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The effects of graphene oxide nanosheets localized on F-actin filaments on cell-cycle alterations

María-Concepción Matesanz [a], Mercedes Vila [b,c], María-José Feito [a], Javier Linares [a], Gil Gonçalves [d], María Vallet-Regi [b,c], Paula-Alexandrina A.P. Marques [d], María-Teresa Portolés [a,*]

[a] Department of Biochemistry and Molecular Biology I, Faculty of Chemistry, Universidad Complutense, 28040 Madrid, Spain
[b] Department of Inorganic and Bioinorganic Chemistry, Faculty of Pharmacy, Universidad Complutense, 28040 Madrid, Spain
[c] Networking Research Center on Bioengineering, Biomaterials and Nanomedicine, CIBER-BBN, Spain
[d] TEMA-NRD, Mechanical Engineering Department, University of Aveiro, 3810-193 Aveiro, Portugal

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A B S T R A C T

Graphene oxide (GO) is considered to be a promising nanomaterial for biomedical applications due to its small two-dimensional shape besides its electrical and mechanical properties. However, only a few data concerning the cell responses to this material have been described and the GO biocompatibility has not been yet fully assessed. In the present study, graphene oxide nanosheets (GOs) decorated with 1-arm (1-GOs) and 6-arm (6-GOs) poly(ethylene glycol-amine) (PEG) have been incubated with cultured Saos-2 osteoblasts, MC3T3-E1 preosteoblasts and RAW-264.7 macrophages to analyze several key cell markers for *in vitro* biocompatibility evaluation. The results demonstrate that, after internalization, GO nanosheets are localized on F-actin filaments inducing cell-cycle alterations, apoptosis and oxidative stress in these cell types. The observed GOs effects must be considered in further studies focused on photothermal cancer therapy as a synergistic factor.

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1. Introduction

Nanomedicine is focused in searching new nanosystems for biomedical applications and the research approaches in this area include various nanoparticles which are being investigated as they offer unique advantages. Among all nanoparticles, graphene is a single-atom-thick sheet of sp^2-hybridized carbon atoms that has attracted great interest in the world of nanotechnology because of its excellent properties as flexibility, elasticity, mechanical resistance and its ability to conduct electricity [1–3]. Although current graphene research is mainly focused on nanoelectronics [4], the use of graphene for biomedical applications such as drug delivery [5,6], tissue engineering [7,8] and hyperthermia cancer therapy [9–11] has been recently proposed. The induction of hyperthermia on graphene for cancer treatment is possible due to the energy transfer produced during the irradiation of the material with light of wavelength in the near infrared (NIR) region (~890 nm), generating vibrational energy. Its strong NIR absorption thus generates sufficient heat for cell destruction (40 °C) [10,11] and it is called photothermal therapy. Graphene is classified depending on its properties such as surface area, number of layers, lateral dimension, surface chemistry and purity [2]. Graphene oxide (GO) is one of the most important graphene derivatives in terms of applications, and has been extensively studied in the recent years [2]. GO is known to have carboxylate groups on the periphery that provide pH dependent negative surface charge and colloidal stability [12]. The basal surfaces contain hydroxyl and epoxide functional groups as well as hydrophobic domains capable of π–π interactions. Although GO colloids are soluble in water, they need further functionalization with molecules like poly(ethylene glycol-amine) (PEG) which let the material become dispersible and highly stable in aqueous solutions [5,13]. Recent studies demonstrate the applicability of graphene with magnetic properties and fluorescence as a multifunctional marker for *in vitro* and *in vivo* studies with HeLa cells and zebrafish respectively [14]. *In vivo* tests in mice show that, after intravenous administration, GO is mainly located in lung, liver and spleen, causing some toxicity in these tissues [15]. However, other authors showed no toxicity after three months of injection of GO (20 mg/kg) in this animal model [16]. In human lung epithelial cells, GO doses over 50 μg/mL show no toxicity *in vitro* [17], but higher GO concentrations cause a dose-dependent oxidative stress in these cells and a slight loss of cell viability. In fact, most studies agree that GO promotes cytotoxicity mainly through ROS generation in a dose-dependent manner [14,18–20]

* Corresponding author. Fax: +34 1 394 41 59.
E-mail address: portoles@quim.ucm.es (M.-T. Portolés).

