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Original Research

Synthesis and evaluation of bactericidal properties of CuO nanoparticles against *Aeromonas hydrophila*

Sayedeh Fatemeh Shaffiey[1], Maryam Shapoori[1*], Abbas Bozorgnia[2], Mohammad Ahmadi[1]

[1].Department of Natural resources, Savadkooh Branch, Islamic Azad University, Savadkooh, Iran
[2] Department of Fishery, Qaemshahr, Branch, Islamic Azad University, Qaemshahr, Iran

Abstract

Objective(s): CuO is one of the most important transition metal oxides due to its captivating properties. It is used in various technological applications such as high critical temperature superconductors, gas sensors, in photoconductive applications, and so on. Recently, it has been used as an antimicrobial agent against various bacterial species.
Materials and Methods: Here, we synthesized CuO nanoparticles (NPs) and explored the antibacterial activity of CuO NPs preparation.
Results: Single crystalline nanoparticles of copper oxide having almost uniform particle size of 5-6 nm has been synthesized by a facile and versatile route. XRD spectra confirmed the formation of single phase CuO NPs. Transmission electron microscopy results corroborate well with XRD results. The technique employed is free from toxic solvents, organics and amines, is based on a simple reaction of copper sulfate and de-ionized water (DI), and their bactericidal effects against of *Aeromonas hydrophila* ATCC 7966T bacteria were investigated. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) with liquid culture for all of the *Aeromonas hydrophila* culture Medias was done.
Conclusion: Present study confirms that Copper oxide nanoparticles have great promise as antimicrobial agent against *Aeromonas hydrophila*.

Keywords: *Aeromonas hydrophila*, Bactericidal effects, CuO nanoparticle

***Corresponding author:** Maryam Shapoori, Department of Natural resources, Savadkooh Branch, Islamic Azad University, Savadkooh, Iran.*
Email: m_shapoori@iausk.ac.ir

Introduction

Aeromonas hydrophila causes disease in fish known as "Motile Aeromonas Septicemia" (MAS), "Hemorrhagic Septicemia", "Ulcer Disease," or "Red-Sore Disease." The many synonyms of this disease relate to the lesions caused by this bacterium which include septicemia where the bacteria or bacterial toxins are present within numerous organs of the fish, and ulcers of the fish's skin. *Aeromonas hydrophila* is a ubiquitous gram-negative rod-shaped bacterium which is commonly isolated from fresh water ponds and which is a normal inhabitant of the gastrointestinal tract. The disease caused by this bacterium primarily affects freshwater fish such as rainbow trout and catfish (1). Copper oxide (CuO) is a semiconducting compound with a monoclinic structure. It is the simplest member of the family of copper compounds and exhibits a range of potentially useful physical properties such as high temperature conductivity, superconductivity, electron correlation effects and spin dynamics. Therefore, it finds a wide application (2-3). CuO crystal also has photo catalytic or photovoltaic properties and photoconductive functionalities (4). There is limited information available about the antimicrobial activity of nano CuO. As CuO is cheaper than silver, easily mixes with polymers and relatively stable in terms of both chemical and physical properties, it finds a wide application (5). It is suggested that highly ionic nanoparticulate metal oxides, such as CuO, may find potential application as antimicrobial agents as they can be prepared with extremely high surface areas and unusual crystal morphologies (6). CuO nanoparticles (NPs) were effective in killing a range of bacterial pathogens involved in hospital-acquired infections. But a high concentration of nano CuO is required to achieve a bactericidal effect (7). It has been suggested that the reduced amount of negatively charged peptidoglycans makes Gram-negative bacteria such as *Pseudomonas aeruginosa* and *Proteus* sp. less susceptible to such positively charged antimicrobials.

Studies have been conducted to assess the potential of nano CuO embedded in a range of polymer materials.

A lower contact-killing ability was observed in comparison with release killing ability against MRSA strains. This suggests that a release of ions into the local environment is required for optimal antimicrobial activity (7, 8).

Copper NPs have a high antimicrobial activity against *B. subtilis*.

This 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.

Copper ions released may also interact with DNA molecules and intercalate with nucleic acid strands. Copper ions inside bacterial cells also disrupt biochemical processes (9).

In this study, we studied the synthesis, characterization and antibacterial activity of copper oxide nanoparticles as a new class of agents against *A. hydrophila* and compare new drug effects with Tetracycline as reference antibacterial drug.

