

Original Article

INVESTIGATION OF THE SYNERGISTIC ANTIBACTERIAL ACTION OF COPPER NANOPARTICLES ON CERTAIN ANTIBIOTICS AGAINST HUMAN PATHOGENS

M. SELVARANI*

Department of Zoology, V. V. Vanniaperumal College for Women (Autonomous), Virudhunagar 626001, Tamil Nadu, India
Email: selvarani.msrani@gmail.com

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ABSTRACT

Objective: Resistance to antibacterial agents by pathogenic bacteria has emerged in recent years and is a major challenge for the healthcare industry. Copper nanoparticles (CuNPs) are known to be one of the multifunctional inorganic nanoparticles with effective antibacterial activity. Hence the present investigation has been focused on synthesizing and evaluating the bactericidal effect of copper nanoparticles.

Methods: CuNPs were synthesized by reducing the aqueous solution of copper sulfate with sodium borohydride. The synthesized particles were characterized by x-ray diffractogram (XRD), scanning electron microscopy (SEM) and energy dispersive spectroscopy (EDS) techniques to analyze size, morphology and quantitative information respectively. The antibacterial activity of CuNPs was examined by agar well diffusion method. Synergistic effect of CuNPs with broad-spectrum antibiotics was determined by the agar disc diffusion method.

Results: Color change of reaction mixture from blue to dark brown indicated the formation of CuNPs. SEM image clearly demonstrated that the synthesized particles were spherical in shape and its size was found to be 17.85 nm. EDS report confirmed the presence of elemental copper in the resultant nanoparticles and its accounts for major proportion (96%) of the mass of nanoparticles. Bacterial effect of CuNPs revealed that *Pseudomonas aeruginosa* showed the highest antibacterial sensitivity (16.00±1.63 mm), whereas least susceptibility (9.67±0.47 mm) was noticed against *Staphylococcus aureus*. An enhanced antibacterial activity of commercial antibiotics was also noticed when it combined with CuNPs. A minimum zone of inhibition was increased from 0.67±0.47 mm to 10.66±0.24 mm when the nanoparticles and antibiotics were given together.

Conclusion: It was observed that copper nanoparticles exhibited profound activity against all the tested bacterial strains which shows that CuNPs may serve as a better option for use in medicine in the future.

Keywords: Copper nanoparticles, Bacterial pathogens, Antibacterial activity, Zone of inhibition, Antibiotics, Synergistic effect

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INTRODUCTION

Emerging infectious diseases and the increase in the incidence of drug resistance among pathogenic bacteria have made the search for new antimicrobials inevitable. Nanotechnology offers opportunities to re-explore the biological properties of already known antimicrobial compounds by manipulating their size to alter the effect [1]. In recent years, metal nanoparticles (Me-NPs) have received tremendous scientific and practical interest which exhibit novel chemical and physical properties owing to their extremely small dimensions and special surface area [2]. Among the noble metal nanoparticles, copper nanoparticles (CuNPs) have attracted great attention because of their catalytic and optical properties, high electrical and heat conductivity [3]. Copper (Cu) has also long been known to have antimicrobial activity and is used in drinking water treatment and transportation. It has been recognized by the American environmental protection agency (EPA) as the first metallic antimicrobial agent in 2008 [4]. Though numerous methods are available for the synthesis of CuNPs, chemical reduction in aqueous or organic solvents exhibits the greatest feasibility because of simple equipment, short process, low cost and easy industrial production [5]. Bactericidal effect of CuNPs has been attributed to their small size and high surface to volume ratio, which allows them to interact closely with microbial membrane and is not merely due to the release of metal ions in solution [6]. Copper ions are known to penetrate bacteria and disrupt molecular pathways important for their survival. The antibacterial property of CuNPs was attributed mainly to adhesion with bacteria because of their opposite electrical charges, resulting in a reduction reaction at the bacterial cell wall [7]. The copper-fluoro polymer nano-composite is employed as bioactive coatings that are capable of inhibiting the growth of target microorganisms such as *Saccharomyces cerevisiae*, *Escherichia coli*, *Staphylococcus aureus* and *Listeria* [8]. Copper nanoparticles showed more inhibitory effect in bacteria than fungus

[9]. Therefore the objective of the present study was to fabricate and investigate the antibacterial efficacy of copper nanoparticles and the combinatorial effect of nanoparticles impregnated along with major broad-spectrum antibiotics.

