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ABSTRACT

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ANTIBACTERIAL AND ANTIBIOFILM ACTIVITY OF CU/CU_2O NPS AGAINST MULTIDRUG-RESISTANT BACTERIA

Introduction. Multidrug-resistant (MDR) bacteria are very dangerous and represent a major problem in all areas of healthcare: they often cause diseases that cannot be treated with antibiotics, which leads to long-term ineffective treatment, complications, and high treatment costs. Searching for new antimicrobials is one of the key components of a successful fight against infections caused by MDR. This study was designed to elucidate the antimicrobial and antibiofilm activities of copper/copper oxide nanoparticles (Cu/Cu_2O NPs) against MDR bacteria.

Methods. Cubic Cu/Cu_2O NPs were synthesized by the polyol method. The physicochemical characteristics of the nanoparticles were investigated using transmission electron microscope, X-ray diffraction investigation, energy dispersive spectroscopy and Fourier-transform infrared spectroscopy. Laboratory reference bacterial strains (*S. aureus* ATCC 25923. *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853) and MDR clinical strains isolated from patients with a purulent process (*S. aureus*, *E. coli*, *P. aeruginosa*) were used to examine the antibacterial effect of nanoparticles.

Results. Cubic Cu/Cu_2O NPs showed antimicrobial activity against both Gram-negative and Gram-positive bacteria, but the antibiofilm activity of Cu/Cu_2O NPs was more promising for targeting Gram-negative bacteria. Cu/Cu_2O NPs were less effective against MDR strains of planktonic bacteria in comparison to laboratory reference strains. No significant differences were found between the action of the Cu/Cu_2O NPs on biofilms formed with reference laboratory strains or MDR clinical strains.

Discussion. The findings of this research may be useful to develop new drugs and approaches for treating infection caused by MDR microorganisms. Further research is warranted to elucidate the

underlying mechanisms of Cu/Cu_2O NPs action, optimize their formulation, and evaluate their safety and efficacy in preclinical and clinical settings.

Keywords: copper oxide nanoparticles, biofilms, bacteria, bacterial resistance, microorganisms.

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АНТИБАКТЕРІАЛЬНА ТА АНТИБІОПЛІВКОВА АКТИВНІСТЬ НАНОЧАСТИНОК CU/CU$_2$O ВІДНОСНО МУЛЬТИРЕЗИСТЕНТНИХ БАКТЕРІЙ

Вступ. Мультирезистентні бактерії є дуже небезпечними та становлять серйозну проблему в усіх сферах охорони здоров'я: часто викликають захворювання, які не піддаються лікуванню антибіотиками, що призводить до неефективності лікування, ускладнень і високих затрат. Пошук нових антимікробних препаратів є одним із ключових компонентів успішної боротьби з інфекціями, що спричинені мультирезистентними бактеріями. Дана робота присвячена дослідженню антимікробної та антибіоплівкової активності наночастинок оксиду міді (Cu/Cu_2O) відносно мультирезистентних бактерій.

Матеріали та методи досліджень. Кубічні наночастинки Cu/Cu_2O було синтезовано поліольним методом. Фізико-хімічні характеристики наночастинок досліджували за допомогою просвічуючої електронної мікроскопії, рентгенівської дифракції, енергодисперсійної спектроскопії та Фур'є-інфрачервоної спектроскопії. Лабораторні музейні бактеріальні штами (*S. aureus* ATCC 25923. *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853) та клінічні штами мультирезистентних бактерій (*S. aureus, E. coli, P. aeruginosa*), що виділені від пацієнтів із гнійно-запальними процесами використовувалися для вивчення антибактеріальних властивостей наночастинок.

Результати. Кубічні наночастинки Cu/Cu_2O показали ефективність відносно як грамнегативних так і грампозитивних бактерій, але антибіоплівкова активність наночастинок Cu/Cu_2O виявилася більш перспективною по відношенню до грамнегативних бактерій. Наночастинки Cu/Cu2O виявилися менш ефективними відносно мультирезистентних штамів планктонних бактерій порівняно з лабораторними штамами. Не було виявлено істотних відмінностей між дією наночастинок Cu/Cu_2O на біоплівки, що сформовані музейними та клінічними штамами мультирезистентних бактерій.

Обговорення. Результати цього дослідження можуть бути корисними для розробки нових ліків і підходів до лікування інфекцій, спричинених мультирезистентними мікроорганізмами. Необхідні подальші дослідження для з'ясування основних механізмів дії наночастинок Cu/Cu_2O, оптимізації їх складу та оцінки їх безпеки та ефективності в доклінічних і клінічних умовах.

Ключові слова: наночастинки оксиду міді, біоплівки, бактерії, резистентність бактерій, мікроорганізми.

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INTRODUCTION

The ever-increasing antimicrobial resistance to antibiotics is a serious public health challenge [1]. The wide spread of multidrug-resistant (MDR) bacteria reduces antibiotics' effectiveness, increasing morbidity and mortality, leading to longer hospital stays and, consequently, higher treatment costs [2]. According to the US Center for Disease Control (CDC) and Prevention, each year in the USA more than 2.8 million people become ill with resistant infections, and at least 35000 patients die [3]. In Europe yearly the number of infections and deaths caused by multidrug-resistant bacteria (*S. aureus, E. coli, E. faecium, S. pneumoniae, K. pneumoniae and P. aeruginosa*) was estimated approximately 400 000 and 25 000, respectively, in 2007 [4]. CDC estimated financial burden of MDR as $55 billion per year overall that include $20 billion direct healthcare costs and $35 billion indirect costs (loss of productivity) [1, 5]. Therefore, the development of new approaches, materials, and methods for destroying bacteria can help to minimize the drawbacks of growing multi-drug resistance [6, 7].

