ANTIBACTERIAL ACTIVITY OF COMPOSITE OF GRAPHENE OXIDE WITH SILVER NANOPARTICLES

ZUZANA BYTESNIKOVA[1], ZUZANA KOUDELKOVA[1], LUKAS RICHTERA[1,2], PAVEL KOPEL[1,2], VOJTECH ADAM[1,2]

[1]Department of Chemistry and Biochemistry
Mendel University in Brno,
Zemedelska 1, 613 00 Brno,
[2]SIX Centre, Department of Microelectronics,
Brno University of Technology,
Technicka 3058/10, 616 00 Brno
CZECH REPUBLIC

zuzka.bytesnikova@gmail.cz

Abstract: Looking for strategies against the development of antibiotic resistance is a major global object of interest for the public health. This work deals with synthesis of antimicrobial composites of graphene oxide (GO) with metal nanoparticles. GO has been prepared by modified Hummers' method and characterized using scanning electron microscopy, Fourier transform infrared spectroscopy (FT-IR) and differential pulse voltammetry. Composites of GO have been synthetized with silve nanoparticles, which have been characterized. Potential antimicrobial activity of the nanocomposites was tested against *Escherichia coli*, *Staphylococcus aureus* and methicilin resistant *Staphylococcus aureus* (MRSA).

Key Words: graphene oxide, nanocomposite antimicrobial, *Staphylococcus aureus, Escherichia coli*

INTRODUCTION

Pathogens, whose resistance profiles present a new challenge for containing their spread and their impact on human health, are appearing among drug-resistant bacteria (Webb et al. 2005). For example, methicillin-resistant *Staphylococcus aureus* (MRSA) is worldwide a major nosocomial pathogen (Wenzel et al. 1991, Cunha 1998, Witte 1999). The occurrence of bacterial resistance phenotypes has been linked to the clinical use of antimicrobial agents to which the bacteria express resistance (Rice 1999). In spite of antimicrobial therapy, morbidity and mortality associated with these bacterial infections remain high partially as a result of the ability of these organisms to develop resistance to practically all antibiotics. Therefore, new strategies are needed to identify and develop the new generation of drugs or agents to control bacterial infections. Hence, the key for prevention of staphylococcal and related bacterial infections are good standards of hygiene, avoidance of skin trauma, and the use of antibacterial ointments or surface-coating agents with potential antibacterial properties. (Jones et al. 2008). Recent advances in the field of nanotechnology, especially the ability to prepare highly ordered nanoparticulates of any size and shape, have led to the development of new biocidal agents. Several studies have shown that nanoparticles formulations can be used as effective bactericidal agents (Tiller et al. 2001, Lin et al. 2002, Stoimenov et al. 2002, Kuhn et al. 2003, Sawai 2003, Sondi and Salopek-Sondi 2004, Lewis and Klibanov 2005, Rosi and Mirkin 2005, Ma et al. 2006). Nanotechnology provides a good platform for development and modification of nanoparticles based on metal, with promising applications in diagnostics, cell labelling, biomarkers, contrast agents for biological imaging, drug delivery systems, antimicrobial agents, and drugs based on nanotechnology for treatment of various diseases (Marcato and Duran 2008, Singh and Nalwa 2011). Therefore, researchers are focusing on nanoparticles in general and silver nanoparticles in particular to solve the problem with emergence of multi-drug resistant bacteria (Gemmell et al. 2006).

It has been presumed that graphene exhibits antibacterial efficiency against pathogenic bacteria strains via lipid peroxidation. The recent studies have shown that antibacterial effect of graphene is caused by inducing oxidative stress and membrane damage (Krishnamoorthy et al. 2012).

The modifications of graphene with metal nanoparticles render this material more effective against pathogenic bacteria.

MATERIAL AND METHODS

Preparation of graphene oxide

The graphene oxide (GO) was prepared by chemical oxidation of 5 g graphite flakes (Sigma-Aldrich, and 100 mesh, ≥75% min) in a mixture of concentrated H_2SO_4 (670 ml, Sigma-Aldrich) and 30 g $KMnO_4$ (Sigma-Aldrich) according to the simplified Hummer's method (Hummers and Offeman 1958). The reaction mixture was stirred vigorously. After 4 days, the oxidation of graphite was terminated by addition of H_2O_2 solution (250 ml, 30 wt% in H_2O, Sigma-Aldrich). Formed graphene oxide was washed 3 times with 1 M HCl (37 wt% in H_2O, Sigma-Aldrich) and several times with Milli-Q water (total volume used 10 l) until constant pH value (3–4) was achieved.

