ORIGINAL ARTICLE

CONTENTS

Ag/Cu_2O nanocomposites as an alternative antimicrobial agent for multidrug-resistant bacterial infections

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ABSTRACT

Aim: To investigate the physicochemical properties and antibacterial and antibiofilm activity of nanocomposites Ag/Cu_2O synthesized by the polyol method.
Materials and Methods: The nanocomposites Ag/Cu_2O were synthesized using the polyol method, followed by a study of their physicochemical properties. The antibacterial and antibiofilm properties of the nanocomposites Ag/Cu_2O were investigated against multidrug-resistant clinical strains of bacteria *S. aureus*, *E. coli*, and *P. aeruginosa*.

Results: Nanocomposites Ag/Cu_2O, were mostly spherical in shape and had an average size of 135.6±65 nm. X-ray diffraction analysis confirmed the presence of Ag and Ag/Cu_2O phases. Energy-dispersive spectroscopy revealed the following elemental composition: 13.05% silver, 31.81% copper, and 55.14% oxygen. Ultraviolet-visible spectroscopy suggested the potential presence of silver oxides in the structure. The minimum inhibitory concentration and minimum bactericidal concentration values were as follows: for *S. aureus*, the minimum inhibitory concentration was 52.08±18.04 μg/ml, and the minimum bactericidal concentration – 72.92±47.74 μg/ml; for *P. aeruginosa* – 26.04±9.02 μg/ml, and 41.67±18.04 μg/ml, respectively; and for *E. coli* – 83.33±36.08 μg/ml, and 208.30±72.17 μg/ml, respectively. The nanocomposites Ag/Cu_2O NCs also demonstrated antibiofilm activity, being effective against *S. aureus* at 3 and 5 times the minimum bactericidal concentration, against *E. coli* at 1, 3, and 5 times the minimum bactericidal concentration, and against *P. aeruginosa* at 5 times the minimum bactericidal concentration.

Conclusions: The nanocomposites Ag/Cu_2O revealed high antibacterial and antibiofilm properties, which support their use as an alternative to conventional antibiotics in nanomedicine for treating multidrug-resistant infections.

KEY WORDS: Ag/Cu_2O nanocomposites, bacteria, biofilm, multidrug-resistance

Wiad Lek. 2026;79(1):9-14. doi: 10.36740/WLek/217263 DOI

INTRODUCTION

Multidrug-resistant (MDR) bacteria are often the cause of various ranges of infectious diseases, their complications, and the death of patients [1]. Recently, there has been an exponential increase in the number of bacterial strains with multidrug resistance [2]. Each year, approximately 0.7 million people die from MDR bacteria, and the total number of deaths is projected to reach 10 million in 2025 [3]. In some cases, bacteria are resistant to almost all existing antibiotics [4]. In this regard, the World Health Organization has identified this problem as a major global health threat and called for an improved and coordinated global effort to contain antimicrobial resistance [5]. Even more problematic and difficult to treat are infectious diseases with persistent biofilm forms of MDR bacteria. Biofilm forms of bacteria exhibit a 10- to 1,000-fold increase in antibiotic resistance compared to planktonic forms of the same bacteria [3]. About 80% of chronic and recurrent microbial infections in humans are caused by biofilms of MDR bacteria, which lead to high mortality rates [3].

S. aureus, *E. coli*, and *P. aeruginosa* are ubiquitous bacteria that cause a variety of infectious diseases and are often responsible for hospital-acquired infections. *P. aeruginosa*, in particular, has been recognized as one of the most life-threatening bacteria and included by the World Health Organization in its list of priority pathogens for research and development of new antibiotics [6]. Methicillin-resistant *S. aureus* (MRSA) is a widespread pathogen that causes a spectrum of infections from superficial skin problems to severe conditions such as osteoarticular infections and endocarditis, resulting in high morbidity and mortality [7]. Biofilm production is a key aspect of MRSA's ability to invade, spread, and resist antimicrobial treatments [7]. In recent years, an increase in resistance of *E. coli* has also been noted:

the emergence of hypervirulent carbapenem-resistant strains has been reported [8].

