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Comparative in vitro study of single and four layer graphene oxide nanoflakes — Cytotoxicity and cellular uptake

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ABSTRACT

In recent years, graphene and its derivatives have been extensively investigated because of their unique properties, which can be used in many fields including biomedical applications. Therefore, detailed biological study is required. In the current paper the detailed toxicological studies on single and four layer graphene oxide (GO) nanoflakes is presented. The morphology and size of the nanomaterials were characterized via atomic force microscopy. Cytotoxicity, proliferation and internalization study were performed using various methods, including optical, confocal and Raman microscopy imaging, flow cytometry analysis, colorimetric and luminescent cell assays. Our first findings undeniably show that the nanomaterials' functionalization has a considerable impact on their behavior in a biological environment. The cytotoxicity assay confirmed comparable, dose dependent cytotoxicity of single and four layers GO flakes. The differences between these two nanomaterials became more distinct during cell proliferation study and ROS detection. Namely, markedly stronger inhibition of cell proliferation and higher ROS generation by one-layer GO-PEG than four-layer GO-PEG were observed. Cell imaging revealed efficient internalization of the both GO nanoflakes in a time dependent manner. These findings emphasize the role of number of layer and functionalization in GO toxicological characteristics and may provide helpful information for their further biomedical applications.

1. Introduction

Graphene is a part of a bigger class that has been named as graphene family nanomaterials (GFNs) that consist of carbon structures with different chemico-physical properties and different number of layers. GFNs possess extraordinary electronic, optical, thermal and mechanical properties, which can be used in many fields (Sanchez et al., 2012). Moreover, high specific area and facile functionalization make them promising materials for biomedical applications, ranging from drug/gene delivery, biological sensing and imaging, antibacterial materials, scaffold for cells' culture and tissue engineering (Shen et al., 2012; Chung et al., 2013).

The biocompatibility of graphene-based nanomaterials is not only dose and time dependent but also may be connected with physical parameters, number of layers, size, shape, surface functionalization and synthesis (Chatterjee et al., 2015). Therefore, new materials in biomedical applications require detailed studies. Many authors focused on graphene oxide (GO) that demonstrates considerable advantages over other members of GFNs (Chatterjee et al., 2015). In the current paper the biological characteristics of single and four layer GO nanoflakes of similar length is presented. The number of GFNs layers is an important parameter relevant for their biological effects, as it determines specific surface area, bending stiffness and adsorptive capacity. Namely, specific area is inversely proportional to number of layers, thus adsorptive capacity for biological molecules increase significantly as layer number decreases. In contrary, bending stiffness increases with the number of layers (Sanchez et al., 2012; Chatterjee et al., 2015).

Prevention of aggregation is an important factor for graphene sheets because most of their unique properties are only connected with the single layer. Aggregation can be reduced by the attachment of surfactant or by controlled chemical conversion of GO colloids (Li et al., 2008; Wang et al., 2013). Many researchers confirmed that PEGylation (functionalization with polyethylene glycol) made nanomaterials much more dispersible and stable in different solutions, even rich in salts or proteins, such as cell media and serum (Wang et al., 2013; Sun et al., 2008). Moreover functionalization can profoundly change their cytotoxicity by attenuating the hydrophobic interactions between

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graphene or GO with cells and tissues (Zhang et al., 2012). Therefore, in the first step the biocompatibility of the GO nanoflakes in pristine form and after PEGylation was investigated in the present study. Next, more detailed research on less cytotoxic PEGylated nanostructures was continued. Biocompatibility and internalization study were performed using various methods, including optical and confocal microscopy imaging, flow cytometry analysis, colorimetric and luminescent cell assays.

2. Materials and methods

2.1. Nanomaterials synthesis and functionalization

The graphene oxides (GO) synthesis routes were reported previously (Marcano et al., 2010; Wojtoniszak and Mijowska, 2012). The 4-layer GO synthesis proceeded as follows, 1 g of graphite was dispersed in a mixture of perchloric and nitric acids (350 mL, 4:3—volume ratio), and next potassium chromate (6 g) was added. The mixture was then heated to 50 °C for 24 h. The obtained mixture was centrifuged and washed three times with ethanol (200 mL) and 10% hydrochloric acid (200 mL) to remove residual metal ions, and finally with distilled water until pH of the solution reached 7. Finally, the material was dried in air at 60 °C for 24 h. Graphite was purchased from Alfa Aesar (synthetic, 99.9995%, 325 mesh). Potassium chromate was bought from POCH (Poland). Perchloric acid, nitric acid, hydrochloric acid, potassium chromate and ethanol were obtained from Chempur (Poland).

The 1-layer GO synthesis was performed as described below, i.e. prior heating a mixture of graphite, perchloric acid, nitric acid, and K_2CrO_4, an ultrasonication process was performed for 6 h at room temperature. The time and temperature of oxidation process were increased to 48 h and 100 °C, respectively. The obtained graphene oxide samples were PEGylated with 6-arm polyethylene glycol-amine (Sunbio Inc., Korea). Graphene oxide sheets were added to the solution of 6-arm polyethylene glycol-amine (2 mg/mL), and the mixture was sonicated for 5 min. Further *N*-(3-dimethylaminopropyl-*N*′-ethylcarbodiimide) hydrochloride (EDC, Sigma-Aldrich, Germany) was added in to produce a concentration of 4 mmol/L, and allowed to react overnight. The final product was obtained by ultracentrifugation at 45,000 rpm for 1 h and dried under vacuum. Graphene oxide was vortexed and sonicated (not longer than 1 min) every time before cell culture studies.

