BIOCIDAL PROPERTIES OF CuO NANOPARTICLES

ORIGINAL SCIENTIFIC PAPER

Ivana Katarina Ivković✉, Stanislav Kurajica, Marija Vuković Domanovac, Katarina Mužina, Ivan Borić

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University of Zagreb, Faculty of chemical engineering and technology

✉ imunda@fkit.unizg.hr

ABSTRACT:

Biocides are products used to prevent or control the spread of various harmful organisms such as bacteria or viruses. Silver and gold nanoparticles are mostly used as active substances of biocides used in the medical field. However, a more economically acceptable alternatives are different copper compounds, specifically copper(II) oxide. CuO nanoparticles were gained via sonication method from copper(II) acetate in a sodium hydroxide solution. Physical and chemical properties of gained CuO nanoparticles were investigated by X-ray diffraction analysis (XRD), energy dispersive X-ray spectroscopy (EDS), thermogravimetric analysis (TGA) and atomic force microscopy (AFM). Biocidal tests were performed on bacteria *Pseudomonas aeruginosa* and *Bacillus subtilis*, as well on fungi *Candida albicans* and *Aspergillus niger* using the disc diffusion method. The ultrasonic irradiation method was found to yield pure CuO nanoparticles smaller than 70 nm. Also, EDS measurement verified the stoichiometric distribution of copper and oxygen in the sample. Antimicrobial properties were proven excellent for both bacteria and fungi except for *Pseudomonas aeruginosa*, for which CuO nanoparticles seem to have low effect.

KEYWORDS: copper(II) oxide; sonication; antimicrobial properties

INTRODUCTION

In recent years, metallic and semiconductor nanoparticles are considered for use in biocides due to their versatile biocidal toxicity mechanism. [1], [2] Other properties which give added benefits to the use of these materials in nano-medicine field are surface area to volume ratio and physicochemical properties related to size and shape. Silver and gold nanoparticles are mostly used in this field, but copper(II) oxide is proposed as a more economical alternative. CuO nanoparticles possess a combination of high surface area, redox activity, and biocompatibility, rendering them promising candidates for applications in biomedicine, as well as water treatment and beyond. [3]–[6]

The sonochemical method, a powerful and versatile synthesis technique, uses the energy generated by high-frequency ultrasound waves to induce a range of chemical and physical transformations in liquids. The creation process of tiny, rapidly expanding and collapsing bubbles is known as acoustic cavitation and occurs because of the ultrasound waves. [7] These cavitation bubbles generate localized extreme conditions of temperature and pressure, leading to the formation of free radicals and the initiation of chemical reactions that might otherwise require a different synthesis approach. [8] The sonochemical method has found applications across diverse fields, including materials science, nanotechnology, and environmental remediation, due to its ability to facilitate processes like nanoparticle synthesis.

Antimicrobial resistance is a growing global concern due to the fast evolution of resistance mechanisms in microbes. Because of this, traditional biocidal agents, such as disinfectants, are showing diminishing efficiency. [9] In this context, CuO nanoparticles have emerged as a compelling solution due to their ability to induce potent antimicrobial effects through multiple mechanisms. These mechanisms include the generation of reactive oxygen species, disruption of cell membranes, and interference with vital cellular processes. [10]–[12] Such versatility in

their antimicrobial action makes CuO nanoparticles valuable tools for mitigating the spread of infections in healthcare settings, the food industry, and environmental remediation efforts.

This paper delves into the biocidal properties of CuO nanoparticles, exploring their preparation via the sonochemical method, inhibitory effects on various bacteria and fungi, and physical properties.

EXPERIMENTAL

SYNTHESIS

Copper(II) oxide nanoparticles were synthesized by a fast sonochemical method using a ultrasound tip CL-334 (Industrial Sonomechanics, USA) following the preparation method of Sonia et.al. [13]A typical synthesis involves the preparation of 0.02 mol solution of NaOH (p.a.) and 0.01 mol solution of copper(II) acetate (p.a.). The solutions are mixed separately for 30 min to obtain good homogenization and ensure complete dissolution of precursors. Then, the sodium hydroxide solution was added dropwise to the copper(II) acetate solution. The resultant mixture was then sonicated for 1 h at room temperature and a black colored precipitate was obtained. To acquire only CuO nanoparticles, the product was washed with water and ethanol in three cycles using sonification and centrifugation. The final step is the drying of the precipitate overnight at 60°C.