MATERIALS AND METHODS

Materials

Copper sulfate pentahydrate (CuSO₄·5H₂O), sodium citrate (Na₃C₆H₅O₇), sodium borohydride (NaBH₄), ethanol, nutrient broth, Mueller Hinton agar, and standard antibiotic discs were purchased from Himedia (P) Ltd. Mumbai and used as starting materials without further purification. Milli-Q water was used for the fabrication of nanoparticles.

Methods

Preparation of copper nanoparticles

Copper nanoparticles were prepared by aqueous phase chemical reduction of cupric salt solution using sodium borohydride in the presence of sodium citrate as a capping agent [10]. In brief, 10 ml of 0.0125 M CuSO₄·5H₂O solution was purged with nitrogen (N₂) gas for 10 min to remove dissolved oxygen (DO) and then 10 ml of 0.2 M sodium citrate solution was added. The reaction solution was allowed to stir for 30 min. Then 1 ml of aqueous solution of NaBH₄ (0.1 M) was added dropwise to it under constant stirring. The entire experiment was carried out under N₂ atmosphere. The solution color changed to dark brown on the complete addition of reducing agent indicating the formation of citrated capped copper nanoparticles. The particles were then collected by ultracentrifugation of resultant solution at 10,000 rpm for 1 h. The particles thus obtained were washed twice with acetone before being vacuum dried at 50 °C for 12 h to remove water. The dried particles were used for further analysis.

Characterization of copper nanoparticles

Formation of copper nanoparticles in the reaction mixture was primarily monitored by observing the color change of reaction solution. The phase purity and crystalline structure of the synthesized CuNPs were identified by XRD pattern. XRD pattern was recorded with an X'pert PROPAN analytical instrument operated at 40 kV and a current of 30 mA with Cu α radiation ($\lambda=1.54060 \text{ \AA}$). A continuous scan mode was used to collect 2θ data from 10.08° to 79.93° , and the diffraction intensities were compared with the standard JCPDS. Average size of the resultant particles was calculated using Debye-Scherrer's formula, $D = k\lambda/\beta\cos\theta$, Where D is the thickness of the nanoparticle, 'k' constant, ' λ ' is the wavelength of x-rays, ' β ' is the width at half maxima of reflection at Bragg's angle 2θ , ' θ ' Bragg's angle. Surface morphology and size distribution of the prepared CuNPs was examined by SEM (SU 1510) operated at 5 kV with a magnification of about 10 k. The quantitative information and distribution of the elemental copper were investigated by EDS instrument (JSM 35 CF JEOL) in a resolution of 60 \AA , operated at 15.0 kV with a magnification of about 5 k.

Antibacterial screening of copper nanoparticles

The selected pathogenic bacterial strains in the present experiment namely *Bacillus cereus* (MTCC 619), *Escherichia coli* (MTCC 4296), *Pseudomonas aeruginosa* (MTCC 424) and *Staphylococcus aureus* (MTCC 3160) were procured from microbial type culture collection (MTCC), Chandigarh, India. All the cultures were grown on nutrient agar plates and maintained in the nutrient agar slants at 4°C . Overnight culture in the nutrient broth was used for the antibacterial study. The screening of copper nanoparticles for its bactericidal effect was determined by the agar well diffusion method [11]. Disc diffusion assay was adopted to investigate the synergistic effect of CuNPs with commonly used antibiotics [12]. To determine the synergistic effect, each broad-spectrum antibiotic disc (Tetracycline, Rifampicin, Chloramphenicol, Vancomycin, Gentamycin, Streptomycin, Kanamycin, Tobramycin, Penicillin, and Ampicillin) was impregnated with $50 \mu\text{l}$ of freshly prepared CuNPs and was placed onto the sterile Mueller Hinton agar plates inoculated with tested pathogenic strains. Antibiotic discs without CuNPs were served as positive control. These plates were incubated overnight at room temperature. After incubation, results were recorded by measuring the diameter of the inhibition zone (mm). The assay was performed in triplicates. Percentage fold increase of individual antibiotics was calculated using the formula,