The antibacterial properties of copper have been known since ancient times, but the discovery of antibiotics temporarily stopped the use of copper and other metals as antimicrobials. Given the increasing antibiotic resistance, there has recently been renewed interest in copper, especially nanostructured copper, as a good antibacterial alternative. Nanostructured metals are more effective because they can interact closely with microorganisms, thanks to their unique properties such as small size and high surface area/volume ratio [8]. In the current study, copper was the metal of choice as it is more readily available than other noble metals such as silver and gold and is equally potent in its antimicrobial properties.

Previously, it was repeatedly reported that copper nanoparticles exhibit antibacterial activity against Gram-positive and Gram-negative bacteria [9, 10, 11, 12]. The bactericidal properties of copper oxide nanoparticles also were noted against methicillin-resistant *S. aureus, K. pneumonia,* and *E. faecalis* [13, 14, 15]. Raffi M. et al. reported a concentration dependent activity of Cu NPs against reference laboratory strain *E. coli ATCC15224* [16]. However, there is little published information on the activity of Cu/CuO NPs against MDR strains.

Several mechanisms of action of copper nanoparticles on bacterial cells have been reported: nanoparticles reduce transmembrane electrochemical potential, disrupt the integrity of the cell membrane with subsequent leakage of the cytoplasm; nanoparticles release copper ions (Cu^{+} and Cu^{2+}), inactivate proteins inside the cell and damage DNA; generate reactive oxygen species, thereby causing the development of oxidative stress, which leads to apoptosis [17, 18, 19].

Existing research recognizes the critical role played by microbial biofilm in infections. The bacteria in the biofilm matrix possess higher resistance to antibacterial drugs compared to planktonic forms of bacteria [20, 21, 22], as well as drug penetration to target cells is limited [23]. Diseases caused by biofilms are complicated to treat, often take a long course, gradually progress, and have a poor response to antibiotic therapy [24, 25]. Therefore, radical new approaches are needed to treat diseases caused by microbes in biofilms [26], namely the formation of non-toxic and potent antibiofilm agents targeting signaling pathways regulating quorum sensing (QS), extracellular polymeric substance synthesis, biofilm-related genes, microbial motility, adhesion, dispersion, and many more [27]. Although extensive research has been carried out on the antibiofilm effectiveness of copper oxide nanoparticle [28, 29, 30], no single study exists that investigates its activity against reference laboratory and clinical MDR strains.

The current research set out to compare the antimicrobial activity of copper/copper oxide nanoparticles (Cu/Cu_2O NPs) against laboratory reference strains and MDR clinical Gram-positive Gram-negative strains of planktonic bacteria and their biofilms.

MATERIALS AND METHODS

Synthesis of Cu/Cu_2O NPs

Cu/Cu_2O NPs were synthesized by the following method. 12.5 g polyvinylpyrrolidone (PVP) K-30 (Thermo Fisher Scientific, USA) and 4.0 g sodium hypophosphite (Thermo Fisher Scientific, USA) were dissolved in 40 ml of ethylene glycol (Thermo Fisher Scientific, USA) and stirred using a magnetic stirrer until completely dissolved at room temperature. The solution was heated to 90 °C at a rate of 5 °C min^{-1}. Then 2.5 g of copper sulfate (Thermo Fisher Scientific, USA) was dissolved in 10 ml of ethylene glycol in a similar way and slowly added to the PVP/sodium hypophosphite solution with constant stirring. Gradually, the color of the suspension turned brown, indicating the formation of copper nanoparticles. In general, the reaction lasted 15 min. The dispersion was cooled down to room temperature and mixed with a certain amount of isopropanol and distilled water. The

precipitate was collected after centrifugation (8000 rpm for 10 min) and washed with distilled water three times to remove excess PVP and side products, followed by drying at 50 °C for 2 h in a vacuum dryer. Nanoparticles were treated with low-frequency ultrasound (22±1.65 kHz) for 5 minutes (ultrasonic dispergator UZDN-A, SELMI, Ukraine).

Cu/Cu_2O NPs characterization

The morphology of synthesized Cu/Cu_2O NPs was examined by transmission electron microscope (TEM). Samples for TEM were prepared by deposition isopropyl alcohol solution of Cu/Cu_2O NPs on a copper grid covered with a thin carbon film with the subsequent drying in air at room temperature. TEM analyses were performed using "PEM-125K" (Ukraine). In an X-ray diffraction (XRD) investigation of synthesized materials were carried out on the automated diffractometer DRON 4-07 connected to the computer-aided experiment control and data processing system. The Ni-filtered CuKα radiation (wavelength 0.154 nm) was used with a conventional Bragg–Brentano ϑ-2ϑ geometry (2ϑ is the Bragg's angle). The samples were measured in the continuous registration mode (at the speed of 1.0 ◦/min) within the 2ϑ-angle range from 30° to 120°. All data processing procedures were carried out with the use of the program package DIFWIN-1 ("Etalon PTC" Ltd). Phase analysis was carried out by comparing the diffraction patterns from the investigated samples and the reference data JCPDS. Energy dispersive spectroscopy (EDS) elemental analysis of Cu/Cu_2O NPs was carried out on a JEOL JSM–6390LV scanning microscope with an X-ray detector INCA 350 (Jeol, Japan). Fourier-Transform Infrared (FTIR) Spectroscopy analysis spectra were obtained using a spectrophotometer (Nicolet IS20, Thermo Fisher Scientific, USA) in the spectral region of 4000 – 400 cm^{-1} using a resolution of 4 cm^{-1} and 64 coadded scans. Concentration of Cu/Cu_2O NPs in the aqua solution was determined by the method of inductively-coupled plasma atomic spectrometry (ICP-AES) using an iCAP 6300 Duo spectrometer (Thermo Scientific Corporation, USA).