CHARACTERIZATION

The phase composition of the samples was verified by X-ray diffraction on Shimadzu XRD 6000 diffractometer operating in step scan mode with 0.02 °2θ step and 0.6 s retention time. For characterization, CuKα radiation (λ= 0.15405 nm) and a measurement range of 5 to 80 °2θ was used. The Scherrer equation (1):

$$d = \frac{k\lambda}{\beta cos\theta} \quad (1)$$

was implemented for the determination of the crystallite size of the prepared nanoparticles. Scanning electron microscopy enabled insight into sample morphology, which, paired with energy dispersive X-ray spectroscopy, provides the means to observe and determine surface elemental composition. The analysis was accomplished using Tescan Vega 3 scanning electron microscope coupled with Oxford INCA X-sight EDS detector operating at 10 kV. Atomic force microscopy was conducted in ambient conditions using CoreAFM microscope with a Tap300Al-G probe in a tapping mode. Nominal resonant frequency of 300 kHz, nominal spring constant of 40 Nm^{-1} and tip radius less than 10 nm proved best for the acquisition of quality images.

ASSESSMENT OF BIOCIDAL ACTIVITY

The biocidal effect was tested using the well diffusion method. The following microorganisms were used in this work: Gram-positive bacteria *Bacillus subtilis* 3020, Gram-negative bacteria *Pseudomonas aeruginosa* 3011 and the fungi *Aspergillus niger* 405 (mould) and *Candida albicans* 159 (yeast). All microorganisms are kept in the Microorganism Collection of the Faculty of Chemical Engineering and Technology, Zagreb, Croatia. The volume of Mueller-Hinton agar (MHA) poured into a Petri dish was 20 mL with a thickness of approximately 3 mm. 100 µL of a microbial culture suspension (0.5 McFarland) was applied to the surface of the MHA. A hole with a diameter of 6 mm was then punched aseptically with a sterile cork borer and a volume of 50 µg of the antimicrobial suspension was added to the well at various concentrations. The Petri dishes were incubated at 37 °C for 24 hours and the bacterial and fungal inhibition zones were assessed after 24 and 72 hours, respectively.

RESULTS AND DISCUSSION

The success of the sonochemical process for the synthesis of copper(II) oxide material was visible already 5 minutes after the commencement of the synthesis when the starting solution turned from bright blue to dark brown (almost black), indicating that the process of transformation from $Cu(CH_3COO)_2$ to CuO begins. The chemical reactions occurring in the sonochemical process are as follows: [13]

$$\begin{aligned} &Cu(CH_3COO)_2 + 2NaOH \\ &Cu(OH)_2 + 2CH_3COONa \rightarrow \\ &CuO + H_2O + 2CH_3COONa \end{aligned} \quad (2)$$

The diffractogram pattern shown on figure 1 is a match with ICDD card no. 89-2529 which confirms the formation of copper(II) oxide. All

maxima present in this figure are attributed to CuO, where the three highest diffraction maxima are for the Miller indices (111), (022) and (202). Since there are no other unaccounted diffraction maxima, it can be concluded that copper(II) oxide is the only crystalline phase in the sample. It is noteworthy that the maxima for the (111) plane at 35.44 °2θ and (002) plane at 38.58 °2θ show greater intensity than all other maxima in this sample. This usually occurs when a sample has a preferred orientation, i.e. layered structures or some type of elongation along one plane (nanofiber). Value for width at half height of the 38.58 °2θ peak was implemented in the Scherrer equation and 27.37 nm crystallite size was calculated. CuO maxima on figure 1 are narrow and of relatively high intensity, which is in concurrence with the calculated crystallite size.