Materials and Methods

Synthesis of CuO NPs

All reagents were purchased from Merck and Aldrich (Germany) and used without further purification. The reactions were carried out under an atmosphere of air. In a typical synthesis procedure, $CuSO_4.5H_2O$ was dissolved in de-ionized water.

The solution was stirred with a magnetic stirrer at 100°C. About 0.8 g of NaOH was added to solution till pH reaches to 8. With increase pH to 8 large amount of $Cu(OH)_2$ precipitate was formed immediately. The precipitate was filtered and washed 4 times with de-ionized water.

The obtained precipitate was dried in air for 24 h. Then, powders were annealed for

1 hour at temperature of 400°C, to obtain the highly crystalline CuO NPs.

Characterization of CuO NPs

Synthesized CuO NPs were characterized by X-ray diffraction (XRD), Fourier-transform infrared spectroscopy (FT-IR), and transmission electron microscopy (TEM). Crystallinity, structure, and crystallite size of CuO NPs were determined by XRD technique using a Rigaku-Miniflex X-ray diffractometer (Rigaku Corporation, Tokyo, Japan) with Cu-Kα radiations (λ = 0.15406 nm). TEM analysis was carried out using a 200 kV JEOL transmission electron microscope (JEOL Ltd, Tokyo, Japan). FTIR spectra of the samples were obtained using a PerkinElmer FTIR spectrophotometer (PerkinElmer Inc, Waltham, MA).

Disk diffusion test

Antimicrobial activity of the synthesized CuO NPs were determined using Gram-negative bacteria (*Aeromonas hydrophila* ATCC 7966T) following a modified Kirby Bauer disc diffusion method (10). A lawn of bacterial culture was prepared by spreading 100 µL culture broth, having 1.5×10^8 CFU/mL of test organism on solid nutrient agar plates. The plates were allowed to stand for 10–15 minutes, to allow for culture absorption. The 8 mm size wells were punched into the agar with the head of sterile micropipette tips. Wells were sealed with 1 mL of molten agar (0.8% nutrient agar) to prevent leakage from the bottom of the plate. The bacteria were plated onto solid nutrient agar plates. Using a micropipette, 20-100 % (V/V) of 100 µL (50 µg) of the nanoparticles solution sample was poured into each of four wells on all plates. After incubation at 22-25 ± 2°C for 24 hours, the size of the zone of inhibition was measured with a ruler with up to 1 mm resolution. Each experiment was repeated three times, and the resulting bacterial growth on three plates corresponding to a particular sample were averaged and reported ($p < 0.05$). A solvent blank was run as a negative control whereas the antibiotic (tetracycline) was used as a positive control.

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 (11), was determined based on batch cultures containing varying concentration of copper oxide nano particles in suspension (60–300 µg/mL).

Sterile Erlenmeyer flasks (500 mL), each, containing 100 mL peptone water medium 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 1.5×10^8 CFU /mL.

Bacterial growth was measured as increase in absorbance at 625 nm determined using a spectrophotometer (Thermo Spectronic, Helios Epsilon, USA). The experiments also included a positive control (flask containing nanoparticles and peptone water medium, devoid of inoculums) and a negative control (flask containing inoculums and peptone water medium, 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 peptone water media, inoculums and nanoparticles). All the experiments were carried out in triplicate. Copper oxide nanoparticles were tested for bactericidal effect using the microbial culture selected for the study.

The minimum bactericidal concentration (MBC) (11), the lowest concentration of nanoparticles that kills 99.9% of the bacteria was also determined from the batch culture studies.

For growth inhibitory concentration (MIC) the presence of viable microorganisms was

tested and the lowest concentration causing bactericidal effect was reported as MBC.

Results

XRD study

The typical XRD pattern of the CuO NPs annealed at 400°C is shown in Figure 1. The peak positions of the sample exhibited the monoclinic structure of CuO which was confirmed from the International Centre for Diffraction Data (ICDD) card No 801916. Further, no other impurity peak was observed in the XRD pattern, showing the single phase sample formation. The crystalline size was calculated using the Scherrer formula, $D = 0.9 \lambda / \beta \cos\theta$, where λ is the wavelength of X-ray radiation, β is the full width at half maximum (FWHM) of the peaks at the diffracting angle θ.

Crystallite size calculated by the Scherrer formula was found to be 8 nm. Lattice parameters were found to be a = 4. 88 Å, b = 3.42 Å, c = 5.32 Å. These values are in good agreement with the standard values reported by the ICDD Card No 801916. The peaks broadening due to the nano-size effect.