Bacterial Strains and Culture Conditions

To examine the antibacterial effect, we used laboratory reference bacterial strains (*S. aureus* ATCC 25923. *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853) and MDR clinical strains isolated from patients with a purulent process (*S. aureus*, *E. coli*, *P. aeruginosa*). Identification of the clinical strains was performed by examining their morphological, staining, biochemical, and antigenic features. Microorganisms were tested on sensitivity to macrolides, β-lactam, aminoglycosides, fluoroquinolones, tetracyclines, amphenicols, lincosamides, and cephalosporins. The strains resistant to at least one antibacterial drug in three or more antibacterial categories were selected as multi-resistant and stored at the Microbiology Lab (Sumy State University, Ukraine) for further research. The study protocol was approved by the Institutional Ethics Committee (Sumy State University, Protocol № 1/9 from 14 September 2023) after the informed consent collection from the patients.

Media (Mueller-Hinton broth and agar) for the cultivation of microorganisms were purchased at Hi Media (Maharashtra, India). Sigma Aldrich (St. Louis, MO, USA) provided gentian violet. glutaraldehyde.

Antibacterial activity of the Cu/Cu_2O NPs

Antibacterial activity of Cu/Cu_2O NPs was examined with the determination of minimum inhibitory concentration (MIC) by tube serial dilution method according to the international recommendations provided by the Clinical and Laboratory Standards Institute (CLSI) [31]. Overnight pure cultures of microorganisms were diluted with cultivation media to a concentration of 5×10^5 CFU/ml. Then, 0.2 ml of serially diluted Cu/Cu_2O NPs were added to 1.8 ml of the bacterial suspension at a final concentration of 1000 μg/ml to 31.25 μg/ml. Tubes containing growth medium and tested microorganisms were considered as positive controls. The tubes containing growth medium, and Cu/Cu_2O NPs were used as negative control. The tubes with Muller Hinton broth served as a blank control. Then, tubes were incubated aerobically at 37 °C for 24 h. The tube with the lowest concentration of Cu/Cu_2O NPs that completely inhibits visual growth of bacteria (no turbidity) was considered as MIC. After that 100 μL of aliquots from each tube were inoculated onto Muller Hinton agar and incubated at 37 °C for 24 hours. The lowest concentration of Cu/Cu_2O NPs that kills 100% of the initial bacterial/fungal population was recorded as minimum bactericidal concentration (MBC). All measures were triplicate. MBC of Cu/Cu_2O NPs was used as starting point for in vitro time-kill test.

In vitro static time-kill studies were conducted in glass tubes containing 2-ml volumes of Mueller Hinton broth with logarithmically growing cultures. The starting inoculum of microorganisms was 5×10^5 CFU/mL. In the test, we used the concentrations of Cu/Cu_2O NPs equivalent to 1 MBC. In 30 min, 1, 3, 6, 12 and 24 h of incubation 100-μL aliquots from tubes were spotted onto plates with solid media. After that, plates were incubated at 37 °C for 24 h. Viable organisms were counted in $\log_{10}$.

Antibiofilm activity of Cu/Cu_2O NPs

To assess the ability of nanoparticles to reduce biofilm mass, the suspensions of the overnight cultures of microorganisms were placed in polystyrene 96-well plates containing 200 μL Mueller-Hinton broth with

bacteria at 5×10^5 CFU/mL concentration and incubated for 72 h at 37 °C. Then, the nutrient broth was removed and 200 μL of fresh nutrient broth with 1 MBC, 3 MBC, and 5 MBC of Cu/Cu_2O NPs was added to each well and incubated for 24 h at 37 °C. Positive control wells were untreated with Cu/Cu_2O NPs. The Cu/Cu_2O NPs control with 1 MBC, 3 MBC, and 5 MBC without microorganisms, and the Mueller-Hinton broth control without Cu/Cu_2O NPs and microorganisms were also incubated. Then, the culture media with Cu/Cu_2O NPs were discarded, followed by triple rinsing with a 0,9% saline solution. To evaluate the volume of biofilm mass, 0.1% (w/v) gentian violet staining was used. After that, the plates were rinsed and air-dried, and 200 μL of 96% v/v ethanol was put into each well for the dissolving of connected dye. We measured the optical density (OD) of each well at a wavelength of 595 nm using a Multiskan FC spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The assay was repeated 6 times and mean values were calculated.

The cell morphology and arrangement of pathogens in biofilms were assessed using SEM. Small glass slides (0.5×1.0 cm) were immersed in 5 mL Mueller-Hinton broth with bacteria at 5×10^5 CFU/mL concentration and incubated for 72 h at 37 °C. Then, samples were split into four groups. Three groups were added with Cu/Cu_2O NPs diluted in Muller-Hinton broth at the concentration 1, 3, or 5 MBC. To the control group was added Muller-Hinton broth. After that, all samples were incubated for 24 h. Then, the media were discarded. Samples were washed three times with phosphate-buffered saline (pH 7.2 – 7.4, LLC “Genesis”, Ukraine), fixed with glutaraldehyde 2% for 120 min, dehydrated in ethanol-water mixture with increasing ethanol concentrations (30%, 50%, 70%, 80%, 90%, and 96%), and air-dried overnight. Dehydrated specimens were coated with a thin film of silver in a sputter coater. Morphological analysis was performed by the examination of the SEM (SEO-SEM Inspect S50-B; accelerating voltage − 20 kV) images.

Statistical analysis

Statistical significance was determined using an analysis of variance with Graph Pad Prism 9 software, where p-value < 0.05 was considered statistically significant.

RESULTS

Cu/Cu_2O NPs were mostly cubic and distributed in the range of 35-200 nm with a strong tendency to agglomerate (Fig. 1a). Fig. 1c depicts the XRD pattern with peaks indicating the formation of Cu and Cu_2O nanoparticles. The reflections on the XRD pattern can be indexed to that of Cu and Cu_2O according to the literature pattern (JCPDS, card № 004–0836 and 005–0667). The diffraction peaks Cu at 43.32o, 50.46o, 74.14o, 89.94o correspond to the (111), (200), (220), (311) planes, and peaks Cu_2O at 29.6o, 36.44o, 42.32o, 61.38o, 73.54o, 77.38o correspond to the (110), (111), (200), (220), (311), (222) planes respectively. This probably indicates a weak stability of copper nanoparticles before oxidation. EDS spectrum measurement reveals that the samples contain elements of copper and oxygen and exhibit an oxide state (fig. 1b). The absorption bands of PVP are recorded on the FTIR spectrum of the synthesized Cu/Cu_2O NPs which were added to stabilize the suspension in a mass ratio Cu: PVP = 2:1 (fig. 1d).