$$\text{Fold increase (\%)} = \frac{(b-a)}{a} \times 100$$

Where b and a represent the zone of inhibition area of antibiotics with and without copper nanoparticles.

RESULTS AND DISCUSSION

Synthesis and characterization of copper nanoparticles

The appearance of dark brown coloration in the reaction mixture clearly indicated the formation of colloidal copper (fig. 1). These color changes is due to the excitation of plasmon resonance with a significant contribution from interband transition [13]. Shahverdi et al. [14] reported that appearance of dark brown color in the reaction solution indicated the formation of copper nanoparticles and this color arises from excitation of surface plasmon vibration in the metal nanoparticles.



Fig. 1: Formation of dark brown color colloidal copper (right)

The X-ray diffraction pattern recorded for fabricated copper nanoparticles in the present study is shown in fig. 2. The diffraction peaks observed at 2θ value of 43.47° , 53.37° , and 74.50° belong to the face-centered cubic (fcc) metallic Cu. The diffraction peaks appear at an angle 43.47° , 53.37° and 74.50° correspond to (111), (200) and (220) sets of lattice planes of fcc structure of metallic copper ions revealing that the fabricated CuNPs were composed of pure crystalline copper. XRD pattern obtained in the present investigation was found to be in good agreement with the earlier report by Mukhopadhyay *et al.* [15] who examined the XRD peaks at 2θ value of 43.36° , 50.48° and 74.15° corresponding to (111), (200) and (220) lattice planes of fcc structure of CuNPs respectively. Similarly, three Bragg's diffraction peaks at 2θ values of 42.47° , 51.73° and 73.42° which could be indexed as (111), (200) and (220) reflection planes of fcc structure of zero-valent CuNPs [16]. Moreover, the obviously broadened diffraction peaks noticed in the given XRD pattern suggest that the resultant nanoparticles should have a very small crystallite size and its size was found to be 17.85 nm.

