

Modern techniques of increase the antibacterial properties of the instruments

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Complication caused by resistant bacteria require qualified technical and technological measures since bacteria proliferation can occur in operations where aseptically environment is required. Constituents used as decontaminating compositions can be either bactericidal inorganic or organic substances like chemotherapeutics and antibiotics. In research special attention is paid to the problem of bacterial resistance toward antibiotics. A possible solution to this issue consists in the employment of ions and metal complexes such as silver nitrate or new nanoparticles (silver, copper), whose antibacterial properties are known over centuries. In the focus of this research is the preparation of a composite material, consisting of graphene oxide and nanoparticles of zinc oxide (ZnO), silver phosphite (Ag_3PO_4) and silver. Graphene oxide was prepared by Hummers method, involving the oxidation of graphite flake in sulfuric acid with permanganate with subsequent addition of the necessary components for the formation of metal nanoparticles. Physicochemical methods are considered for their characterisation. The size of nanoparticles ranges from 50 – 200 nm. Subsequently, the antibacterial effect of composites was tested by disk method on bacterial cultures of *S. aureus*, *E. coli*, methicillin-resistant *S. aureus* (MRSA). Selenium nanoparticles exhibit the highest antibacterial activity from selected nanoparticles containing graphene oxide composite with an inhibitory zone with the size 5 ± 1 mm. Silver nanoparticles display also a distinguishable antibacterial effect with inhibition zones of 2 ± 1 mm. Graphene oxide which is modified by zinc oxide nanoparticles shows no inhibitory effect. The obtained results show suitability of the prepared composite materials as candidates for alternative antimicrobial materials.

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1. Introduction

Nowadays food and textile industries as well as health centers have employed advanced technologies and precaution measures which prevent bacteria proliferation and distribution among staff, consumers and patients.¹⁻³ These technologies and measures aren't always effective to fulfill the aseptic requirements. One of the main objectives to aseptic conditions is related to the necessity for the treatment of surfaces, tools and working materials by decontaminants. The application of chemotherapeutics or antibiotics isn't always a good choice since

they may be in direct contact with the product, or may migrate and cause the transfer of contaminants into the product³. Some organic compounds used for disinfection purposes pose some disadvantages, including their toxicity for the human body⁴. Therefore there is an increasing interest in using of inorganic disinfectants, such as metal nanoparticles (NPs)⁵. Furthermore, a crucial factor related to the use of chemotherapeutics and antibiotics is the enhancement of resistant bacteria toward these decontaminant agents⁶. Therefore it's necessary to develop new compounds which would

show bactericide properties also toward bacterial species immuned with special resistance or multiresistance to antibiotics^{6,7}. Bacteria with enhanced resistance are often found in hospital operations as a consequence of long-term antibiotics administration to patients, leading to genetic mutations and formation of various defense systems against damaging mechanism of antibiotics⁸. The most resistant form which evolved from the bacteria *S. aureus* is methicillin-resistant *S. aureus* (MRSA). Enhanced resistance to antibiotics or other bactericidal agents in staphylococ is present due to the variable genetic element such as pathogenicity islands genome, plasmids, transposons and others. The responsible gene for resistance enhancement in MRSA if found in the staphylococcal chromosomal cassette mec (SCCmec)⁹.

The integration of SCCmec into the genome of *S. aureus* leads to methicillin-resistant strain (MRSA) which is resistant to nearly all β -lactam antibiotics. The resistance toward methicillin is dedicated to the presence of penicillin-binding protein 2a (PBP2a) and the β -lactamase enzyme that cleaves β -lactam antibiotics (penicillin-based antibiotics), and is encoded by the *mecA* gene¹⁰. The PBP2a is an analogue of PBP transpeptidases whose presence is essential for normal cell, as they play an important role in cell-wall synthesis¹¹. The presence of β -lactam antibiotics inhibit their function, and the cell loses its ability to produce cell wall and consequently dies. However PBP2a exhibits a low affinity toward β -lactam antibiotics, therefore is not subject to their inhibition⁹. All of this lead to cost uneffective and time consuming therapy for hospitalized patients with MRSA¹². The treatment of surfaces, tools, fabrics or packages with antibacterial agents in the above mentioned areas is an absolute necessity, and it is therefore it is necessary to ensure an effective treating agent with pronounced antibacterial effect. Such a property is prone of metal nanoparticles¹³, graphene oxide (GO) or biofunctionalized graphene¹⁴. Graphene with antimicrobial effect is used in containers for food packaging or for the coating of biomedical devices, wherein the bacterial colonization on the surface are unwanted¹⁵. Modification of

graphene metal nanoparticles with antibacterial effect streamlines this effect.

