ANTIBACTERIAL GRAPHENE OXIDE AND METAL PARTICLES NANOCOMPOSITES FOR INHIBITION OF PATHOGENIC BACTERIA STRAINS

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ABSTRACT

The most effective means to protect against bacterial invasion and to reduce the risk of dangerous infections are antibacterial components synthesis. It was determined that hybrid graphene oxide and metal nanoparticles composite coatings have long-term bactericidal effect on wide spectrum of standard bacterial strains. Metal nanoparticles are known as an effective antimicrobial agent, while graphene oxide also shows high antibacterial activity. In this study the possibilities to use hybrid graphene oxide and metal nanoparticles composites for the inhibition of clinically important bacteria strains, which have developed multiple resistances to antibiotics have been investigated. These bacteria due to the acquired resistance to many classes of antibiotics, the ability to obtain resistance to all antibiotics through mutations often cause dangerous infections in medical institutions of many countries. Hybrid graphene oxide and metallic (silver, copper) nanostructures composite bactericidal efficiency was increased by using higher concentrated anisotropic shape nanoparticles colloids. Nanocomposite samples were characterized by TEM and SEM microscopy. The antibacterial activity was evaluated against standard gram-negative and gram-positive bacteria strains by solutions dilution method. The clinical antibiotics resistant *A. baumannii* strains was investigated, also. The investigations demonstrated that hybrid nanocomposite shows synergistic mechanism of action. Besides, this nanocomposite has excellent antibacterial activity against multidrug-resistant bacteria, such as *A. baumannii* bacteri strains.

1 INTRODUCTION

The continuous increasing demand and use of antibiotics by general population, in hospitals, and for veterinary purposes has led to accumulation of antibiotics in the environment. Continual exposure of the bacterial fauna to even small concentrations of antibiotics or active metabolites could lead to the development of antibiotic resistant bacteria strains [1]. The infections by these bacteria are causing cost health care systems tremendous amount of expenses, continuously increasing over the years. Thus, the development of novel antibacterial agents looks promising in this context. For instance, silver (Ag) nanoparticles are known to exhibit combinations of mechanisms [2], e.g. disruption of cell morphology, DNA condensation, inhibition of ribosome interaction, accumulation at lethal concentration in cell, protein denaturation, loss of DNA replication, depletion of adenosine triphosphate, modulation of cellular signalling, generation of reactive oxygen species, oxidative stress, etc. Copper (Cu) nanoparticles are also proved to have bactericidal effect, the mechanisms of which may involve disruption of DNA helical structure through copper ion cross-linking within and between nucleic acid strands and related mechanisms which intervene cell biochemical processes [3]. Graphene and graphene oxide (GO) were also reported to exhibit bacterial toxicity effect [4]. The suggested toxicity mechanisms include cutting off intracellular metabolic routes, oxidative stress and rupture of

cell membrane. Thus, the assembly of the mentioned nanoderivatives into nanocomposite materials was proved to be even more effective with enhanced bactericidal effect [5, 6].

In this paper, the antibacterial hybrid nanocomposite obtained by silver and copper nanoparticles precipitation on graphene oxide sheets was investigated. The morphology and antibacterial activity of the nanocomposite was evaluated.

2 EXPERIMENTAL

2.1 Materials

Highly concentrated GO dispersion in water (concentration – 5 g/L; flake size – 0.5–5 μm) was obtained from Graphene Laboratories Inc. and used as received. Other chemicals were purchased from Sigma-Aldrich. The metal nanoparticles were synthesised by simple and cost-effective chemical reduction methods. The Cu nanoparticles colloidal solution (~2.5 mg/L) was prepared using appropriate amount of copper(II) chloride dehydrate as precursor and L-ascorbic acid as redactor. High concentrated Ag nanoparticles colloidal solution (~12 mg/L) was prepared by silver nitrate reduction with polyvinylpyrrolidone (M_n=10 000). The GO–Ag–Cu nanocomposite solutions were prepared by mixing highly concentrated GO dispersion with Cu and Ag nanoparticles colloidal solutions with 1:1:1 ratio, respectively. The corresponding thin nanocomposite films were assembled on the glass cover slips using same technique and technological parameters as described in [7].