Synthesis of composite of graphene oxide with silver nanoparticles (AgNPs)

A solution of silver nitrate (50 ml, $AgNO_3$, 1 mM, 2 mM, 4 mM, 8 mM resp., Sigma-Aldrich) was added dropwise to the GO solution (1 ml, 5 mg/ml) under vigorous stirring. After that sodium borohydride (40 mg $NaBH_4$, Sigma-Aldrich) was added slowly to the reaction mixture and the resulting mixture was stirred intensively for 24 h at room temperature to allow reduction. The prepared composite was washed with Milli-Q water several times.

Differential Pulse Voltammetry

The electrochemical determination of silver by differential pulse voltammetry was performed using a CH Instruments Electrochemical Workstation (CH Instrument Inc., Austin, TX, USA), using glass measuring cell with three electrodes. The glassy carbon electrode was the working electrode, an Ag/AgCl/3 M KCl was the reference and a platinum wire was the auxiliary one. The parameters of this method were as follows: initial potential −0.2 V, end potential 0.5 V, modulation amplitude 0.05 V, step potential 1 mV. 0.2 M acetate buffer (pH 5) was used as a supporting electrolyte. The volume of injected sample was 20 µl; the volume of electrolyte was 1980 µl. For results evaluation, the software CHI 440A (CH Instrument Inc., Austin, TX, USA) was used.

Fourier transform infrared spectroscopy

Fourier transform infrared spectroscopy (FT-IR) spectra were collected using a Nicolet iS10 FT-IR spectrometer with a diamond ATR attachment (Thermo Electron Inc., San Jose, USA). IR spectra were recorded from 4000 to 650 cm^{-1} at a resolution of 4 cm^{-1}. Each spectrum was acquired by adding together 32 interferograms. Spectra were taken at 22 °C. The OMNIC™ software was used for IR spectra recording, and the JDXview v0.2 software was used for further spectra evaluation.

Scanning Electron Microscopy

The structures of the GO with AgNPs composites were characterized by scanning electron microscopy (SEM). For documentation of the nanoparticles structure, a MIRA3 LMU (Tescan, Brno, Czech Republic) was used. This model is equipped with a high brightness Schottky field emitter for low noise imaging at fast scanning rates. The SEM was fitted with In-Beam SE detector. For automated acquisition of selected areas a TESCAN proprietary software tool called Image Snapper (Tescan, Brno, Czech Republic) was used. The software enabled automatic acquisition of selected areas with defined resolution. An accelerating voltage of 15 kV gave satisfactory results regarding maximum throughput.

Cultivation of bacteria strains

Staphylococcus aureus (NCTC 8511), *Escherichia coli* (NCTC 13216) and MRSA (ST239) were obtained from the Czech Collection of Microorganisms, Faculty of Science, Masaryk University (Brno, Czech Republic). Cultivation media (Mueller Hinton) (Oxoid) were inoculated with bacterial culture and were cultivated for 24 h on a shaker at 600 rpm and 37 °C.

Colony-Forming Capability Test

Bacterial cultures (*E. coli*, *S. aureus*, MRSA) were diluted with MH medium to an absorbance of 0.1 measured using a spectrophotometer Ultrospec 10 (Biochrom, Cambridge, United Kingdom) at a wavelength of 600 nm. After that cultures were diluted by decimal dilution (to 10^{-7} cells per millilitre) and incubated at 37 °C for 2 h. After being exposed to different concentrations of composites of GO with AgNPs at 37 °C for 2 h. 100 µl of the cell suspension was spread onto MH agar plates. The number of the colonies was counted after agar plates were incubated at 37 °C in the dark overnight. The survival percentage was used to evaluate the antimicrobial effect of composites of GO with AgNPs and it was defined as the following formula:

$$\text{Survival\%} = \frac{\text{Colony numbers of treated bacteria}}{\text{Colony numbers of control bacteria}} \times 100\%$$