2. Material and Methods

2.1 Preparation of graphene oxide and metal nanoparticles

Preparation of graphene oxide (GO)

Graphene oxide was prepared by Hummers method using potassium permanganate. This occurred by transferring 46 ml of concentrated H_2SO_4 into an ice bathed beaker under continuous stirring of 2 g of graphite, 1 g $NaNO_3$ and 6 g $KMnO_4$. After it, the beaker was removed from the ice bath and placed at room temperature. Later on, 92 ml of water were added into the resulting solution, followed by 15 minutes of still standing, and the addition of 280 ml of warm water. Finally a 3% H_2O_2 was transferred into the prepared solution until until it exhibits a bright yellow color.

Preparation of zinc oxide nanoparticles (ZnO)

Zinc acetate dihydrate (assay $\geq 98\%$) was used as the raw material to synthesize ZnO nanostructures. According to this method 1 g of zinc acetate dihydrate was placed in an oven and then it was heated up for 2 h at 300 °C. The heating rate seemed to be a very important parameter in the amount of the product and the undesired phases which might appear during the heating. So the heating rate was set to 10 °C/min and the samples were annealed at 300 °C for 2 h, and then cooled to room temperature for 12 h. It was observed that two different kinds of products were obtained after sample annealing. A white porous powder covering the crucible walls was denoted as sample No. 1 a gray black powder sticking to the bottom of the crucible denoted as sample No. 2. After microwaving and naturally cooling down, the samples were transferred in eppendorf tubes and washed with ethanol. After that the precipitate at the bottom of the eppendorf tube was washed 3 times with sufficient amounts of MilliQ water. The obtained sample was kept at ambient conditions 12 hours drying, yielding thereafter a total amount of 72 mg.

Preparation of silver nanoparticles (SPNPs)

1 ml of AgNO_3 (850 mg of AgNO_3 in 10 ml of MiliQ) was mixed with 25 ml of MiliQ and separately 1 ml of Na_2HPO_4 (850 mg of Na_2HPO_4 in 10 ml of MiliQ) was mixed with 25 ml of MiliQ. Immediately after preparation, these two solutions were mixed together and mixed a few minutes on magnetic stirrer until its color turned to light yellow. Then, 500 mg of cekol was added into this complex under continuous mixing of 2 hours.

Preparation of selenium nanoparticles (SeNPs)

5 ml of Na_2SeO_3 (Na_2SeO_3 263 mg/ 50 ml of MiliQ) was dissolved in 45 ml water followed by adding 80 μl of 3-mercaptopropionic acid (MPA). Its pH was adjusted to 9 by the addition of 1 M NaOH (0.2 ml). The reaction mixture was stirred for 2 hours yielding to a red color. Then these two solutions were mixed under magnetic stirring for 30 minutes. The color of solution was black and its pH was 6. Finally, 1 g of cekol was added into the solution under continuous mixing for two hours⁶.

2.2 Cultivation of *S. aureus*, methicillin-resistant *S. aureus* and *E. coli*

S. aureus (NCTC 8511), methicillin-resistant *S. aureus* (7111 2/A8) and *E. coli* were obtained from the Czech Collection of Microorganisms, Faculty of Science, Masaryk University, Brno, Czech Republic. Cultivation media (LB = Luria Bertani) were inoculated with bacterial culture for 24 hours on a shaker at 130 rpm and 37°C. The bacterial culture was diluted by cultivation medium to $\text{OD}_{600} = 0.1$ for the following experiments.

2.3 Determination of antimicrobial properties

Inhibition zones and growth curves

Agar surface in a Petri dish was covered with a mixture of 100 ml of 24 hour grown culture of *S. aureus* or *E. coli* and 3 ml of LB medium (Luria Bertani medium). The circles with a diameter of 1 cm were delivered by the factory (Vyzkumny ustav pletarsky in Brno) and mixed

with SPNPs with a concentration of 300 μM in complexes with hyaluronic acid (8.3 mM) or chitosan (9.7 mM). Petri dishes were incubated in a thermostat at 37 °C for 24 hours. The antimicrobial effect of tested compounds was measured by measuring the absorbance using the apparatus Multiskan EX (Thermo Fisher Scientific, Germany). The culture was diluted with LB medium to obtain absorbance 0.1 AU at 600 nm. In the microtitration plate *S. aureus* or *E. coli* culture was mixed with various concentrations of SPNPs (0, 10, 25, 50, 75, 150, 225 and 300 μM) and constant concentration of hyaluronic acid (8.3 mM) and chitosan (9.7 mM). Total volume in the microtitration plate wells was always 300 μl .