2.2 Characterization

TEM images were acquired on Tecnai G2 F20 X-TWIN (FEI) equipped with field emission electron gun. TEM accelerating voltage was 200 kV. Elemental analysis was performed using an energy dispersive X-ray (EDX) spectrometer. Samples with graphene oxide and nanoparticles were prepared for TEM by diluting colloidal solutions in ethanol and placing a drop of solution on a Lacey carbon grid and left overnight at 21 °C.

SEM micrographs were acquired using field emission scanning electron microscope Quanta 200 FEG (FEI) and e-line plus multi-application nanoengineering workstation (Raith).

2.3 Antibacterial activity

The antibacterial activity of hybrid GO–Cu–Ag nanocomposite was evaluated against gram-negative *Escherichia coli* (*E. coli*) (ATCC 25922), *Pseudomonas aeruginosa* (*P. aeruginosa*) (ATCC 27853), *Klebsiella pneumoniae* (*K. pneumoniae*) (ATCC 700603), which developed resistance to b-lactams, fluoroquinolones and aminoglycosides. The gram-positive *Staphylococcus aureus* (*S. aureus*) (ATCC 25923) and methicillin-resistant *Staphylococcus aureus* (MRSA) that show resistance to b-lactams, fluoroquinolones and macrolides were used, also. Additionally, for investigations 79 clinical *Acinetobacter baumannii* (*A. baumannii*) strains have been separated from the various clinical materials in microbiology laboratory using standard procedures.

The bacteria were cultured in Tryptone Soya Broth (TSB) solution (30 g/L). Bacteria were first cultured in a tube with 30 mL of TSB. The incubation was performed at 37 °C and oscillated at a frequency of 150 rpm for 24 h to obtain the overnight phase of the bacteria. Following that, about 1 mL amount of the bacteria were pipetted from the overnight phase into another tube with freshly prepared 30 mL of TSB, respectively. Bacteria were grown at 37 °C in the incubator shaker for another 5 h to obtain the bacteria with higher activity. The stationary phase bacterium was diluted to concentration ca. 10^3 CFU/mL with NaCl solution (0.85%) and mixed with GO dispersion and nanoparticles colloidal solution with 1:1 ratio. Afterwards, the samples were taken and placed on petri dishes surface, and incubated at 37 °C for 24 h. Bacteria survival was recorded over a 6 h period.

3 RESULTS AND DISCUSSIONS

3.1 Morphology of nanocomposite

At first, the morphology and antibacterial activity of GO was evaluated. TEM image of GO-Cu-Ag nanocomposite is shown in Fig. 1a and 1b.