We isolated several strains of the pathogens from patients and tested them on sensitivity to antibiotics. For further investigation, we selected one multi-resistant strain of each species. Antibiotic resistance profiles of the selected strains are presented in table 1.

Antibiotic profile of laboratory reference strains showed that *S. aureus* ATCC 25923, *E. coli* ATCC 25992, and *P. aeruginosa* ATCC 27853 were sensitive to all the antibiotics listed in the table 1.

MIC and MBC of Cu/Cu_2O NPs against laboratory reference strains were similar mostly for all tested microorganisms (125 μg/mL), except *E. coli* with an MBC of 250 μg/ml. MIC and MBC of Cu/Cu_2O NPs against all examined MDR microorganisms were determined at a concentration of 250 μg/ml (fig. 2)

Time-killing assay demonstrated similar speed for laboratory and MDR strains killing. *S. aureus* were destroyed in 3 hours after the start of incubation, while *E. coli* and *P. aeruginosa* lost viability in 6 and 12 hours, respectively (fig. 3).

Examination of the antibiofilm activity did not show any significant difference in the activity of Cu/Cu_2O NPs at concentrations 1 MBC, 3 MBC, and 5 MBC against biofilms formed with laboratory reference strains and MDR *S. aureus* compared to the positive control (fig. 4).

The treatment of the biofilms formed by laboratory reference strains and clinical MDR strains *E. coli* with Cu/Cu_2O NPs caused a significant decrease in optical density compared to positive control almost at all used concentrations (fig. 5).

Use of Cu/Cu_2O NPs for treatment of the biofilms formed with both types of *P. aeruginosa* also demonstrated sufficient reduction of the optical density at all applied concentrations of nanoparticles in comparison to non-treated wells with biofilms (fig. 6).

Table 1 – Profile of MDR strains sensitivity to antibiotics

Antibiotics		Strains / registration number in the laboratory		
		S. aureus / 73	*E. coli* / 54	*P. aeruginosa* / 254
Macrolides	Azithromycin	R[1]	R	R
	Clarithromycin	R	R	R
β-lactams	Amoxicillin	R	R	R
	Ampicillin	R	R	R
	Oxacillin	R	R	R
Aminoglycosides	Amikacin	R	R	R
	Gentamicin	R	R	R
	Kanamycin	R	R	R
Fluoroquinolones	Gatifloxacin	S[2]	R	R
	Levofloxacin	S	S	S
	Ofloxacin	S	S	R
	Ciprofloxacin	S	R	S
	Lomefloxacin	S	S	S
Tetracyclines	Doxycycline	R	R	R
Amphenicols	Levomycetin	R	R	R
Lincosamides	Lincomycin	R	R	R
Cephalosporins	Cefazolin	R	R	R
	Cephalexin	R	R	R
	Cefepime	S	S	S
	Cefixime	R	R	S
	Cefotaxime	R	R	R
	Cefpodoxime	R	R	R
	Ceftazidime	R	R	R
	Ceftriaxone	R	S	S

Note: [1]R – resistant, [2]S – sensitive

Figure 7 demonstrates the effectiveness of the Cu/Cu_2O NPs against *S. aureus* biofilm. There were no any reductions in the bacteria cell numbers after the treatment of the biofilm with Cu/Cu_2O NPs. However, SEM images showed the cell deformation and decrease in the cell size (marked with a red arrow). In addition, analysis of Fig. 7b, 7c, and 7d indicates the absence of an exopolymer matrix, which is the main structural component of the biofilm and provides quorum sensing. The data obtained suggest the lack of a full-fledged mature biofilm of *S. aureus* under the influence of Cu/Cu_2O NPs and, accordingly, the quorum sensing system, which regulates the virulence of these bacteria. This contributes to the lower survival of *S. aureus* when exposed to nanoparticles.

Treatment of biofilms formed by *E.coli* with

Cu/Cu_2O NPs caused a dose-dependent decrease in biofilm mass that is shown in figure 8. Bacterial cells treated with 1 MBC of Cu/Cu_2O NPs were deformed, and the arrangement of cells in space relative to each other changed. Biofilms formed by *E. coli* and treated with 5 MBC Cu/Cu_2O NPs were mostly demolished and consisted of single spherical-shape cells with damaged cell walls and leakage of cytoplasmic contents. Red arrows point the serious damage.

The influence of Cu/Cu_2O NPs on the structure of mature biofilms formed by *P. aeruginosa* is reflected in fig. 9. We found a similar effect of Cu/Cu_2O NPs on biofilms formed by *P. aeruginosa* and *E. coli*. There was a decrease in the *P. aeruginosa* biofilm mass and bacteria cell destruction after treatment with Cu/Cu_2O NPs at concentrations 3 and 5 MBC. The cells exposed to these nanoparticles displayed a ruptured and shrunken appearance, resulting in a loss of cellular components.

Taken together, these results suggest that there is a relation between bacteria features and the effectiveness of Cu/Cu_2O NPs antibiofilm activity.