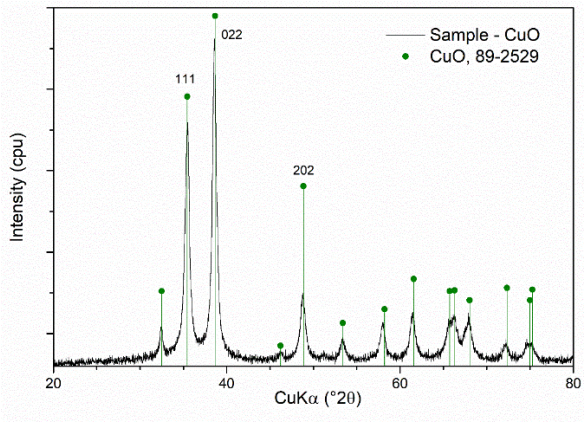

Figure 1. X-ray diffraction pattern for the sonochemically prepared sample

The easiness of the preparation of CuO nanoparticles via the sonochemical method is seen in the timing of the synthesis, which is only 1 h. As for the washing of the percipitate, cheap and easy to access chemicals are used and the longest part of the synthesis is the 24 h drying. Figure 2 shows the EDS mapping of a small part of the sample. Figure 2a refers to the sample and shows that areas of agglomeration of CuO nanoparticles appear. It can also be seen that these agglomerates are not compact but, on further investigation, look fluffy. EDS is a good tool for determination of the elemental composition of the sample surface.

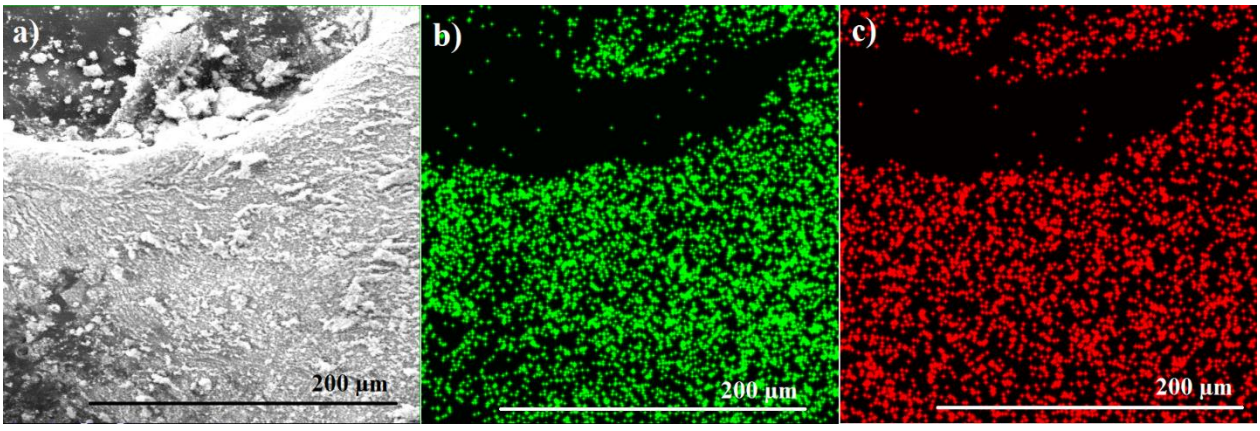

Figure 2. a) SEM micrograph, EDS mapping and distribution of: b) copper c) oxygen

In the images obtained by EDS mapping of the sample CuO, it can be discerned that the elements copper (2b) and oxygen (2c) are present in the sample. Trace amounts of gold, palladium and carbon are also present due to sputter coating of the sample with Au and Pd, and its deposition on carbon tape.

Table 1. Elemental composition of copper (II) oxide sample gained by EDS mapping

Element	Cu	O
Atomic number	29	8
Mass percentage (%)	47.40	25.60
Atomic percentage (%)	42.26	57.74
Relative error (%)	3.34	3.57

It can be seen that the areas of occurrence of copper and oxygen coincide, which is a confirmation of the chemical composition of the obtained crystalline phase. In addition, the results of the analysis are given in a tabular view (table 1). When viewing table 1, an interesting phenomenon is noticed. The atomic ratio of copper to oxygen should be 1:1 because of the chemical formula, CuO, however, there is a slight increase in the percentage of oxygen compared to copper. This is probably due to the nature of nanoparticles which, thanks to their great specific surface area, are susceptible to contamination in the form of adsorption of water and carbon dioxide from ambient air. AFM imaging was used to get insight into particle size of the prepared CuO nanoparticles.