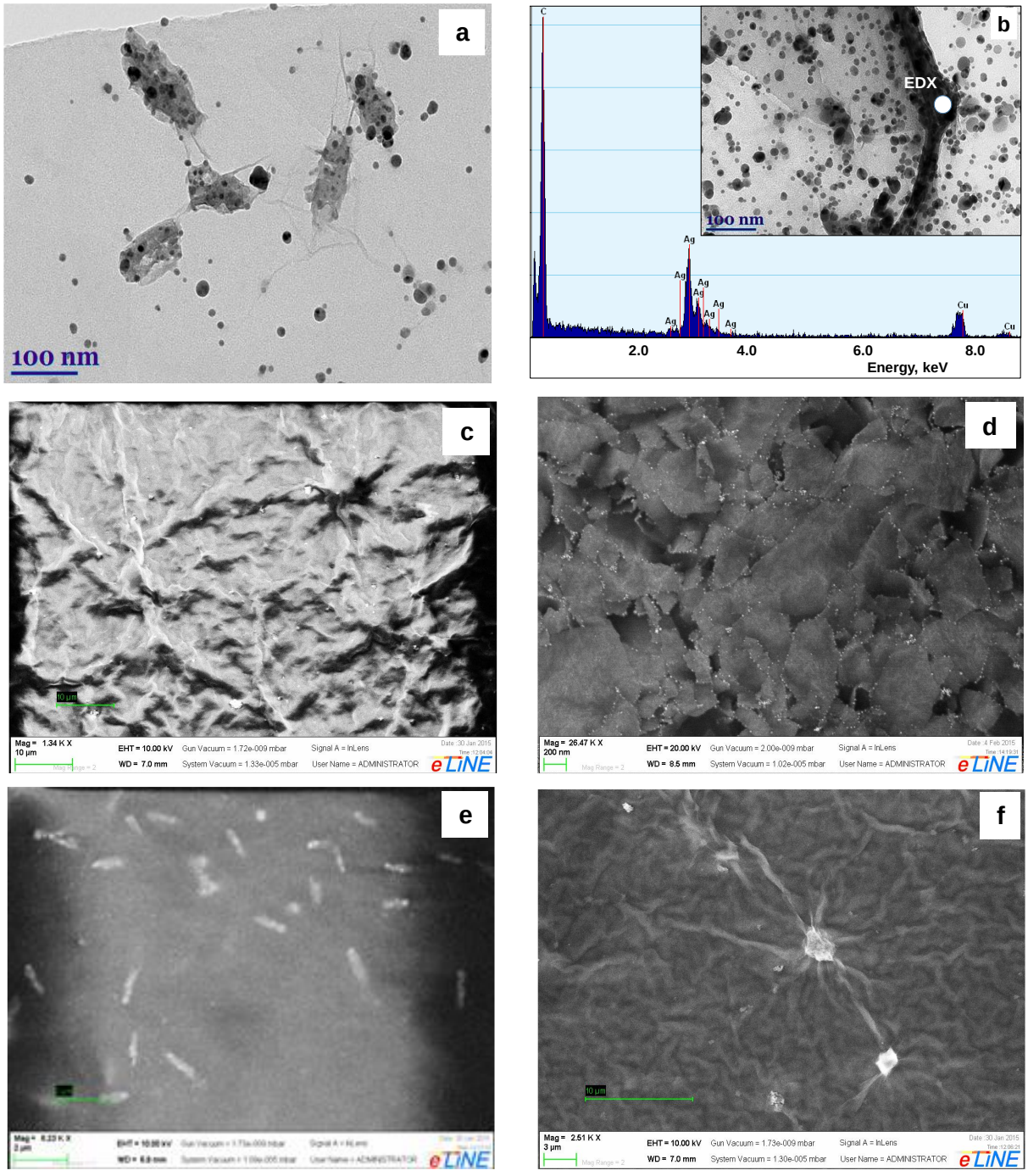

Figure 1: (a) TEM images and EDX spectrum of hybrid GO–Cu–Ag nanocomposite; (b) SEM of GO–Cu–Ag nanocomposite; (c) killed *E.coli* bacteria after interaction with GO–Cu–Ag nanocomposite; (d) bacteria microencapsulated with GO sheets.

It clearly illustrates that Ag and Cu nanoparticles are precipitated on graphene oxide sheets. The nanoparticles are well dispersed across the GO surface with relatively low density of assembled aggregates observable. In order to quantify the nanoparticle presence, EDX point analysis on the GO wrinkle with high concentration of nanoparticles was performed (Fig. 1b). The EDX spectrum confirms the presence of Ag and Cu on GO nanosheet surface. Fig. 1c shows SEM image of GO multilayer film assembled on glass coverslip surface using vertical dip-coating technique. The GO