3. Results and Discussion

3.1 Characterization of particles

Average size of nanoparticle and distribution curves were determined by a dynamic light scattering with a device Zetasizer Nano (Malvern) (Fig. 1). The average size of the nanoparticles was GO 200 nm, ZnO 50 nm, SPNPs 100 nm and SeNPs 15 nm. The composition of the nanoparticles was also demonstrated by using X-ray fluorescence spectrometry (XRF) instrument Spectro XEPOS. In the spektra were found peaks corresponding to lines of zinc, silver, phosphorus and selenium.

For using graphene oxide modified by nanoparticles of zinc oxide to creating to inhibition zones around the diffusion dishes in any of the tested cultres. A similiar study Leung (2012) dealt with the GO modified by ZnO nanoparticles, when the researchers demonstrated the antibacterial effect of this composite applications to surface materials. Diferent results are caused by the different preparation, size and surface finish of GO and ZnO nanoparticles¹⁶. Graphene oxid is modified with silver nanoparticles and it was used as the second potential antibacterial components. For these particles were give 2 mm of inhibition zone during incubation with *E. coli*, *S. aureus* was inhibited by

formation of 3 mm inhibition zone and MRSA was inhibited by formation only 1 mm inhibition zone. Method of measuring the inhibition zones is useful as a primary test the inhibitory effect of substances¹⁷. In the third composite with GO were chosen selenium nanoparticles (SeNPs). Selenium nanoparticles are new alternative growth inhibition of bacterial cultures, the effect of which dealt with the study⁶, when were compared the effect SeNPs and SPNPs. For SPNPs were measured inhibition zones of 3 mm, while SeNPs inhibition zone of size 7 mm. Graphene oxide modified by SeNPs worked on bacterial cultures in the most efficient inhibition effect of the test substances. For bacterial *E. coli* culture was made 4 mm inhibition zone from the diffusion disk with inhibitory substance. In *S. aureus* was measured 6 mm inhibition zone and in MRSA was measured 5 mm zone of inhibition. Graphene oxid was modified with selenium nanoparticles and it can be classified as an effective inhibitory components on G⁺, G⁻ and resistant bacteria.

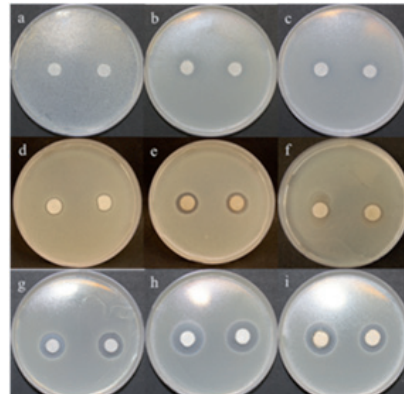


Figure 2. Testing the antibacterial action by method of inhibition zones. While we tested GO modified by ZnO avoid to the formation of inhibition zones around any of the test bacterial cultures *E. coli* (a), *S. aureus* (b) a MRSA (c). In GO modified by SPNPs have been created 2 mm inhibition zone in *E. coli* (d), 3 mm in *S. aureus* (e) a 1 mm in MRSA (f). In modification GO by SeNPs particles leads to the formation of 4 mm inhibition in *E. coli* (g), 6 mm in *S. aureus* (h) and 5 mm in MRSA (i).