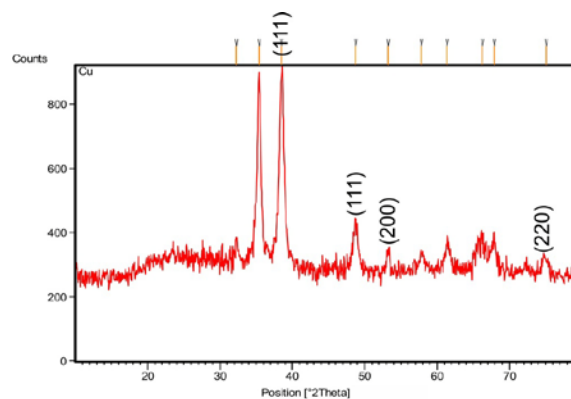


Fig. 2: Powder X-ray diffraction pattern of copper nanoparticles

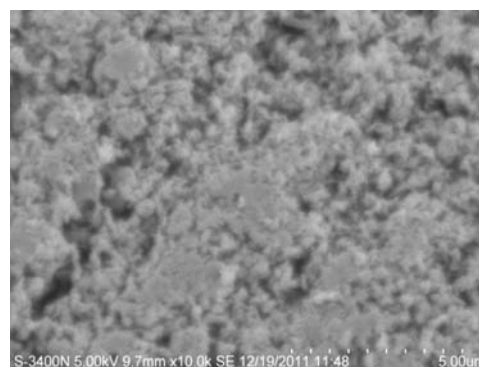


Fig. 3: Scanning electron micrograph of copper nanoparticles

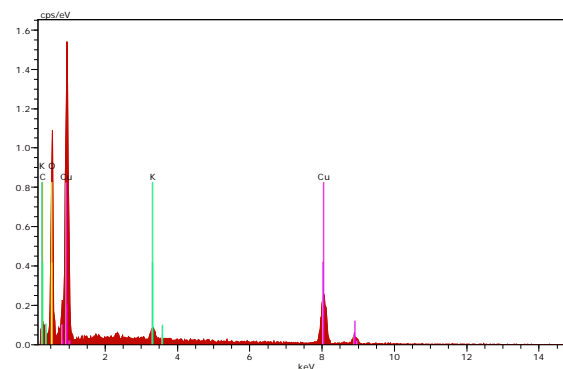


Fig. 4: Energy dispersive spectrum of copper nanoparticles

Scanning electron micrograph of the synthesized copper nanoparticles is given in fig. 4. The micrograph shows that the appearance of the nanoparticles is spherical in shape. The resultant nanoparticles do not appear as a discrete one but form much larger particles. The observations of such larger nanoparticles were composed of van der waals clusters of smaller entities [6]. The size and shape of the nanoparticles may depend on many parameters such as the choice of reduction technique, the concentration of metal precursors, reductant and capping agents used [17].

Fig. 4 explains the atomic surface distribution and chemical composition of copper nanoparticles. The given EDS spectrum revealed the presence of an elemental copper signal. Strong signals from copper atoms (97.07% in mass) while weaker signals from potassium (K), carbon (C) and oxygen (O) were also observed. The

resultant EDS spectrum of CuNPs clearly indicating that copper was in major proportion accounting for about 96% of the mass of nanoparticles. In accordance with these, the same type of elemental Cu signal in the EDS spectrum of CuNPs was observed in an earlier report [18]. The formation of an elemental copper signal in the EDS spectrum is due to the surface plasmon resonance where CuNPs shows absorption peaks of higher counts [19].

Antibacterial screening of copper nanoparticles

The emergence of nanoscience and nanotechnology in the last decade presents opportunities for exploring the bactericidal effect of metal nanoparticles. In the present experiment, colloidal copper showed highly potent antibacterial activity towards tested pathogenic bacterial strains. Resultant zone of inhibition is ranged from 9.67 ± 0.47 mm to 16.00 ± 1.63 mm (table 1).

Table 1: Bactericidal effect of nano copper against selected pathogens

Bacterial pathogens	Zone of inhibition (mm)
<i>Escherichia coli</i>	13.00±2.83
<i>Pseudomonas aeruginosa</i>	16.00±1.63
<i>Bacillus cereus</i>	12.17±0.24
<i>Staphylococcus aureus</i>	9.67±0.47

Each value is the mean±SD of triplicate analysis

Diameter of inhibition zone (DIZ) reflects the magnitude of susceptibility of the bacterial species. Among the different pathogens tested, *Pseudomonas aeruginosa* offered maximum inhibitory zone (16.00 ± 1.63 mm) whereas least susceptibility was noticed against *Staphylococcus aureus* (9.67 ± 0.47 mm). The excellent antibacterial activity against all the selected bacterial pathogens is due to the multi-target nature of copper. The multi-target nature of copper makes resistance extremely unlikely as copper kills bacteria very quickly and leaves no survivors. Even though the exact mechanism behind the bactericidal effect of copper nanoparticles is not clearly known, but the action of copper on bacteria occurs in two different steps generally. 1)

Direct interaction between the surface and bacterial outer membrane causing the membrane to rupture. 2) Formation of holes in the outer membrane through which the cell loses vital nutrients and water causing a general weakening of the cell. Normally bacteria, viruses, and fungi all depend on an enzyme to metabolize oxygen to live. Copper interferes with the effectiveness of these enzymes and disables the uptake of oxygen and thereby killing the microbes [6]. It is also supposed that microorganisms carry a negative charge while metal nanoparticles carry a positive charge. This creates an electromagnetic attraction between the microbe and particles surface. Once the contact is made, the microbe is oxidized and dead instantly [20].