One promising strategy for combating antimicrobial resistance is based on the use of nanostructured metals, the most powerful of which are silver and copper [9, 10]. However, resistance to them has already been noted [11]. The problem of the toxic effects of metal nanoparticles on human cells also remains unresolved, which limits their use in high concentrations [12, 13]. Taking into account the above, it is necessary to direct efforts to the study of new combined nanomaterials based on silver and copper.

AIM

To investigate the physicochemical properties and antibacterial and antibiofilm activity of nanocomposites Ag/Cu_2O (Ag/Cu_2O NCs) synthesized by the polyol method.

MATERIALS AND METHODS

SYNTHESIS OF AG/CU_2O NCS

The synthesis of Ag/Cu_2O NCs was carried out by the polyol method in a diethylene glycol medium. 1.2 g of copper (II) acetate monohydrate and 6.0 g of polyvinylpyrrolidone were dissolved in 50 ml at 50 °C in a 250 ml three-necked round-bottom flask on a flask heater with magnetic stirring for 10 min. The reaction mixture was kept at 220 °C for 1 hour under an argon flow. Then 0.68 g of silver nitrate was added to the reaction mixture cooled to 180 °C and kept for 2 h. After synthesis, the suspension was cooled, and 20 ml of isopropanol and 10 ml of distilled water were added to the flask. The resulting composite was centrifuged at 8000 rpm, followed by washing with distilled water 3 times the collected precipitate and centrifugation. The washed composite was dispersed in 30 ml of distilled water using an ultrasonic bath (Skymen JP-008, China) for 10 min, 40 kHz.

AG/CU_2O NCS CHARACTERIZATION

The morphology of synthesized Ag/Cu_2O NCs was examined by transmission electron microscopy (TEM) (electron microscope "PEM-125K", Ukraine). An X-ray diffraction (XRD) investigation of synthesized materials was carried out on the automated diffractometer DRON 4-07 connected to the computer-aided experiment control and data processing system. Energy dispersive spectroscopy (EDS) and scanning electron microscopy (SEM) of Ag/Cu_2O NCs were carried out on a scanning electron microscope JEOL JSM-6390LV (JEOL Ltd., Japan) with an Oxford INCA X-ray microanalysis detector. Ultraviolet-visible (UV-vis) spectroscopy was measured using a spectrophotometer Cary 4000 (Varian, USA). The concentration of Ag/Cu_2O NCs in the aqua solution was determined by the method of atomic absorption spectroscopy on a C-115-M1 spectrophotometer (NGO "Selmi", Ukraine).

ANTIBACTERIAL ACTIVITY OF THE AG/CU_2O NCS

To evaluate the antibacterial activity of Ag/Cu_2O NCs, MDR clinical isolates of *S. aureus*, *E. coli*, and *P. aeruginosa* were employed. The antibiotic resistance characteristics of the isolates have been previously reported [10]. The antibacterial efficacy of Ag/Cu_2O NCs was assessed by determining their minimum inhibitory concentration (MIC) using the tube serial dilution technique, following the guidelines of the Clinical and Laboratory Standards Institute [14]. Overnight bacterial cultures were adjusted in nutrient medium (HiMedia, India) to achieve a final concentration of 5×10^5 CFU/ml. Subsequently, 0.2 ml of serially diluted Ag/Cu_2O NCs suspensions were mixed with 1.8 ml of the bacterial inoculum, resulting in final concentrations ranging from 1000 to 15.63 µg/ml. Tubes containing only the growth medium and bacterial cultures served as positive controls, while tubes with medium and Ag/Cu_2O NCs acted as negative controls. All tubes were incubated aerobically at 37 °C for 24 h. The MIC was defined as the lowest concentration of Ag/Cu_2O NCs that completely inhibited visible bacterial growth. For determination of the minimum bactericidal concentration (MBC), 100 µl aliquots from each tube were plated on Mueller–Hinton agar (HiMedia, India) and incubated at 37 °C for 24 h. The lowest concentration that resulted in complete (100%) bacterial killing was recorded as the MBC. All experiments were performed in triplicate.

