoparticles may be attributed to greater abundance of amines and carboxyl groups on cell surface of *B. subtilis* and greater affinity of copper towards these groups [38]. Copper ions released subsequently may bind with DNA molecules and lead to disorder of the helical structure by cross-linking within and between the nucleic acid strands. Copper ions inside bacterial cells also disrupt biochemical processes [39,40]. The exact mechanism behind bactericidal effect of copper nanoparticles is not known and needs to be studied further.

4. Conclusions

Growth studies of different microbial cultures were performed in the presence of nanoparticles to observe their effect on the growth profile. This study shows that silver and copper nanoparticles have great promise as antimicrobial agent against *E. coli*, *B. subtilis* and *S. aureus*. MIC, MBC and disk diffusion test suggest that for all cultures of *E. coli* and *S. aureus*, the antimicrobial action of the silver nanoparticles were superior. Although an oxide layer was formed on the copper nanoparticles, these nanoparticles demonstrated better antimicrobial activity towards *B. subtilis*. The MIC/MBC determined in batch cultures with varying concentration of silver and copper nanoparticles reflected the strain specificity with respect to silver and copper nanoparticles. We assume that copper nanoparticles have greater affinity to surface active groups of *B. subtilis*, which may have led to its greater bactericidal effect. The mechanism of action of the silver and copper nanoparticles is not yet fully established. Combination of silver and copper nanoparticles may give rise to more complete bactericidal effect against mixed bacterial population. Before commercialization, detailed research and comparative study of strain-specific variability is required to determine the bactericidal efficiency of metal nanoparticles.

Acknowledgements

The authors gratefully acknowledge the National Doctoral Fellowship (NDF) awarded by All India Council for Technical Education (AICTE), New Delhi, India, which provided funds for student support. The authors would like to acknowledge Department of Metallurgical Engineering and Material Science, IIT Bombay, for the XRD analysis, SAIF (sophisticated analytical instrument facility) IIT Bombay, for the EDS, ICP-AES and TEM analysis, and CRNTS (Centre for Research in Nanotechnology and Science) IIT Bombay, for DLS analysis. The anonymous reviewers are acknowledged for providing valuable comments and insights for improving the manuscript.

References

[1] Kolar M, Urbanek K, Latal T. Antibiotic selective pressure and development of bacterial resistance. Int J Antimicrob Ag 2001;17:357–63.

[2] Kim JS et al. Antimicrobial effects of silver nanoparticles. Nanomedicine 2007;3:95–101.