Table 2: Synergistic effect of different antibiotics with and without nano copper against gram-negative bacteria

Antibiotics (µg/disc)	<i>Escherichia coli</i>		Increased zone size (mm)	Fold Increase (%)	<i>Pseudomonas aeruginosa</i>		Increased zone size (mm)	Fold Increase (%)
	Antibiotic alone	Antibiotic+CuNPs			Antibiotic alone	Antibiotic+CuNPs		
<i>Tetracycline</i> (T ₃₀)	14.33±0.47	20.67±0.47	6.33±0.47	44.17	13.00±0.41	19.67±0.94	6.67±1.31	51.31
<i>Rifampicin</i> (R ₅)	6.00±0.00	10.83±0.24	4.83±0.24	80.50	12.67±1.25	17.33±0.47	5.00±1.41	39.46
<i>Chloramphenicol</i> (C ₃₀)	22.33±1.69	26.83±0.24	4.50±1.78	20.15	26.00±0.41	27.17±0.85	1.17±0.62	45.00
<i>Vancomycin</i> (VA ₃₀)	6.00±0.00	12.00±0.00	6.00±0.00	100.00	11.00±0.82	18.33±0.62	7.33±1.18	66.64
<i>Gentamycin</i> (G ₁₀)	16.40±0.99	20.33±1.25	3.93±0.74	23.96	15.33±0.24	19.00±1.63	3.67±1.84	23.94
<i>Streptomycin</i> (S ₁₀)	11.67±1.25	21.00±0.82	9.33±2.05	79.95	6.00±0.00	8.67±0.62	2.67±0.62	44.50
<i>Kanamycin</i> (K ₃₀)	14.67±0.94	17.67±1.25	3.00±0.82	20.45	12.83±0.94	14.17±0.62	1.33±1.55	10.37
<i>Tobramycin</i> (TB ₁₀)	11.33±0.34	19.17±0.24	7.83±0.53	69.11	11.33±1.03	17.50±1.22	6.17±1.25	54.46
<i>Penicillin</i> (P ₁₀)	6.00±0.00	11.00±0.82	5.00±0.82	83.33	9.33±0.62	12.67±0.94	3.33±0.62	35.69
<i>Ampicillin</i> (A ₁₀)	11.17±0.85	14.00±0.82	2.83±0.62	25.34	13.17±0.85	16.50±1.22	3.33±0.47	25.28
	Overall synergistic bactericidal effect (%)			54.70	Overall synergistic bactericidal effect (%)			39.64

Each value is the mean±SD of triplicate analysis

Table 3: Synergistic effect of different antibiotics with and without nano copper against gram-positive bacteria

Antibiotics (µg/disc)	<i>Bacillus cereus</i>		Increased zone size (mm)	Fold Increase (%)	<i>Staphylococcus aureus</i>		Increased zone size (mm)	Fold Increase (%)
	Antibiotic alone	Antibiotic+CuNPs			Antibiotic alone	Antibiotic+CuNPs		
<i>Tetracycline</i> (T ₃₀)	17.83±0.85	28.50±0.82	10.66±0.24	59.79	25.33±1.25	32.67±2.05	7.33±1.89	28.94
<i>Rifampicin</i> (R ₅)	8.50±0.41	15.17±0.85	6.67±1.25	78.47	19.33±0.94	22.00±1.63	2.67±1.89	13.81
<i>Chloramphenicol</i> (C ₃₀)	25.67±0.62	29.83±0.85	4.17±1.25	16.24	20.00±0.00	23.00±1.47	3.00±1.47	15.00
<i>Vancomycin</i> (VA ₃₀)	6.00±0.00	12.00±0.00	6.00±0.00	100.00	18.33±0.62	20.00±1.63	1.67±1.02	9.11
<i>Gentamycin</i> (G ₁₀)	17.67±1.25	21.83±1.43	4.17±0.24	23.60	21.17±1.03	22.67±1.25	1.50±0.41	7.09
<i>Streptomycin</i> (S ₁₀)	13.00±0.82	16.17±0.85	3.17±1.03	24.38	20.67±0.47	23.00±1.63	2.33±1.70	11.27
<i>Kanamycin</i> (K ₃₀)	11.00±1.63	18.50±0.82	7.50±0.82	68.18	23.33±1.03	25.17±1.03	1.83±1.03	7.84
<i>Tobramycin</i> (TB ₁₀)	12.17±0.85	19.67±1.03	7.50±1.78	61.63	21.00±0.82	21.67±0.47	0.67±0.47	3.19
<i>Penicillin</i> (P ₁₀)	6.00±0.00	10.00±0.82	4.00±0.82	66.67	24.00±0.82	25.50±0.82	1.50±0.00	6.25
<i>Ampicillin</i> (A ₁₀)	12.17±1.31	14.33±0.62	2.17±1.25	17.83	30.33±1.03	32.33±0.62	2.00±1.41	6.59
	Overall synergistic bactericidal effect (%)			51.68	Overall synergistic bactericidal effect (%)			10.91