ANTIBIOFILM ACTIVITY OF AG/CU_2O NCS

The antibiofilm activity of Ag/Cu_2O NCs was evaluated by measuring their capacity to reduce biofilm biomass. Overnight bacterial cultures were adjusted to a concentration of 5×10^5 CFU/mL in Mueller–Hinton broth and inoculated into 96-well polystyrene microplates (200 µl per well). The plates were incubated at 37 °C for 72 h to allow biofilm formation. After incubation, the culture medium was carefully removed, and 200 µL of fresh broth containing Ag/Cu_2O NCs at concentrations equivalent to 1×, 3×, and 5× MBC was added to each well. Plates were then incubated for an additional 24 h at 37 °C. Wells containing untreated bacterial cultures served as positive controls, while wells

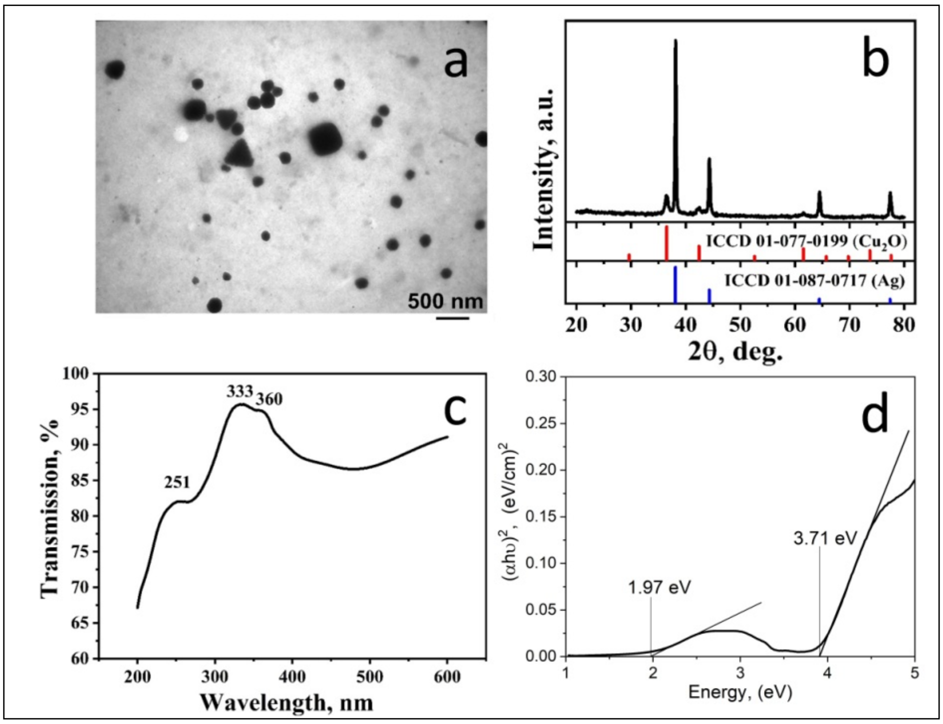

Fig. 1. Physico-chemical characteristics of Ag/Cu20 NCs: a – TEM, b – XRD, c – UV-vis, d – determination of the band gap width
Picture taken by the authors

with Ag/Cu_2O NCs (1×, 3×, and 5× MBC) but no bacteria, and wells with only Mueller–Hinton broth, served as negative and blank controls, respectively. Following treatment, the contents were removed, and the wells were washed three times with 0.9% saline solution. The remaining biofilms were stained with 0.1% (w/v) gentian violet solution. After staining, the wells were rinsed, air-dried, and 200 µl of 96% (v/v) ethanol was added to solubilize the bound dye. The absorbance of each well was measured at 595 nm using a Multiskan FC spectrophotometer (Thermo Fisher Scientific, USA). Each assay was performed six times, and the mean optical density (OD) values were calculated.