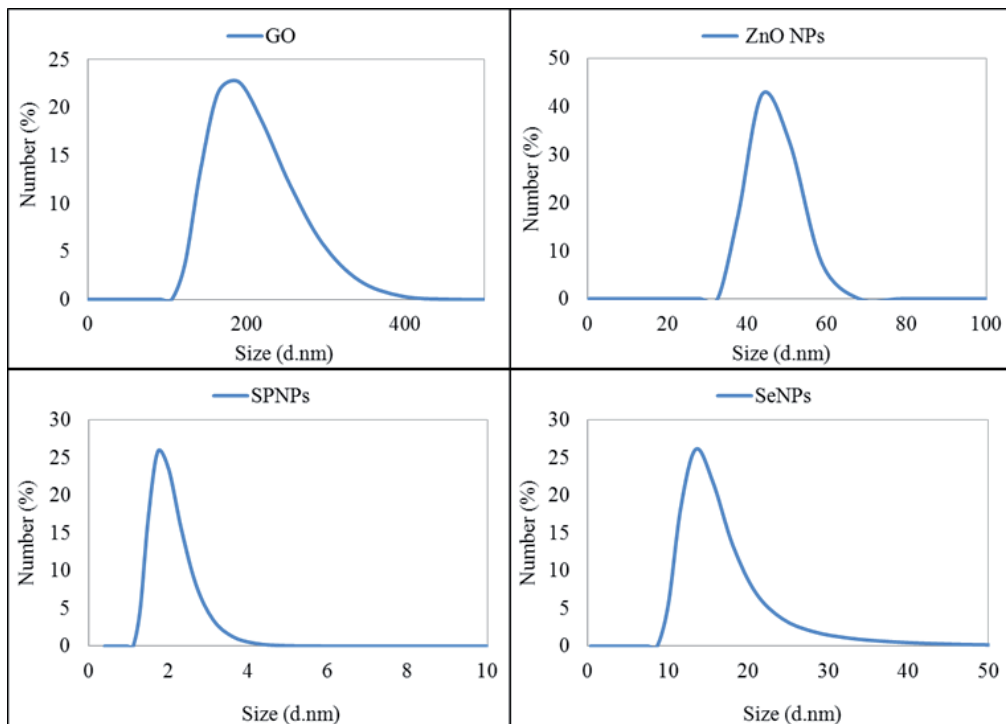


Figure 1. Results of measurement of particle size by using dynamic light scattering.

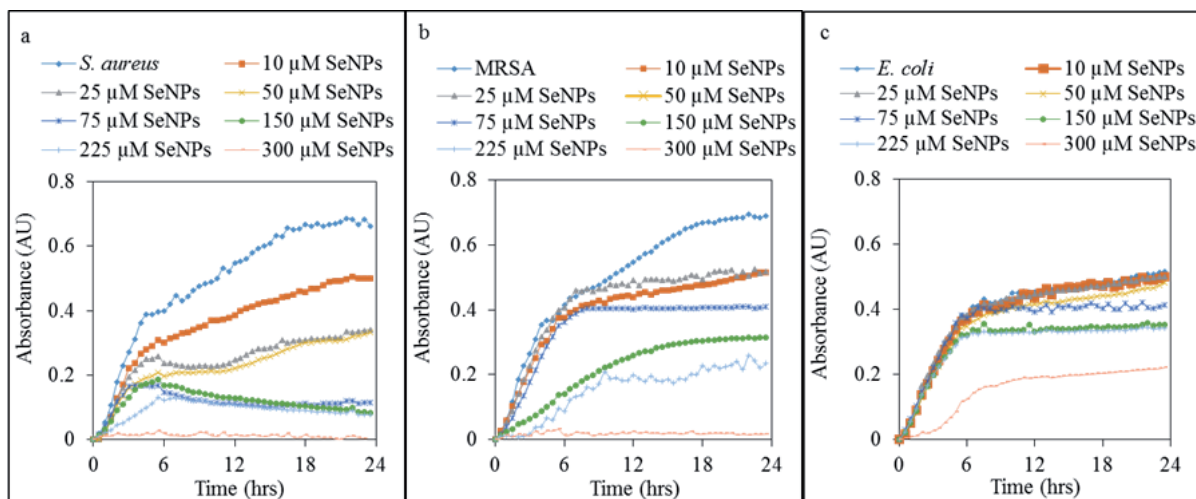


Figure 3. Growth curves of bacterial cultures after application graphene oxide with different concentrations of selenium nanoparticles. Using concentration of graphene oxide was stationary for all different concentrations of selenium nanoparticles. *S. aureus* (a) and *MRSA* (b) growth in a similar downward trend with increasing concentrations of selenium nanoparticles in complex with graphene oxide (1 mg/ml). For *E. coli* was not too significant downward trend (c).

An inhibition effect was different for diversity of the selected bacteria. Gram negative *E. coli* has a stronger cell wall due to the presence of the outer membrane resulting in a poor transport of nanoparticles inside cells¹⁸. High inhibition effect on *S. aureus* is due to absence of lipopolysaccharide and outer membrane layer in cell wall¹⁹. The mechanism causing resistance to β -lactam antibiotics fails to protect the resistant bacteria to antibiotics against complex of graphene oxide and selenium nanoparticles²⁰.

From the above results we can conclude that the GO modified by SeNPs act by inhibitory effect in all the cultures, while the GO modified by ZnO doesn't provide an inhibitory effect on any of these cultures. A similar Leung study (2012) dealt with the GO modified by ZnO nanoparticles, when researchers proved antibacterial effect of this composite applications on surface materials. Different results may be caused by different prepare GO and ZnO nanoparticles¹⁶.

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The authors declare they have no potential conflicts of interests concerning drugs, products, services or another research outputs in this study.

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