[3] Lin YE, Vidic RD, Stout JE, Yu VL. Individual and combined effects of copper and silver ions on inactivation of *Legionella pneumophila*. Water Res 1996;30:1905–13.
[4] Lin YE, Vidic RD, Stout JE, Mccartney CA, Yu VL. Inactivation of *Mycobacterium avium* by copper and silver ions. Water Res 1998;32:1997–2000.
[5] Blanc DS, Carrara P, Zanetti G, Francioli P. Water disinfection with ozone, copper and silver ions, and temperature increase to control Legionella: seven years of experience in a university teaching hospital. J Hosp Infect 2005;60:69–72.
[6] Morones JR, Elechiguerra JL, Camacho A, Holt K, Kouri JB, Ramirez JT, et al. The bactericidal effect of silver nanoparticles. Nanotechnology 2005;16:2346–53.
[7] Vaseashta A, Dimova-Malinovska D. Nanostructured and nanoscale devices, sensors and detectors. Sci Technol Adv Mater 2005;6:312–8.
[8] Comini E. Metal oxide nano-crystals for gas sensing. Anal Chim Acta 2006;568:28–40.
[9] Raveh A, Zukerman I, Shneck R, Avni R, Fried I. Thermal stability of nanostructured superhard coatings: a review. Surf Coat Technol 2007;201:6136–42.
[10] Long TC, Saleh N, Tilton RD, Lowry GV, Veronesi B. Titanium dioxide (P25) produces reactive oxygen species in immortalized brain microglia (BV2): implications for nanoparticle neurotoxicity. Environ Sci Technol 2006;40:4346–52.
[11] Porter AE, Muller K, Skepper J, Midgley P, Welland M. Uptake of C_{60} by human monocyte macrophages, its localization and implications for toxicity: studied by high resolution electron microscopy and electron tomography. Acta Biomater 2006;2:409–19.
[12] Sondi I, Salopek-Sondi B. Silver nanoparticles as antimicrobial agent: a case study on *E. coli* as a model for gram-negative bacteria. J Colloid Interf Sci 2004;275:177–82.
[13] Siva Kumar V, Nagaraja BM, Shashikala V, Padmasri AH, Madhavendra SS, Raju BD, et al. Highly efficient Ag/C catalyst prepared by electro-chemical deposition method in controlling microorganisms in water. J Mol Catal A Chem 2004;223:313–9.
[14] Jain P, Pradeep T. Potential of silver nanoparticle-coated polyurethane foam as an antibacterial water filter. Biotechnol Bioeng 2005;90:59–63.
[15] Cho K, Park J, Osaka T, Park S. The study of antimicrobial activity and preservative effects of nanosilver ingredient. Electrochim Acta 2005;51:956–60.
[16] Cioffi N et al. Copper nanoparticle/polymer composites with antifungal and bacteriostatic properties. Chem Mater 2005;17:5255–62.
[17] Williams DN, Ehrman SH, Holoman TRP. Evaluation of the microbial growth response to inorganic nanoparticles. J Nanobiotechnol 2006;4:3.
[18] Ruparelia JP, Duttagupta SP, Chatterjee AK, Mukherji SM. A comparative study on disinfection potential of nanosilver and nanonickel. Technical poster, Proceedings of the 9th Annual Conference of the Indian Environmental Association (Envirovision-2006), entitled "Advances in Environmental Management and Technology", Goa, India, September 21–23, 2006.
[19] Yoon K, Byeon JH, Park J, Hwang J. Susceptibility constants of *Escherichia coli* and *Bacillus subtilis* to silver and copper nanoparticles. Sci Total Environ 2007;373:572–5.
[20] Li Z, Lee D, Sheng X, Cohen RE, Rubner MF. Two-level antibacterial coating with both release-killing and contact-killing capabilities. Langmuir 2006;22:9820–3.
[21] Son WK, Youk JH, Park WH. Antimicrobial cellulose acetate nanofibers containing silver nanoparticles. Carbohydr Polym 2006;65:430–4.
[22] Esteban-Cubillo A, Pecharroman C, Aguilar E, Santaren J, Moya JS. Antibacterial activity of copper monodispersed nanoparticles into sepiolite. J Mater Sci 2006;41:5208–12.
[23] Panacek A et al. Silver colloid nanoparticles: synthesis, characterization, and their antibacterial activity. J Phys Chem B 2006;110:16248–53.
[24] Pal S, Tak YK, Song JM. Does the antimicrobial activity of silver nanoparticles depend on the shape of the nanoparticle? A study of the gram-negative bacterium *Escherichia coli*. Appl Environ Microbiol 2007;73:1712–20.
[25] He S et al. Formation of silver nanoparticles and self-assembled two-dimensional ordered superlattice. Langmuir 2001;17:1571–5.
[26] Case CL, Johnson TR. Laboratory experiments in microbiology. California: Benjamin Cummings Pub Inc; 1984, p. 126–9.
[27] Qi L, Xu Z, Jiang X, Hu C, Zou X. Preparation and antibacterial activity of chitosan nanoparticles. Carbohydr Res 2004;339:2693–700.
[28] Avadi MR, Sadeghi AMM, Tahzibi A, Bayati Kh, Pouladzadeh M, Zohuriaan-Mehr MJ, et al. Diethylmethyl chitosan as an antimicrobial agent: synthesis, characterization and antibacterial effects. Eur Polym J 2004;40:1355–61.
[29] Hatchett DW, White HS. Electrochemistry of sulfur adlayers on the low-index faces of silver. J Phys Chem 1996;100:9854–9.
[30] Yanagi H, Ohno S, Kamiya T. Magnetic and carrier transport properties of Mn-doped *p*-type semiconductor LaCuOSe: an investigation of the origin of ferromagnetism. J Appl Phys 2006;100:033717.
[31] Li P, Li J, Wu C, Wu Q, Li J. Synergistic antibacterial effects of β-lactam antibiotic combined with silver nanoparticles. Nanotechnology 2005;16:1912–7.
[32] Gogoi SK, Gopinath P, Paul A, Ramesh A, Ghosh SS, Chattopadhyay A. Green fluorescent protein-expressing *Escherichia coli* as a model system for investigating the antimicrobial activities of silver nanoparticles. Langmuir 2006;22:9322–8.
[33] Lok C et al. Proteomic analysis of the mode of antibacterial action of silver nanoparticles. J Proteome Res 2006;5:916–24.
[34] Jacob JA, Kapoor S, Biswas N, Mukherjee T. Size tunable synthesis of silver nanoparticles in water–ethlene glycol mixtures. Colloid Surface A 2007;301:329–34.
[35] Gan X, Liu T, Zhong J, Liu X, Li G. Effect of Silver nanoparticles on the electron transfer reactivity and the catalytic activity of myoglobin. Chembiochem 2004;5:1686–91.
[36] Cioffi N et al. Analytical characterization of bioactive fluoropolymer ultra-thin coatings modified by copper nanoparticles. Anal Bioanal Chem 2005;381:607–16.
[37] Sarkar S, Jana AD, Samanta SK, Mostafa G. Facile synthesis of silver nanoparticles with highly efficient anti-microbial property. Polyhedron 2007;26:4419–26.
[38] Beveridge TJ, Murray RGE. Sites of metal deposition in the cell wall of *Bacillus subtilis*. J Bacteriol 1980;141:876–87.
[39] Kim J, Cho H, Ryu S, Choi M. Effects of metal ions on the activity of protein tyrosine phosphatase VHR: highly potent and reversible oxidative inactivation by Cu^{2+} ion. Arch Biochem Biophys 2000;382:72–80.
[40] Stohs SJ, Bagchi D. Oxidative mechanisms in the toxicity of metal ions. Free Radic Biol Med 1995;18:321–36.