STATISTICAL ANALYSIS

MICs and MBCs data are presented as the mean ± standard deviation. In a study of antibiofilm activity, one-way ANOVA with multiple comparisons was used to assess the difference between groups using GraphPad Prism 9.0 software, where a p-value < 0.05 was considered statistically significant.

ETHICS

This article was written in accordance with ethical principles and industry standards.

FRAMEWORK

This work was supported by the National Research Foundation of Ukraine (grant No. 0124U004801); and Ministry of Education and Science of Ukraine (grant No. 0124U000540).

RESULTS

PHYSICO-CHEMICAL CHARACTERISTICS OF AG/CU_2O NCS

Ag/Cu_2O NCs were predominantly round in shape, with an average size of 135.6±65 nm, without a tendency to agglomerate (Fig. 1a). The peaks in the diffractograms according to reference data (JCPDS, card No. 01-077-0199 and 01-087-0717) correspond to the sum of reflections from the Ag and Cu_2O phases. The diffraction peaks at angles 36.41, 42.50, and 61.63 correspond to reflections from the crystallographic planes (111), (200), (220) of Cu_2O, and the peaks at 38.19, 44.37, 64.50, 77.54 correspond to reflections from the planes (111), (200), (220), (311) of Ag (Fig. 1b). UV-vis spectroscopy indicated the presence of peaks at wavelengths 251, 333, and 360 nm (Fig. 1c). To confirm the presence of oxide phases in the suspension, the band gap width of nanomaterials E_g was determined. For this purpose, dependences were constructed in the coordinates $(ahv)^2$ - hv (Fig. 1d). The linear section of these dependences is approximated by a straight line at the point of intersection with the energy axis, which allows the value of E_g to be determined. The suspension contains materials with a band gap E_{g1} = 1.97 eV and E_{g2} = 3.71 eV. The first value of the band gap width corresponds to copper dioxide Cu_2O, which is characterized by reference values of E_g = (2.1-2.6) eV. The second value may be associated with the

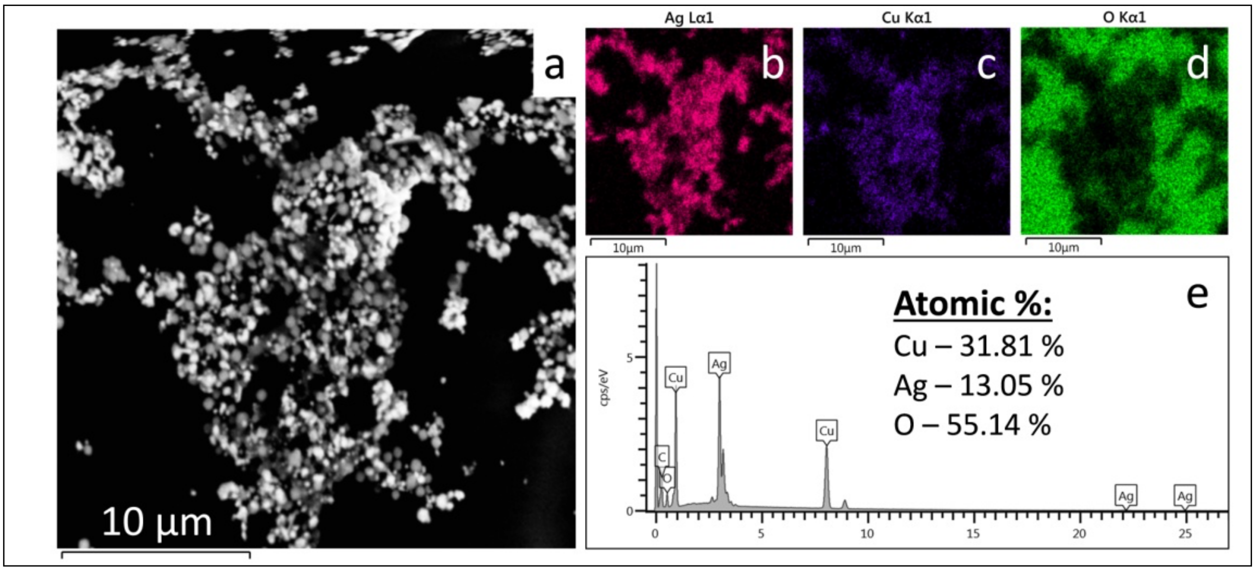

Fig. 2. SEM image (a), EDS (e) and EDS mapping of Ag/Cu20 NCs (b – Ag, c – Cu, d – 0)
Picture taken by the authors