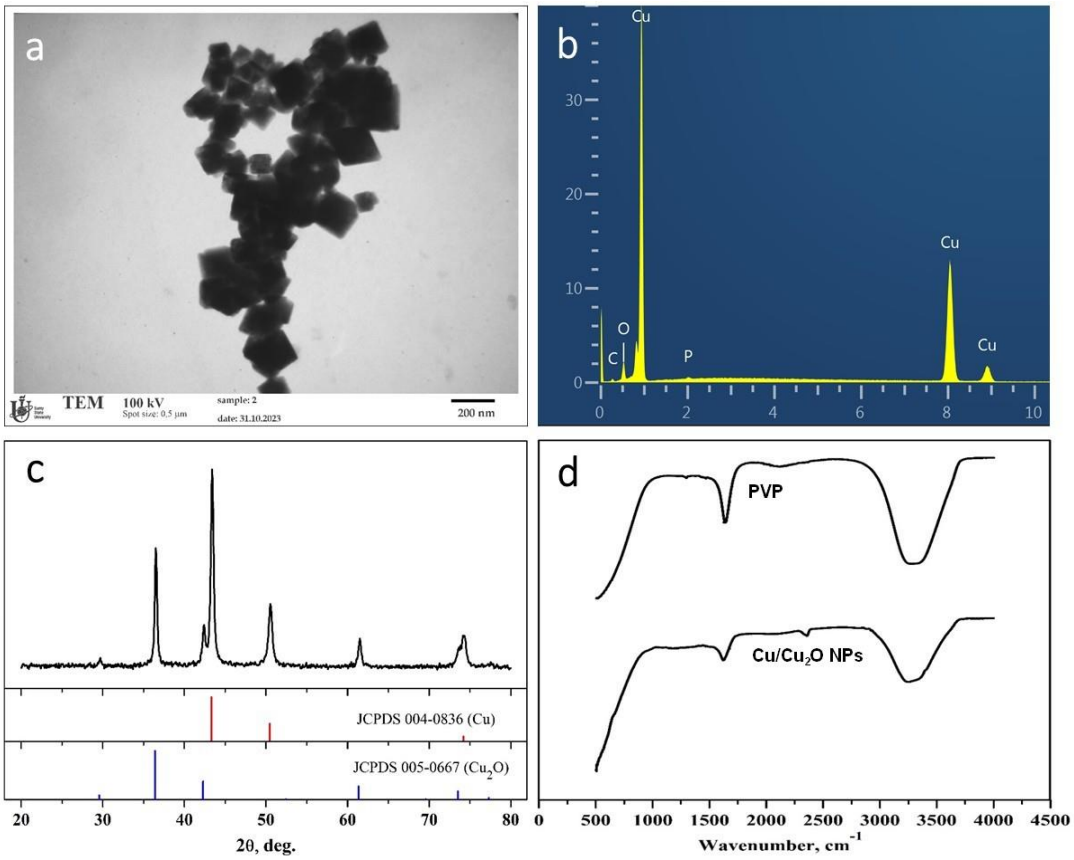

Figure 1– Physicochemical characteristics of Cu/Cu_2O NPs: a) TEM, b) EDS, c) XRD, d) FTIR

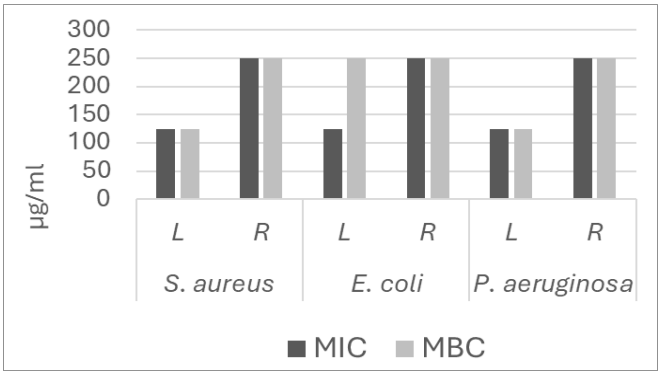

Figure 2 – MIC and MBC values of Cu/Cu_2O NPs against laboratory reference bacterial strains (L) and multidrug-resistant clinical (R) bacteria

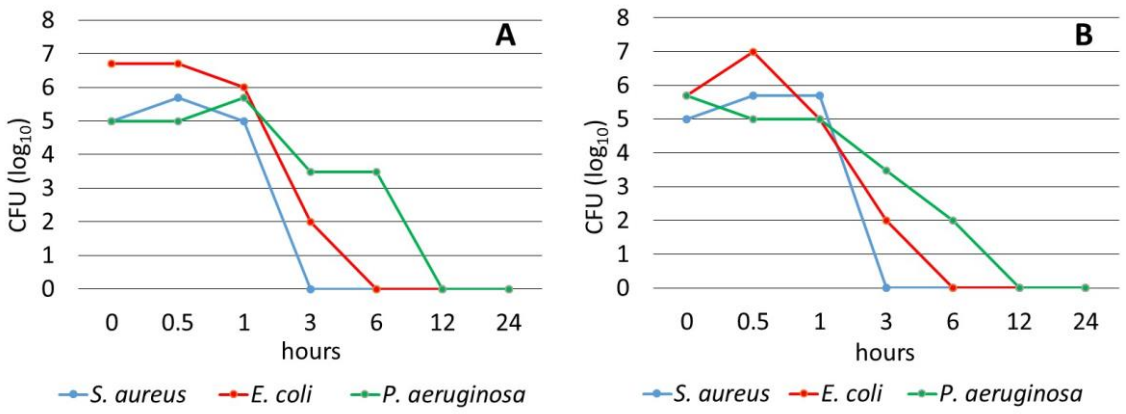

Figure 3 – Time-dependent antibacterial activity of the Cu/Cu_2O NPs against laboratory reference bacterial strains (A) and MDR clinical strains (B)