Each value is the mean±SD of triplicate analysis

Combined effect of copper nanoparticles with antibiotics

The combined effect of copper nanoparticles with different commercial antibiotics was investigated against the selected bacterial pathogens. Diameter of inhibition zone around different antibiotics with and without CuNPs against the tested strains is given in tables 2 and 3.

A minimum zone of inhibition was increased from 0.67±0.47 mm to 10.66±0.24 mm when nanoparticles and the antibiotics were given together. The antibacterial activities of all the antibiotics have been increased in the presence of copper nanoparticles against tested pathogens. The highest fold increase in area was observed for *Streptomycin* (9.33±2.05 mm), *Vancomycin* (7.33±1.18 mm) and *Tetracycline* (10.66±0.24 mm and 7.33±1.89 mm) against *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus cereus*, and *Staphylococcus aureus* respectively. The lowest fold increase in area (0.67±0.47 mm) was observed against *Staphylococcus aureus* for the antibiotic *Tobramycin*. Among the tested strains, *Escherichia coli* exhibited maximum synergistic bactericidal effect (54.70%) followed by *Bacillus cereus* (51.68%), *Pseudomonas aeruginosa* (39.67%) and *Staphylococcus aureus* (10.91%). The observed enhancement of antibacterial activity in the present experimental condition could be due to the antibiotic-nanoparticle combination and not to the effect of CuNPs itself. There are several studies have been carried out on the antibacterial efficacies of nanoparticle-antibiotic combinations, but little is known about interactions between antibiotics and copper nanoparticles. Interactions between different antibiotics and silver nanoparticles produced by bacteria and fungi were investigated earlier [21, 22]. Combining nanoparticles with antibiotics not only reduce the toxicity of both agents towards human cells by decreasing the requirement for high dosages but also enhances their bactericidal properties. Synergistic effect of copper nanoparticles with antibiotics may be due to the certain complex formation which becomes more effective in the inhibition of a particular species of microorganisms either by inhibiting the cell wall synthesis or by causing its lysis or death [23].

CONCLUSION

Nanobiotechnology is an important area of research that deserves all our attention owing to its potential application to fight against antibiotic-resistant pathogens. The present study illustrates a simple, convenient and significant method for the synthesis of copper nanoparticles through the reduction of copper salts using sodium borohydride as a reducing agent. From this work, it is concluded that copper nanoparticles impregnated with antibiotics exhibit profoundly stipulated inhibitory effects. This enhancement in the combined effect is preferably due to the difference in the mechanism of inhibition followed by nanoparticles and antibiotics. Hence our results suggest that copper nanoparticles may be suitable for combating pathogenic microorganisms.

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AUTHORS CONTRIBUTIONS

The Author Dr. M. Selvarani designed and carried out the experiment as well as prepared the manuscript.

CONFLICT OF INTERESTS

The author declares that there is no conflict of interests

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