presence of silver oxide in the suspension. However, among several oxide phases, the most thermodynamically stable is the Ag_2O phase, which has a band gap width depending on the preparation method, stoichiometry, and oxidation temperature E_g = (1.2 - 3.4) eV. The increased band gap width of this oxide E_{g2} = 3.71 eV can be associated with quantum mechanical effects in the nanostructured state and its doping with copper. However, the content of this phase is relatively small because, unlike the other two, it is not recorded by a diffractometer.

This conclusion is confirmed by the data of determining the chemical composition of the nanomaterial. Fig. 2 shows SEM images of the surface of the film obtained by dripping a suspension of Ag/Cu_2O NCs onto the substrate after drying (Fig. 2a) and the results of mapping its chemical composition (Fig. 2b, 2c, 2d). EDS revealed that the nanomaterial contained 13.05 at. % silver, 31.81 at. % copper, and 55.14 at. % oxygen (Fig. 2e). The ratio of the concentrations of the components $\gamma_1 = C_{Ag}/C_{Cu}$ was 0.41, and $\gamma_2 = (C_{Ag} + C_{Cu}) / C_O$ = 0,81. That is, there are no uncontrolled impurities in the material that could form an additional oxide.

ANTIBACTERIAL AND ANTIBIOFILM ACTIVITY OF AG/CU$_2$O NCS

Ag/Cu_2O NCs demonstrated the strongest antibacterial effect against the MDR *P. aeruginosa* (MIC – 26.04±9.02 µg/ml, and MBC – 41.67±18.04 µg/ml). For MDR *S. aureus*, MIC and MBC of Ag/Cu_2O NCs were higher – 52.08±18.04 µg/ml and 72.92±47.74 µg/ml, respectively. The highest concentration of Ag/Cu_2O NCs was required for the complete killing of MDR *E. coli* (MIC – 83.33±36.08 µg/ml, MBC – 208.30±72.17 µg/ml).

Ag/Cu_2O NCs showed antibiofilm activity against *S. aureus* at 3 and 5 MBC, against *E. coli* at 1, 3, and 5 MBC, and *P. aeruginosa* at 5 MBC (Fig. 3). There was no statistical difference between the antibiofilm activity of Ag/Cu_2O NCs using 1, 3, or 5 MBC, but it was observed compared to positive controls (biofilm growth without Ag/Cu_2O NCs). So, a stable antibiofilm effect was observed against all bacteria only when using 5 MBC of Ag/Cu_2O NCs.

DISCUSSION

Ag/Cu_2O NCs synthesized by the polyol method with a size of 135.6±65 nm showed antibacterial and antibiofilm activity against gram-positive and gram-negative MDR bacteria. Even though the Ag/Cu_2O NCs were most effective against planktonic *P. aeruginosa*, the study of antibiofilm properties showed that biofilms formed by *P. aeruginosa*, compared to other tested bacteria, are more resistant to destruction and require higher, fivefold bactericidal concentrations of Ag/Cu_2O NCs. At the same time, to kill planktonic *E. coli*, a significantly higher concentration of Ag/Cu_2O NCs (208.3±72.17 µg/ml) was initially required, but this concentration was already sufficient to destroy its biofilm. These gram-negative bacteria are particularly important to practicing clinicians and require further detailed research on their interaction with nanometal complexes.