multilayer film was found to be uniform with a dense distribution of microscopic wrinkles on its surface. It is known [7] that GO nanosheets may tend to yield wrinkled assemblies due to the solution phase properties, such as pH, and, thus, are solution phase property dependent. Ag and Cu nanoparticles colloid disrupt GO sheets and sharp edges were formed (Fig. 1d). The nanocomposite exhibits Ag and Cu nanoparticles decorated GO sheets. It is reported that sharp edges of sheets could cut through the bacterium's cell membrane causing intracellular matrix to leak, eventually leading to the GO toxicity mechanism. Fig. 1e shows killed *E. coli* bacteria strains after contact with GO-Cu-Ag nanocomposite as a bactericidal agent. In some cases, bacteria are microencapsulated with GO sheets (Fig. 1f). Similar GO behavior was observed by Zeng et al. with *E. coli* bacteria [8].

3.2 Antibacterial activity

Graphene oxide (GO) and metal nanoparticles composites show toxicity toward different bacteria strains, including antibiotics resistant strain. Fig. 2 shows survival curves of gram-negative and gram-positive bacteria strains during 6 h period with GO-Cu-Ag nanocomposite as a bactericidal agent.

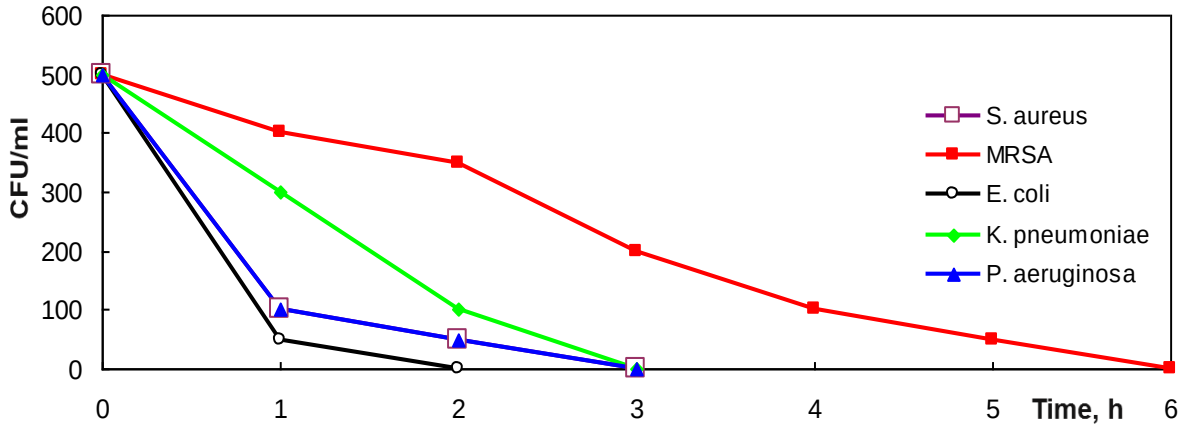

Figure 2: Survival curves of different bacteria strains during 6 h period with GO–Cu–Ag nanocomposite.

The GO–Cu–Ag nanocomposite markedly affected *S. aureus*, *E. coli*, *K. pneumoniae* and *P. aeruginosa* cellular viability during 3 h of incubation period. Though MRSA showed higher resistance, but this bacterium did not survive 6 h period. It is a good indication that synergistic effect of these nanoparticles with multiple toxicity mechanisms (due to the morphological diversity) produces more effective bactericidal effect.

Based on obtained results, the predicted bacterium inactivation mechanism via interaction with GO–Cu–Ag nanocomposite was devised [9]. The positively charged Cu and Ag nanoparticles mainly adsorb on the negatively charged GO nanosheet surface (Fig. 1a). It was demonstrated that GO nanosheets have the tendency to wrap bacteria (Fig. 1f). Thus, it is expected that Cu and Ag nanoparticles are able to interact with entire bacterium's surface. This is specifically relevant in regard to the effect that nanoparticles had on gram-negative bacteria (*E. coli, P aeruginosa, and K. pneumonia*), which have a larger surface area compared to gram-positive bacteria represented by *S. aureus* and MRSA, which are more spherical and have less surface area. Furthermore, MRSA resistance to Cu and Ag nanoparticles could be the product of a decreased cross-linking of peptidoglycan strands which leads to the exposure of more D-Ala-D-Ala residues resulting in areas of bind and trap for nanoparticles. It is predicted that the NP interactions with bacterium's cell membrane are mainly caused due to electrostatic attraction which increases the membrane permeability eventually leading to rupture and leakage of intracellular component, resulting in bacterium inactivation. It is also possible that nanoparticles interaction with cell membrane could also cause the