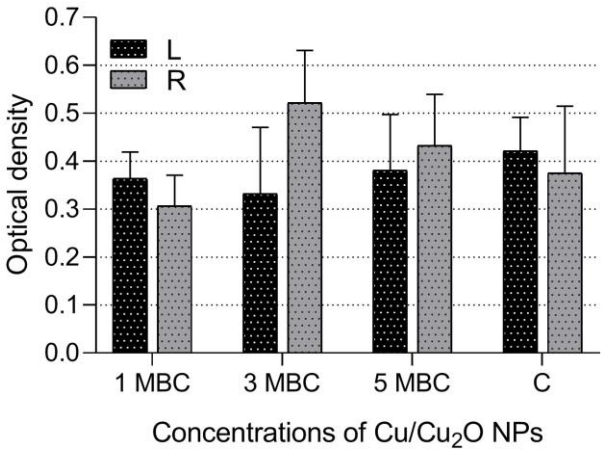

Figure 4 – Antibiofilm activity of Cu/Cu_2O NPs against S. aureus: L – laboratory reference strains; R – MDR clinical strains; C – positive control

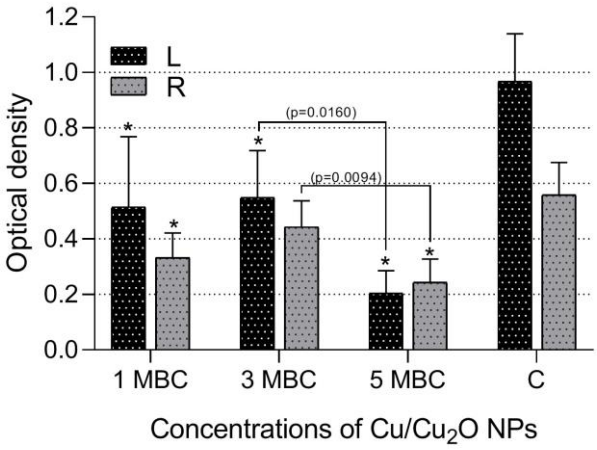

*Figure 5 – Antibiofilm activity of Cu/Cu_2O NPs against E. coli: L – laboratory reference strains of bacteria; R – multidrug-resistant clinical bacteria; C – positive control; * – statistical difference compared to the positive control*

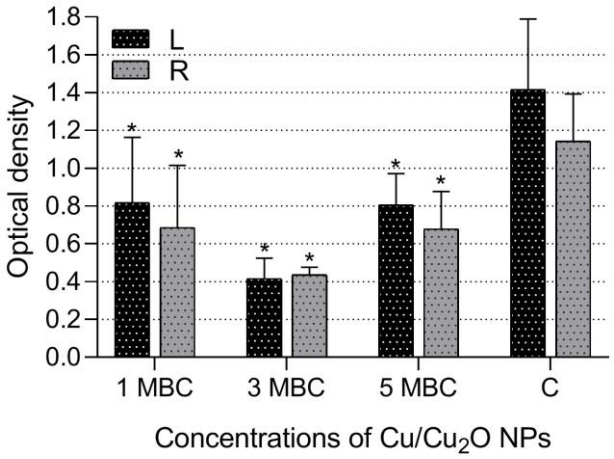

*Figure 6 – Antibiofilm activity of Cu/Cu2O NPs against P. aeruginosa: L – laboratory reference strains of bacteria; R –multidrug-resistant clinical bacteria; C – positive control; * – statistical difference compared to the positive control*

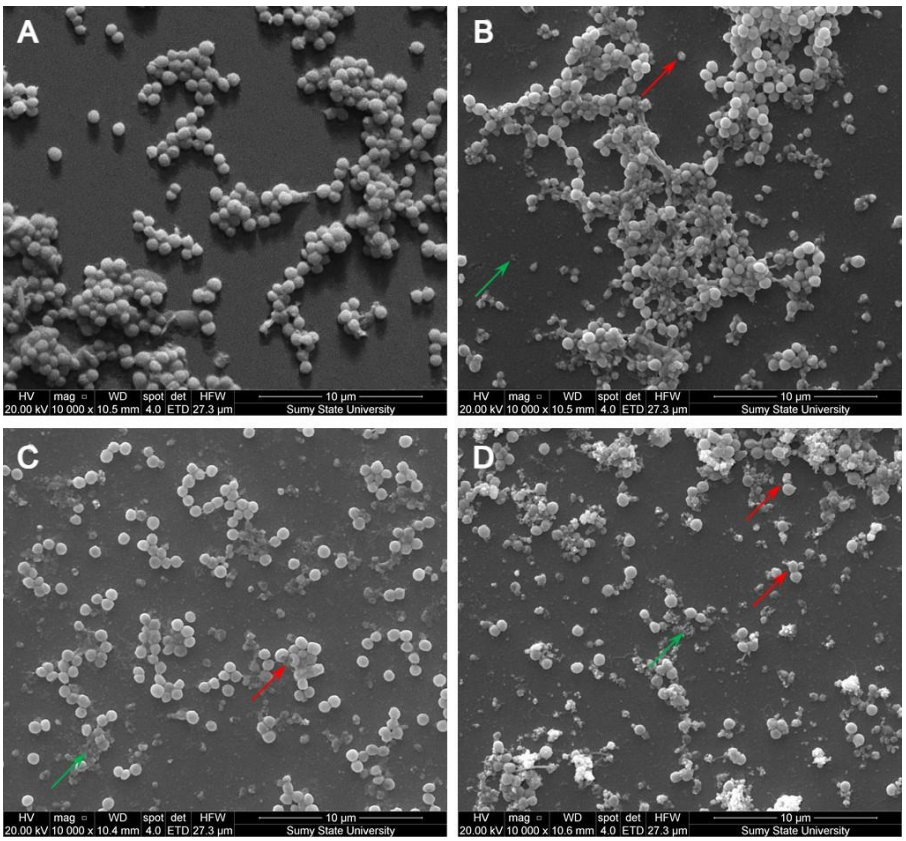

Figure 7 – The influence of Cu/Cu_2O NPs on the structure of mature biofilms formed by S. aureus (SEM): a – control, b – 1 MBC, c – 3 MBC, d – 5 MBC. The red arrows demonstrate cell changes, green arrows – Cu/Cu_2O NPs