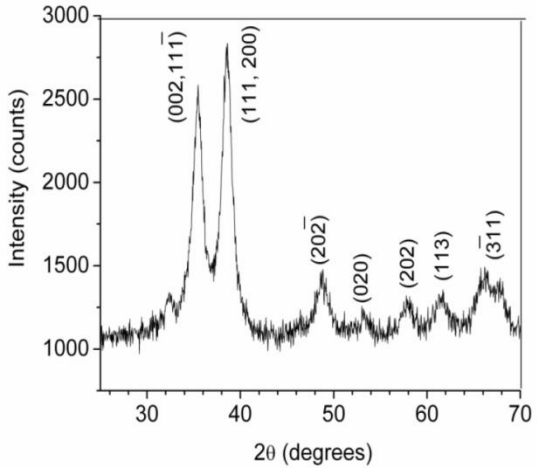

Figure 1. XRD pattern of CuO nanoparticles.

SEM and TEM study

Figure 2 shows the SEM image of prepared CuO NPs. It shows that the CuO NPs are in rectangular shape. Figure 3 shows the TEM image of prepared nanoparticles.

The size of particle observed in TEM image is in the range of 5-6 nm which is in good agreement with calculated by Scherrer formula using XRD.

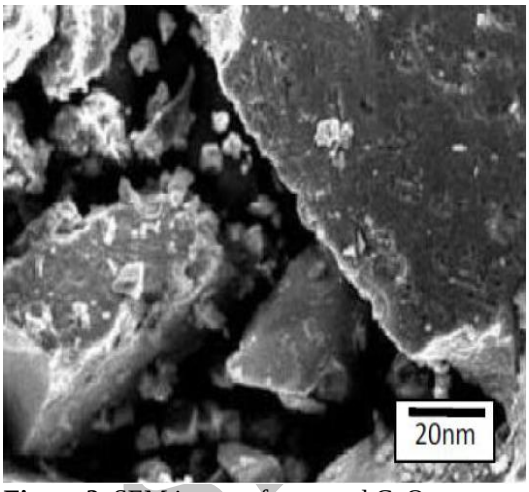

Figure 2. SEM image of prepared CuO nano particles.

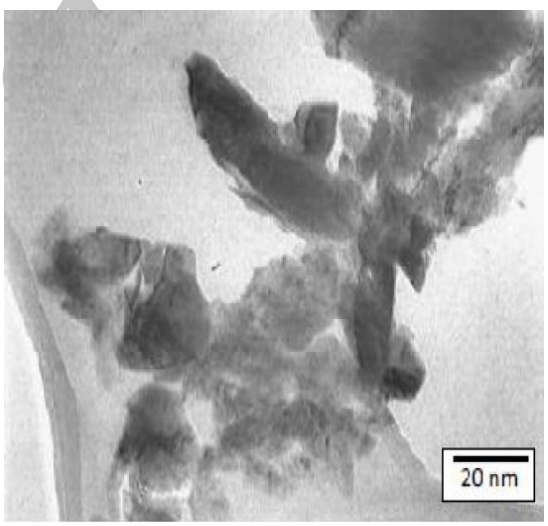

Figure 3. TEM image of prepared CuO nano particles.

FT-IR study

FT-IR spectra were recorded in solid phase using the KBr pellets technique in the range of 3500–400 cm^{-1}. FT-IR spectra of CuO NPs treated at 400°C are shown in Figure 4.

FT-IR spectra exhibit only three vibrations: occurring at approximately 480 cm^{-1}, 530 cm^{-1}, and 580 cm^{-1} for all the samples, which can be attributed to the vibrations of Cu-O, confirming the formation of highly pure CuO NPs.

A weak band at around 2300 cm^{-1} may be attributed to the vibrations of atmospheric CO_2. These assignments are in agreement with the values available in literature (12-14).