deterioration of the energy metabolism by interfering with the electron transport (respiratory) chains. The gram-negative bacteria were more susceptible to nanoparticles which can be explained in part by the difference in the presence of peptidoglycans present in *S. aureus*. Furthermore, relative resistance of MRSA is possibly explained by the fact that this bacterium has a much thicker cell wall which is in part responsible for their resistance to Vancomycin. Furthermore, the enfolded GO sheet fragments with sharp edges could instantly cut through the bacterium's cell membrane upon contact. This mechanism likely explains the fact that MRSA, bacteria with a thick cell wall, seems to be more resistant that the other bacteria strains tested. Finally, another potential mechanism of the GO sheets on gram-negative bacteria such as *E. coli* and *P. aeruginosa* is damaging of the flagellum, a vital organelle that confers virulence to these bacteria. This can result in bactericidal properties and immobilization of bacteria. The morphological diversity of nanoparticles and GO construct the synergistic bactericidal effect (Fig. 2), which is found to be more effective than individual nanoderivatives. Thus, it is important to consider the use of GO–Ag–Cu nanocomposite in the applications that require materials with high antibacterial effectiveness.

The influence of GO–Ag–Cu nanocomposites on the antibiotic resistant bacterium strains was evaluated as well as [10]. *A. baumannii* strains has acquired resistance mechanisms for the most of antibiotics to be tested. It was obtained that 22.8% of strains were resistant to all tested antibiotics. As can be seen from Table 1, after 1 h of incubation GO–Ag–Cu nanocomposites showed better performance on strains that were susceptible, than resistant to ceftazidime, and ciprofloxacin, doxycycline, carbapenems (imipenem and meropenem), gentamicin, tigecycline. After 2 h of incubation graphene GO–Ag–Cu nanocomposites effect on strains that were resistant and susceptible to antibiotics became similar.

Antibiotic	Susceptible to antibiotics		Resistant to antibiotics	
	1 h	2 h	1 h	2 h
Ceftazidime	100	66.7	37.0	76.7
Ciprofloxacin	100	83.3	37.0	75.3
Carbapenem	77.8	77.8	37.1	75.7
Doxycycline	57.6	51.5	30.4	93.5
Gentamicin	69.2	92.3	36.4	72.7
Amikacin	56.2	93.8	61.9	71.4
Tigecycline	63.3	80.0	34.7	73.5

Table 1: Effect of hybrid GO–Cu–Ag nanocomposite on multi-drug resistant A. *baumannii* strains.

On the other hand, some of effected *A. baumannii* strains showed recovery after 2 h of incubation.

4 CONCLUSIONS

Nanoderivatives, such as Cu and Ag nanoparticles participated on GO sheets, is markedly effective bactericidal agent against wide range of gram-negative and gram-positive bacteria strains due to the possible synergy of the multiple toxicity mechanisms. A systematic morphology analysis of the corresponding nanoderivatives was performed employing SEM and TEM and provided guideline information for addressing toxicity mechanisms.

GO–Cu–Ag nanocomposite has shown very significant effect on antibiotic resistant *Acinetobacter baumannii* strains that are susceptible to beta-lactams (ceftazidime, carbapenems) and ciprofloxacin after a short exposition, but it is needed longer incubation time to effect resistant *Acinetobacter baumannii* strains. GO–Cu–Ag nanocomposite inhibits growth of aminoglycoside (gentamicin, amikacin) and tygecycline susceptible strains as well as resistant after 2 h of exposition.

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