For comparison, core-shell Ag-Cu nanostructures, 20-70 nm in size, prepared by S. F. Sabira et al. showed similar results to those described in the current study: MICs for *S. aureus* and *E. coli* were of 75 µg/ml, and against *P. aeruginosa*, MICs were lower – 20 µg/ml [15]. Significantly worse antibacterial properties were shown

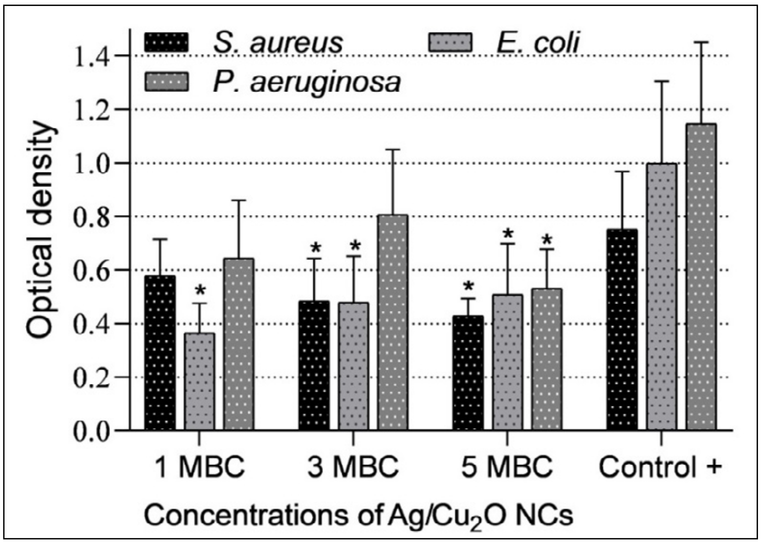

Fig. 3. Antibiofilm activity of Ag/Cu20 NCs against *S. aureus, E. coli, P. aeruginosa*. Control + – positive control. Note. Statistical difference compared to the positive control, $p < 0.05$
Picture taken by the authors

in the study of Ag/Cu nanoparticles (70 and 100 nm in size), where the MICs for *S. aureus* and *E. coli* were > 500 μg/ml, and for *P. aeruginosa* > 125 μg/ml [16]. M. Valodkar et al. reported the successful synthesis of silver-copper nanoparticles (size 20±5 nm) in a ratio of 1/1 and their antibacterial activity: for *S. aureus* the MIC was 0.33 mg/l, MBC – 1.32 mg/l, for *E. coli* the MIC was 0.23 mg/l, MBC – 0.71 mg/l [17]. However, as the authors themselves note, the stability of the solution of these nanoparticles is maintained only for several days. The difference in results can be attributed to various approaches to synthesis, characteristics, different research methods, and different strains of bacteria used.

The antibacterial effect of Ag/Cu_2O NCs is due to both the presence of Ag, which primarily affects the bacterial plasma membrane, and Cu, which can denature nucleic acids and other internal biomolecules and cellular structures. It has also been suggested that the released Cu ions enhance ROS production, leading to DNA damage and lipid peroxidation, which inactivates bacteria [18, 19]. Some authors indicate that Ag^+ ions, which also have a detrimental effect on bacteria, are released in significantly higher quantities in solutions with higher Cu^{2+} ion concentrations [20], explaining the enhanced synergistic antibacterial properties of the nanocomposites.

A more detailed study of the effect of oxide components on the interaction between composites and bacteria, as well as their biofilms, and the determination of the synergistic antibacterial potential of structural components, would be promising.

CONCLUSIONS

Ag/Cu_2O NCs are effective against planktonic and biofilm forms of MDR bacteria *S. aureus*, *E. coli*, and *P. aeruginosa*. Thus, Ag/Cu_2O NCs can be implemented as an alternative to antibiotics in various areas of nanomedicine to combat infections with multiple drug resistance.

COMPETING INTERESTS

The authors have no relevant financial or non-financial interests to disclose.

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CONFLICT OF INTEREST

The Authors declare no conflict of interest

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A – Work concept and design, B – Data collection and analysis, C – Responsibility for statistical analysis, D – Writing the article, E – Critical review, F – Final approval of the article

RECEIVED: 08.09.2025
ACCEPTED: 29.12.2025

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