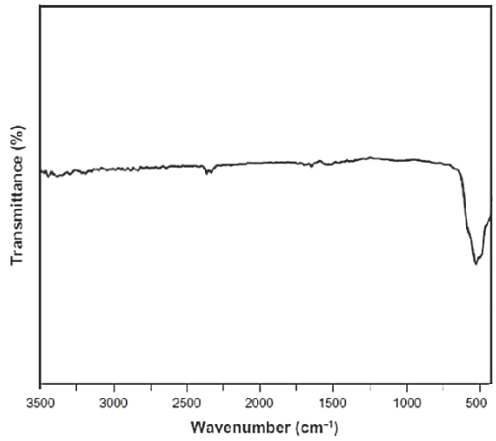

Figure 4. FT-IR spectra of CuO nanoparticles annealed at 400°C**.**

Antimicrobial properties

In batch studies, a greater lag phase and lower maximum absorbance (at 625 nm) were observed as the concentration of nanoparticles increased. As concentration of nanoparticles increased to MIC of the respective strain, no growth was observed in the flask. The bactericidal effect of nanoparticles is dependent on the concentration of nanoparticles and the initial bacterial concentration (16). In this study, the initial bacterial concentration was almost constant at 1.5×10^8 CFU /mL irrespective of nanoparticles concentration and microbial strain. The MIC observed in this study for copper oxide nanoparticles is 80 µg/mL for *Aeromonas hydrophila* ATCC 7966T and MBC value for CuO NPs is 300 µg/mL (Table 1). Figure 5 exhibit the zone of inhibition of CuO NPs synthesized and positive control, a known antibiotic tetracycline, against Gram-negative bacteria (*Aeromonas hydrophila*).

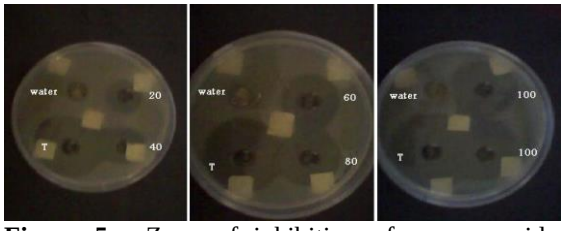

Figure 5. Zone of inhibition of copper oxide nanoparticles.

Table 1. The diameter of inhibition zone (DIZ) and MBC of copper oxide nanoparticles (annealed at 400°C temperature) against *Aeromonas hydrophila*.

CuO Nanoparticles Concentration (µg/mL)	DIZ (mm)	MBC (Log CFU/mL)
Negative Control[a]	0	6.7±0.0
60	3	6.1±0.0
120	5	4.3±0.0
180	6	2.8±0.0
240	8	1.2±0.0
300	8	NCD[b]

Discussion

We have successfully synthesized CuO NPs using aqueous precipitation method. XRD spectra confirmed the formation of single phase CuO NPs. From SEM and TEM study, it is found that particles are rectangular in shape with average size of 5-6 nm. TEM results corroborate well with XRD results. FT-IR spectra also validated the purity of CuO NPs. The copper oxide nanoparticles showed remarkable antibacterial activity against *Aeromonas hydrophila* as Gram-negative bacteria. A few studies have been performed to elucidate the mechanism of bactericidal action of nanoparticles. It is difficult to distinguish between the bactericidal activities of nanoparticles from the ions released by the nanoparticles themselves (15). Ruparelia et al. estimated the concentration of released ions for 10 mg of copper nanoparticles suspended in 100 mL nutrient media and distilled water (16). They found that the concentration of Cu^{2+} ions released in nutrient media was 17 mg/L after 24 hours of incubation in a rotary shaker, while in distilled water under the same conditions over a period of 24 hours; the concentration of ions released was 0.5 mgL^{-1}. These results indicate that the nutrient media can facilitate the release of Cu^{2+} ions.

The considerably greater release of Cu^{2+} ions in the nutrient media is possibly due to the interaction of the media chloride

ions with the oxide layer of the nanoparticles (16). Consequently, the bactericidal effects observed in this study might have been influenced by the release of Cu^{2+} ions in solution. The presence of nanoparticles in suspension would ensure continuous release of ions into the nutrient media (17). There are a few mechanisms of nanoparticle toxicity suggested by other works. For example, 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 (18). Copper ions inside the bacterial cells may bind to deoxyribonucleic acid molecules and become involved in cross-linking within and between the nucleic acid strands, resulting in the disorganized helical structure. In addition, copper ion uptake by the bacterial cells has also been found to damage important biochemical processes (19, 20). Gram-negative bacteria like *Aeromonas hydrophila* have a special cell membrane structure which possesses an important ability to resist antimicrobial agents (21).

Conclusion

In conclusion, this study reports the successful synthesis of copper oxide nanoparticles. The antimicrobial screening studies were also performed in the study. The antimicrobial screening suggests that synthesized CuO NPs exhibited moderate activity toward *Aeromonas hydrophila*. One unique observation was that CuO NPs synthesized at 400°C with the smallest particle size demonstrated the maximum zone of inhibition in the case of *Aeromonas hydrophila***.** Moreover, minimum inhibitory concentration and minimum bactericidal concentration of CuO nanoparticles annealed at 400°C was lowest for the bacterial strain.

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