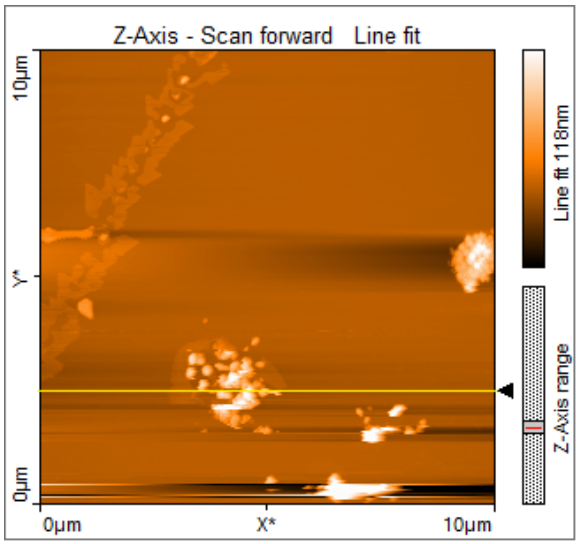

Figure 3. AFM micrograph of prepared CuO sample

Figure 3 represents the morphology of the sample where a very small portion of the powder sample was mixed with ethanol, deposited on a mica surface and left to air dry. The feature in the upper left corner is a crack created during the separation of mica layers, while the particulate parts in the image (in the middle and on the right) represent the CuO nanoparticles. Due to great surface energy, nanoparticles are prone to agglomeration and therefore, in this case, AFM turned out to be unsuitable for particle size estimation. On the other hand, the z-axis profile, due to a technique used for sample deposition, could provide insight into particle size. A z-axis height profile is given on figure 4 representing the height profile of the sample taken across the yellow line in figure 3.

The average height of CuO nanoparticles calculated from the z-profile is 44.5 nm.

Along with the particle height, which is an indication of particle size, assuming spherical particles, this figure also gives insight into particle distribution on the surface. Taking the x-axis dimension into consideration on figures 3 and 4 the claim on particle agglomeration gains credibility.

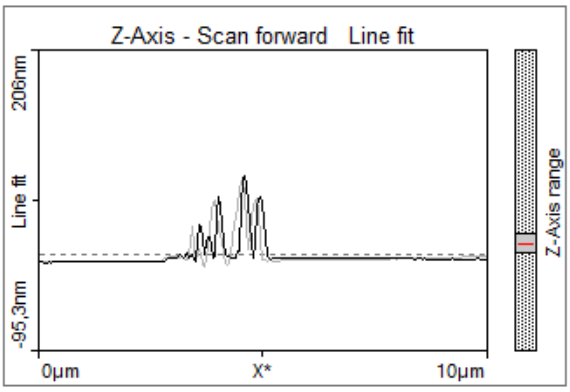

Figure 4. Height profile of particles across the yellow line in figure 3

CuO nanoparticles are known to have good biocidal properties, they mostly manifest great inhibition of Gram-positive bacteria, such as *Bacillus subtilis* or different fungi. Inhibition zones of prepared nanoparticles were tested on *Pseudomonas aeruginosa* 3011 and *Bacillus subtilis* 3020, as well as on *Candida albicans* 159 and *Aspergillus niger* 405. For the tests, different concentrations of CuO were dispersed in water. Table 2 shows the results for concentrations up to 100 mg L^{-1} for all tested microbes. As can be observed in Table 2, *Bacillus subtilis* 3020 and *Candida albicans* 159 show the greatest sensitivity to copper(II) oxide nanoparticles, as seen from the widest inhibition zones during disc diffusion tests. Given that microbial pores are greater than nanoparticle size, it is assumed that nanoparticles are able to infiltrate the cell membrane of this microbe, cause damage and disrupt cross-linking between nucleic acid strands inside the cell. [14] On the other hand, *Pseudomonas aeruginosa* 3020 seems to be

completely insensitive to copper(II) oxide nanoparticles, while *Aspergillus niger* 405 is sensitive only to the greatest concentration of nanoparticles. It is noteworthy that even the greatest concentration has no effect on the Gram-negative *Pseudomonas aeruginosa* 3011. That was a surprise given the work of Khashan et.al [15], whose findings indicated that CuO nanoparticles with average particle size of about 15 to 20 nm have greater inhibition effect on Gram-negative (*E. coli*, *P. aeruginosa* and *P. vulgaris*) than on Gram-positive bacteria. However, the concentration used in the work of Khashan et.al [15] was much greater than that used in this work, so another set of tests was conducted with greater concentrations. Indeed, the greatest concentration used, 1000 mgL^{-1} proved to be effective for the Gram-negative bacteria.