RESULTS AND DISCUSSION

Characterization of composites

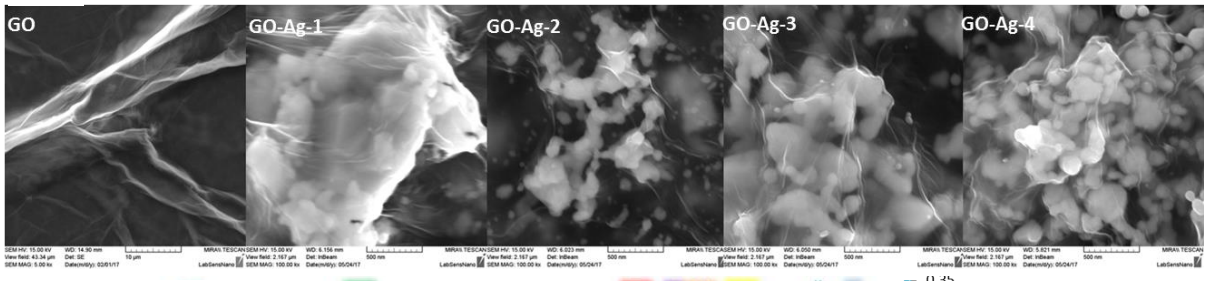
Silver was detected as Ag(I) by glassy carbon electrode, and the electrochemical signal of Ag(I) was observed at the potential +0.18 V. Based on the electrochemical determination, it can be confirmed that the silver content was significant. The silver content in individual samples is shown in Table 1.

Table 1 Determination of silver content using Differential Pulse Voltammetry

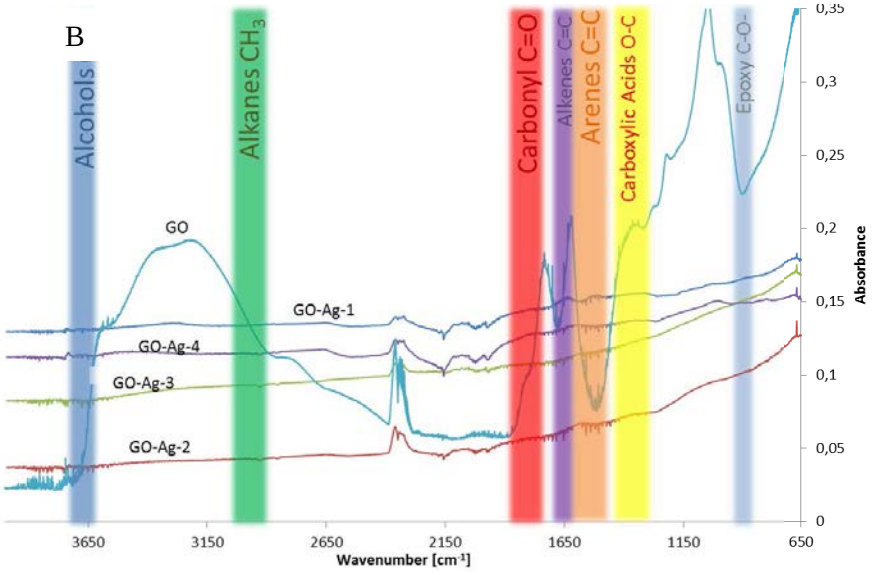
Name of sample	Concentration of Ag in sample [mg/ml]
GO-Ag-1	1.36
GO-Ag-2	1.46
GO-Ag-3	2.74
GO-Ag-4	4.08

Figure 1 Characterization of GO and composites of GO-AgNPs by using A) SEM and B) FT-IR