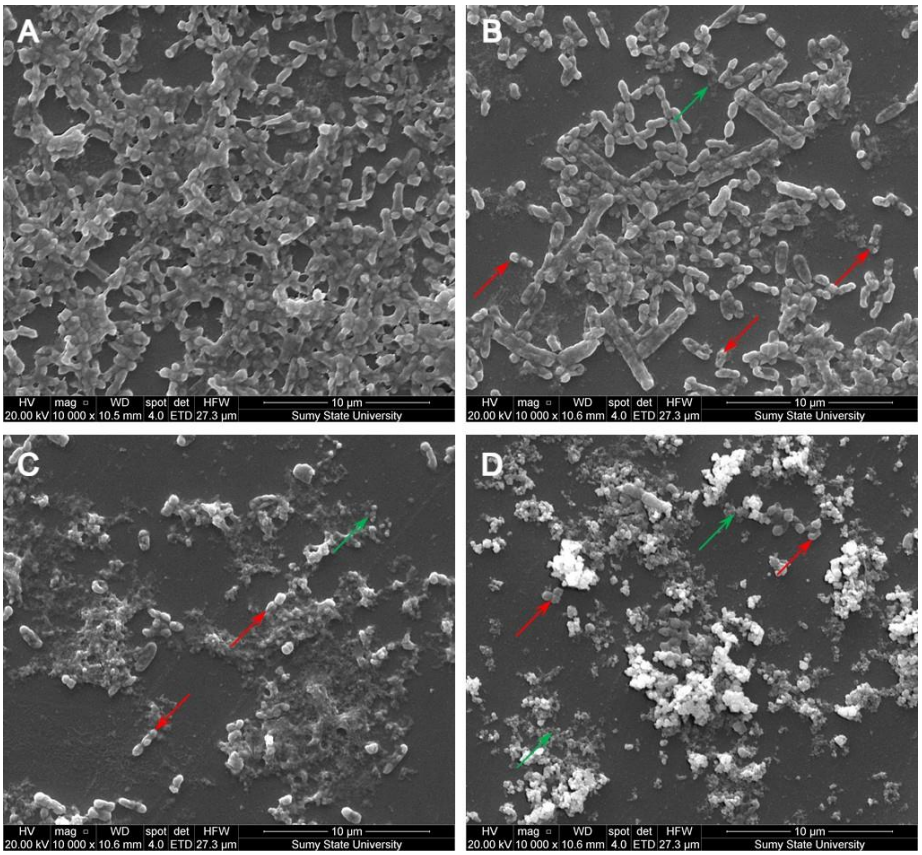

Figure 8 – The influence of Cu/Cu_2O NPs on the structure of mature biofilms formed by E. coli (SEM): a – control, b – 1 MBC, c – 3 MBC, d – 5 MBC. The red arrows demonstrate cell changes, green arrows – Cu/Cu_2O NPs

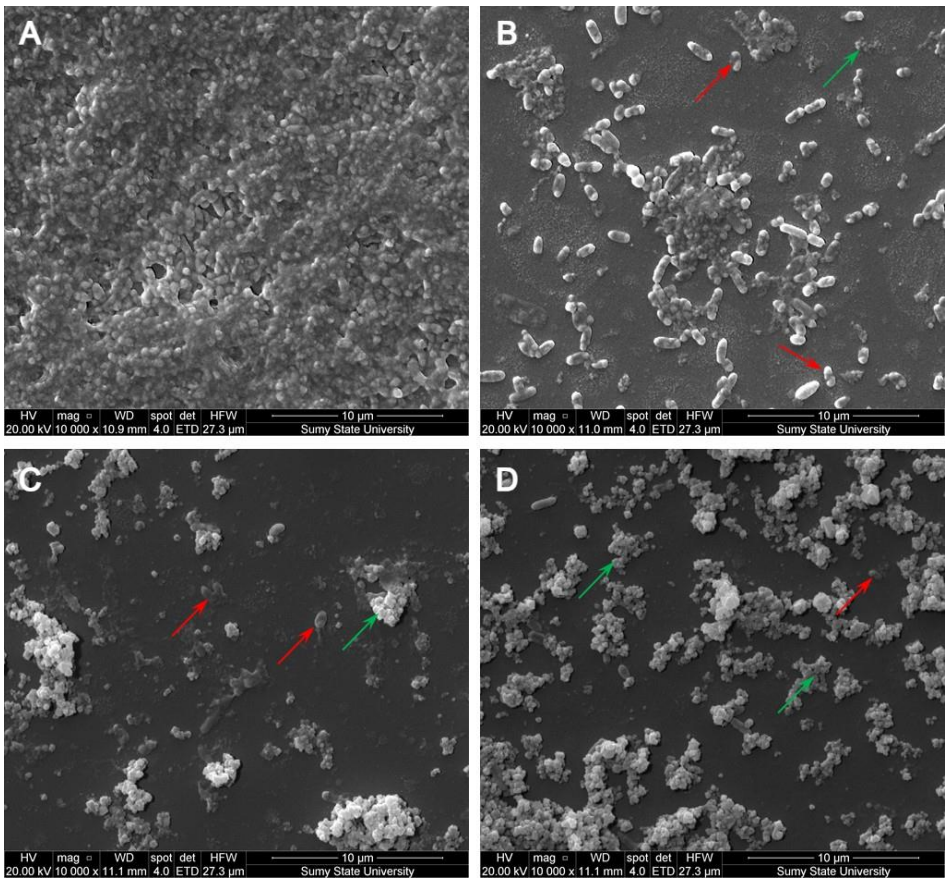

Figure 9 – The influence of Cu/Cu_2O NPs on the structure of mature biofilms formed by P. aeruginosa (SEM): a – control, b – 1 MBC, c – 3 MBC, d – 5 MBC. The red arrows demonstrate cell changes, green arrows – Cu/Cu_2O NPs

DISCUSSION

As mentioned in the literature review, searching for new antimicrobials is one of the key components of a successful fight with infections. It is now well established from a variety of studies that nanometals are promising alternatives to existing antibiotics. However, several questions regarding the treatment of infectious pathology using metal nanoparticles remain relevant.