Functionalization of the PEGylated GO nanoflakes with the fluorescence dye - Alexa Fluor 647 Succinimidyl Ester (Thermo Fisher, USA), was carried out by dispersion PEG-GO in 5 mL of dye solution (w/v Alexa Fluor in DMF 1:10) and gentle stirring for 24 h (in the dark). Next, the suspension was centrifuged and washed with fresh DMF. Finally, the precipitate was washed with water as long as all free dye was removed and dried under vacuum at room temperature. As prepared PEGylated and Alexa Fluor 647-functionalized GO was dispersed in PBS solution in order to obtain a concentration of 1 mg/mL, and afterwards sonicated for 12 h (100 W, 20 kHz) under the constant cooling to produce homogeneous dispersion using Vibra-Cell Ultrasonic Processor.

2.2. Nanomaterial characterization

The morphology and size of the nanomaterials were characterized via atomic force microscopy AFM (Nanoscope V MultiMode 8, Bruker, Germany). AFM images were obtained from the individual flakes deposited on SiO_2/Si wafer. Raman measurements were performed in In-Via Raman microscope (Renishaw, UK) with excitation laser wavelength of 785 nm. For the in vitro Raman measurements, cells incubated with the nanomaterials (according to the procedure described in the Cellular uptake, confocal microscope imaging section) were fixed in the glass plates. The recorded maps show the intensity of G band, that arises from stretching of the C—C bond in graphitic materials (from 1581 to 1600 cm^{-1}).

2.3. Cell culture

Human Caucasian breast adenocarcinoma cells (MCF7, catalogue no. 86012803) was purchased from European Collection of Authenticated Cell Cultures (ECACC). During experiments the cell line authentication was confirmed (Human Cell Line Authentication, Multiplexion, Germany). Cells were cultured in a humidified incubator (5% CO_2, 37 °C) in culture medium (Dulbecco's Modified Eagle Medium, High Glucose, Sigma-Aldrich, Germany) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma, Germany), L-glutamine (2 mM, Sigma-Aldrich, Germany) and 0.4% penicillin-streptomycin (Sigma-Aldrich, Germany).

2.4. Cell morphology

Cell morphology was recorded using Smart Fluorescent Cell Analyzer Microscope JuLi™ (Korea) and FV1000 Confocal system with Olympus IX81 inverted microscope in transmitted light channel (Germany) after 48 h incubation with 100 μg/mL of 1-layer and 4-layer GO and GO-PEG. The cells cultured in medium without the nanomaterials were used as controls.

2.5. Cytotoxicity assay

The cytotoxicity of the GO nanoflakes was evaluated using the WST-1 assay (Sigma-Aldrich, Germany). The WST-1 test is based on the reduction of the tetrazolium salt WST-1 to a soluble red formazan by mitochondrial dehydrogenase. The amount of formazan dye is directly correlated to the number of metabolically active cells. In the current study, MCF7 cells were seeded in 96-well plates (4×10^3 cells/well in 100 μl medium). After 24 h the culture medium was removed and the cells were treated with GO nanoflakes dissolved in the culture medium at different concentrations: 3.125, 6.25, 12.5, 25.0, 50.0, and 100.0 μg/mL for 48 h. Cells without GO nanoflakes were used as controls. After 48 h, WST-1 reagent was added, incubated with cells for 30 min and absorbance was measured at 450 nm (with 620 nm background correction), using a spectrophotometric microplate reader (Infinite 200 Pro, Tecan, Switzerland). The interaction between nanomaterials (without cells) and WST-1 reagents was also determined (Ablank). Results were normalized to the control cells, and the cell viability was calculated using the following formula: number of viable cells (% of control) = [(Atest − Ablank) / (Acontrol − Ablank)] × 100%.

2.6. Membrane integrity

In the same cell culture conditions the LDH assay of PEG-functionalized GO was carried out. The LDH assay is based on the measurement of lactate dehydrogenase activity in the extracellular medium. The LDH release into the culture medium is an indicator of irreversible cell death due to cell membrane disruption. The GO unexposed cells were used as the negative control, and cells treated with Lysis Solution as the positive control (maximum LDH release). For the assay, MCF7 cells were plated in 96-well plates (4×10^3 cells/well) and then cultured as above for 24 h. After incubation time, the culture medium was removed and the cells were treated with the PEG-nanoflakes dissolved in the medium at different concentrations: 3.125, 6.25, 12.5, 25.0, 50.0, and 100.0 μg/mL. After 48 h of incubation, the activity of lactate dehydrogenase (LDH) in the medium was assessed using a CytoTox96 Non-Radioactive Cytotoxicity Assay (LDH, Promega, USA) according to manufacturer's instructions using a spectrophotometric microplate reader (Infinite 200 Pro, Tecan, Switzerland). Results were normalized to the negative control, and the percentage of cell viability was calculated using the following formula: cell viability [%] = 100% − [(experimental LDH release – negative control) / (positive control – negative control)] × 100%. The interaction between the nanomaterials (without cells) and assay components was also evaluated.