A



B



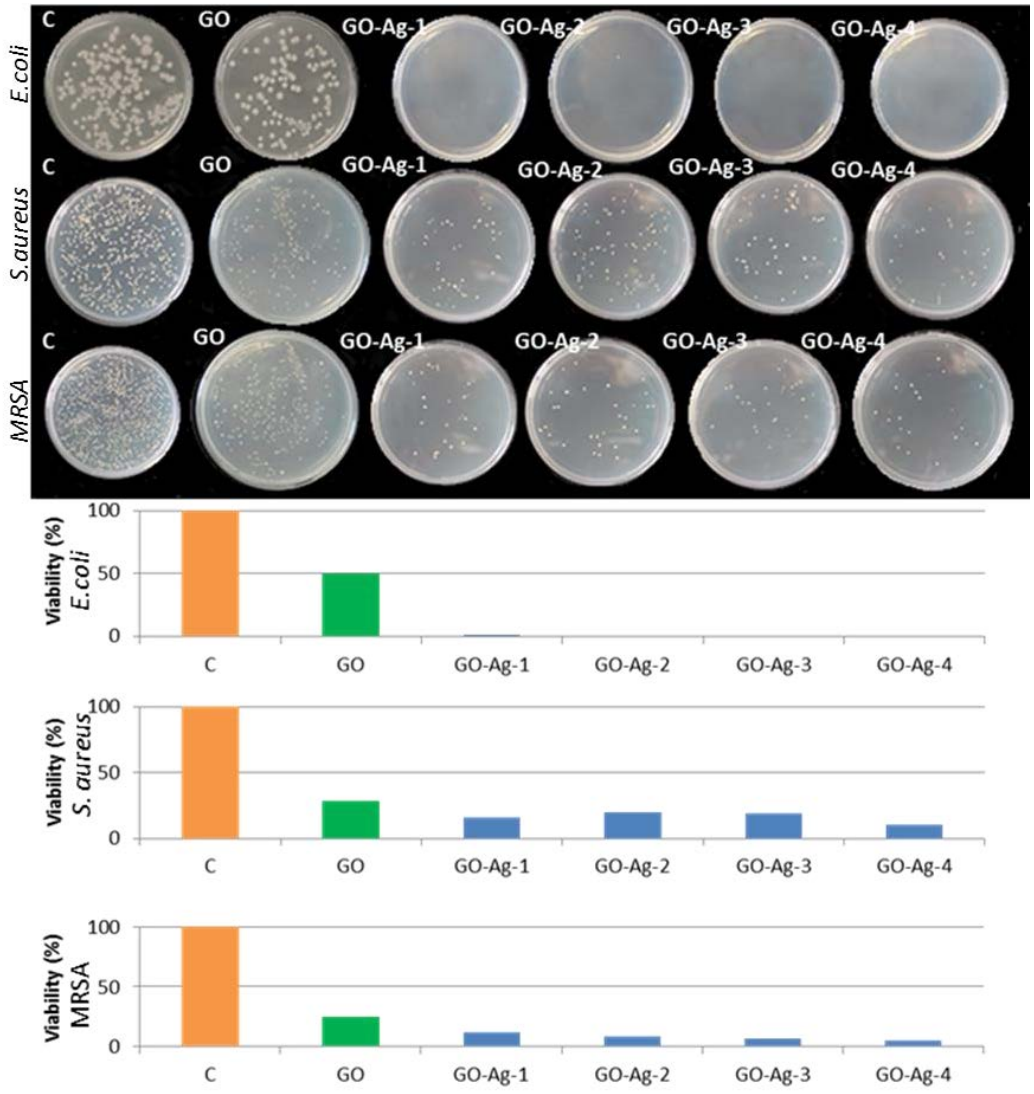
The SEM micrographs (Figure 1A) confirmed the preservation of the original structure of the large area, which remained maintained in comparison with starting material. This method also enabled determining the degree of exfoliation, which is crucial for nanoparticle character. The SEM micrographs also allowed the rating of AgNPs adhesion to GO-based carrier.

The efficiency of GO modifications with AgNPs loading was investigated by FT-IR (Figure 1B). The addition of Ag NPs to the GO resulted in an increase in C=C moiety signals in the 1500–1650 cm^{-1} region. Another increase has occurred in C=O signals in the 1700–1800 cm^{-1} region, O−H signals in the 3580−3650 cm^{-1} region, and O–C signals in the 1320 cm^{-1} region. Increase in overall intensity around 1500 cm^{-1} can be connected with the oxidation. The degree of drying may affect the occurrence and number of O−H groups. Hydrogen bonds in the 3200–3500 cm^{-1} region may be affected by degree of drying. The FT-IR analysis showed the presence of hydroxyl, carbonyl and epoxy groups on the GO sample surface.

The composites influence on pathogenic microorganisms

Antibacterial activity of GO and GO with AgNPs was determined using colony-forming capability test and expressed in terms of the colonies after incubation. GO and composites of GO with AgNPs were tested based on their antimicrobial effects on *S. aureus*, MRSA and *E. coli* strains. Effect of GO and composites of GO with AgNPs on bacterial strains is shown in Figure 2. The highest inhibitory effect after 24 hours of incubation can be seen after the addition of all composites on *E. coli* (Figure 2). All composites of GO with AgNPs had stronger antimicrobial effect against all used bacterial strains than GO itself.

Figure 2 Colony-Forming Capability Test



Legend: C – control, GO – graphene oxide, GO-Ag-1, GO-Ag-2, GO-Ag-3, and GO-Ag-4 are nanocomposites with different concentration of silver nanoparticles

CONCLUSION

In this study, the composites of graphene oxide with silver nanoparticles were synthetized and characterized by different methods and their effect on bacterial strains was tested. The composite showed inhibitory effect on three selected bacterial strains (*S. aureus; E. coli*; MRSA) and the inhibitory effect were stronger than graphene oxide itself. It can be said that combination of GO with metal nanoparticles seems like a promising way to fight against drug-resistant bacteria.

ACKNOWLEDGEMENTS

Research described in this paper was financed by Czech Ministry of Education, Youth and Sports of the Czech Republic in frame of National Sustainability Program under grant LO1401 and by the Internal Grant Agency of Mendel University in Brno (IP 20/2017). For research, infrastructure of the SIX Center was used.

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