Many recent studies have shown that the shape and size of nanoparticles, temperature of synthesis, bacterial cell wall structure, as well as other characteristics of nanoparticles and microorganisms influence antimicrobial activity [32]. In this study, the antibacterial and antibiofilm activity of Cu/Cu_2O NPs was evaluated against laboratory reference and clinical MDR strains of some ESKAPE isolates. It was reported previously that MIC of Cu NPs varies from 500 µg/ml to 103.5±4.71 µg/mL for *E. coli* and *S. aureus* [33, 34]. Ren G. et al. showed a wide range of MBC values for CuO NPs from 100 to 5000 µg/ml for various resistant and laboratory isolates [9]. However, most of these studies did not assess the effectiveness of Cu/Cu_2O NPs against MDR strain.

We synthesized cubic Cu/Cu_2O NPs with size 35-200 nm, stabilized with PVP. Nanoparticles demonstrated two-fold higher antibacterial activity against almost all laboratory reference strains compared to MDR clinical strains of *S. aureus*, *E. coli*, and *P. aeruginosae*. Previous studies suggest the higher efficacy of Cu/CuO NPs against Gram-negative bacteria than Gram-positive [35] due to the presence of a thicker peptidoglycan layer reduced cellular penetration [17]. However, the findings of the current study do not support these assumptions. We did not find a difference in the Cu/Cu_2O NPs antibacterial activity against Gram-positive or Gram-negative bacteria. Although there was a difference in the speed of bacteria-killing between Gram-positive and Gram-negative microbes, we did not reveal the difference between MDR and antibiotic susceptible laboratory reference strains.

Most previously published studies in the field of Cu/CuO NPs antibiofilm activity have been focused only on the ability of copper nanoparticles to inhibit biofilm formation [36, 37, 38]. In this study we found that examined Cu/Cu_2O NPs effectively destroyed the mature biofilms formed with Gram-negative bacteria at low concentrations (1-5 MBC). However, this result has not previously been described. Such contrast is likely to be related to the morphological and physiological difference of Gram-negative and Gram-positive bacteria and various biofilm structure.

Contrary to expectations, no significant differences were found between the action of the Cu/Cu_2O NPs on biofilms formed with reference laboratory strains or MDR clinical strains. A possible explanation might be that antibacterial and antibiofilm activity of Cu/Cu_2O NPs is performed with the involvement various sets of antimicrobial mechanisms. Although the antibiofilm activity of nanoparticles was higher at relatively lower concentrations than that of antibiotics, we can assume that susceptibility to antibiotics and to nanoparticles are not correlated, just as their antimicrobial mechanisms are not comparable. Espirito Santo et al. suggest contact killing via direct contact of copper with cell membrane [39, 40]. Another possible mechanism is copper ions released from copper nanoparticles [41, 42, 43]. Consistent with the literature, this research found that Cu/Cu_2O NPs attach to the bacteria cell and could be attributed to the destruction of the bacteria biofilm via the contact mechanism. However, these results should be interpreted with caution.

This research's findings might help to develop new approaches for treating infection caused by MDR microorganisms. Further study should be undertaken to investigate the biocompatibility and safety of Cu/Cu_2O NPs-based formulations.

CONCLUSIONS

The results of this investigation show that synthesized Cu/Cu_2O NPs were effective against both Gram-negative and Gram-positive bacteria. The antibiofilm activity of Cu/Cu_2O NPs was more promising for targeting Gram-negative bacteria. Cu/Cu_2O NPs were less effective against MDR strains of planktonic bacteria without difference in biofilms. Thus, our findings support the notion that Cu/Cu_2O NPs could be used to enhance the action of existing antibiotics against Gram-negative and Gram-positive bacteria. Further research is warranted to elucidate the underlying mechanisms of Cu/Cu_2O NPs action, optimize their formulation, and evaluate their safety and efficacy in preclinical and clinical settings.

AUTHOR CONTRIBUTIONS

P.M. proposed the idea, formulated overarching research goals and aims, and conducted experiments in a bacteriological laboratory; conducted a statistical analysis of the studies using GraphPad Prism 8.0 software. Prepared pictures 2, 3, 4, 5 and 6; prepared and wrote the initial draft of the published work (including substantive translation); edited and approved the final version of the manuscript. I.D. was responsible for the management and coordination of the research activity planning and execution; developed the design and the main stages of the experiment; provided financial support for the project leading to this publication; critically revised the article. Edited and approved the final version of the manuscript. T.I. proposed a hypothesis of the effectiveness of Cu/Cu_2O NPs regarding MDR bacteria and their biofilms; conducted experiments in a bacteriological laboratory; sowed and prepared microorganisms for the bacteriological investigations; studied the sensitivity of bacteria to antibiotics; tested a biofilm creation model; prepared table 1; edited and approved the final version of the manuscript. R.P. synthesized Cu/Cu_2O NPs, prepared them in the required concentrations, and conducted a study and analysis of the physicochemical characteristics of Cu/Cu_2O NPs; prepared figure 1; wrote and edited sections Materials and Methods (subsections 2.2 and 2.3) and Results; edited and approved the final version of the manuscript. O.B. developed the design of the experiments, critically revised the article, edited and approved the final version of the manuscript. V.H. made a critical review, commentary, and revision of the manuscript; edited and expanded the literature review and discussion; conducted an optical density study; prepared graphical abstract and figures 7, 8, and 9; analyzed SEM images and OD studies; tested a biofilm creation model; provided financial support for the project leading to this publication; edited and approved the final version of the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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ARTIFICIAL INTELLIGENCE DISCLOSURE

No artificial intelligence (AI) technologies were used during manuscript writing or editing.

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