Table 2. Designations for different concentration of CuO nanoparticles with belonging inhibition zones for *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Candida albicans* and *Aspergillus niger*

Designation	Sample concentration (mgL^{-1})	*Pseudomonas aeruginosa* (mm)	*Bacillus subtilis* (mm)	*Candida albicans* (mm)	*Aspergillus niger* (mm)
A	0.1	-	8	10	-
B	1	-	11	11	-
C	10	-	14	13	-
D	100	-	16	15	15

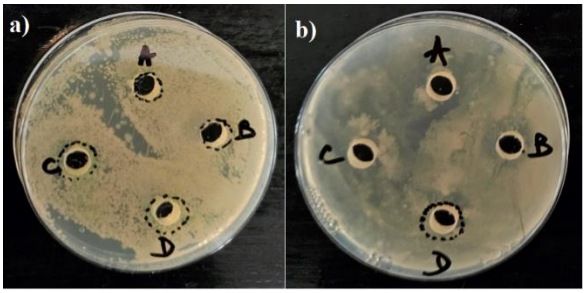

Figure 5. Disc diffusion tests for a) *Candida albicans* and b) *Aspergillus niger* for concentrations given in table 2

Table 3. Designations for different concentration of CuO nanoparticles with belonging inhibition zones for different bacteria strains

Designation	Sample concentration (mgL^{-1})	*P. aeruginosa* (mm)	*B. subtilis* (mm)
A1	250	-	18
B1	500	-	22
C1	750	-	25
D1	1000	20	25

At this concentration, the inhibition zone is greater than inhibition zones of all lesser concentrations for all the microbes tested. This behavior is peculiar because there is a minimum concentration of an antibacterial material below which the material has no effect on that specific microbe. *Bacillus subtilis* 3020 shows an improvement of the inhibition zone with higher concentration, but with stagnation above 750 mg L^{-1}, which can be considered the maximum inhibition zone. The difference between the antibacterial effect on Gram-positive and Gram-negative bacteria could result from a lower interaction potential for Cu^{2+} ions bonding to Gram-negative bacteria having an outer membrane containing lipopolysaccharide, which Gram-positive bacteria lacks. [16]

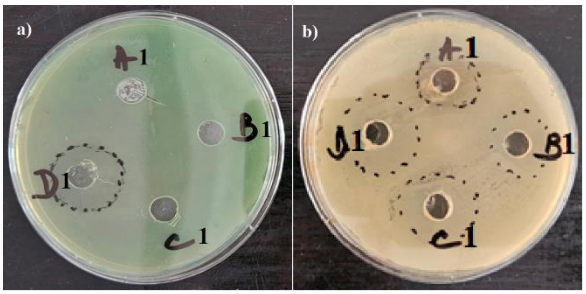

Figure 6. Disc diffusion tests for a) *Pseudomonas aeruginosa* and b) *Bacillus subtilis* for concentrations given in table 3

CONCLUSION

The sonochemical method of synthesis proved to be an effective and fast method of preparing copper(II) oxide nanoparticles. Nanoparticles were proven to be less than 40 nm in size. As for the antimicrobial tests, the prepared nanoparticles showed very good results for *Candida albicans* 159 and *Bacillus subtilis* 3020. CuO nanoparticles have less effect on mold and show a marginal effect on Gram-negative *Pseudomonas aeruginosa* 3011 only at high concentrations.This could be because Cu^{2+} ions bond to the bacterial cell, disrupting the biochemical processes inside the cell.

The poor antibacterial effect on Gram-negative bacteria could be the result of a lower Cu^{2+}ions interaction potential with Gram-negative bacteria due to its outer membrane containing lipopolysaccharide.

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