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inducing oxidative stress. All these results demonstrate that further studies are needed to examine in more detail the interaction between GO and cells before applying this material with medical purposes.

In the present study, different cell types were selected to evaluate their behavior after being in contact with pegylated graphene nanosheets (GOs). Thus, human osteosarcoma Saos-2 cells (as tumor cell line), murine MC3T3-E1 preosteoblasts (as undifferentiated osteoblast-like cells) and murine RAW-264.7 macrophages (as immune cells) were used as experimental models. To know the influence of its surface charge and particle size on cell functions, GOs decorated with 1-arm (1-GOs) and 6-arm (6-GOs) branched PEG were studied. Both GOs were marked with fluorescein isocyanate to measure their uptake and intracellular localization. Different biocompatibility parameters as reactive oxygen species (ROS) production, plasma membrane integrity, cell-cycle phases, apoptosis and light scattering properties were measured by flow cytometry after 1 day treatment. Moreover, the effects of GOs on cell proliferation, cell morphology and cytokine release were also analyzed.

2. Material and methods

2.1. GO nanosheet preparation and characterization

GO nanosheets have been prepared following the method previously published by the authors [21]. Basically, GO nanosheets of c.a. 100 nm were obtained from exfoliation of high purity graphite in acidic medium by a modified Hummers method [22]. The resulting GO suspension was then dialyzed until a pH of 7, activated to promote −COOH groups at its surface, and functionalized by covalent bonding with non-toxic and non-immunogenic polymers poly(ethylene Glycolamine) (PEG) to avoid the intercession with cellular functions or target immunogenicities and to decrease aggregation. Different branched PEG polymer were attached following the same route, 1-arm PEG bis(3-aminopropyl) terminated (1.5 kDa) and 6-arm branched PEG amine (15 kDa). The samples named 1-GOs and 6-GOs respectively were marked with the amine reactive dye fluorescein isocyanate (FITC) covalent bonded to the PEG. Transmission electron microscopy (TEM) was performed on a 200 kV JEOL JEM 2100. GO nanosheets were analyzed by atomic force microscopy (AFM, VEECO multimode; USA.) and Fourier transform infrared spectroscopy (FTIR, Nicolet Nexus spectrometer). Zeta-potential (ζ) and dynamic light scattering particle size analysis DLS measurements were also performed in pH 5 solutions in a Zetasizer Nano Series instrument equipped with a 633 nm "red" laser from Malvern Instruments with reproducibility being verified by collection and comparison of sequential measurements. *Z*-average sizes of three sequential measurements were collected at RT and analyzed.

2.2. Cell culture for FITC-GO nanosheet incorporation and cell proliferation studies

Saos-2 osteoblasts, MC3T3-E1 preosteoblasts and RAW-264.7 macrophages were seeded at a density of 10^5 cells/mL in culture medium supplemented with 10% FBS, 1 mM L-glutamine, penicillin, streptomycin, under a 5% CO_2 atmosphere and at 37 °C for 24 h in contact with 75 μg/mL FITC-GO material. Then, the attached cells were harvested using either 0.25% trypsin-EDTA (in Saos-2 and MC3T3-E1 cells) or cell scrapers (in RAW-264.7 cells) and counted with a Neubauer hemocytometer. The fluorescence of FITC-GO was excited at 488 nm and measured with a 530/30 band pass filter in a FACScalibur Becton Dickinson flow cytometer.

2.3. Intracellular reactive oxygen species (ROS) content and cell viability

After culture with GO material and detachment, cells were incubated with 100 μM 2′,7′-dichlorofluorescein diacetate (DCFH/DA) (37 °C for 30 min). DCF fluorescence was excited at 488 nm and measured with a 530/30 band pass filter in a FACScalibur Becton Dickinson flow cytometer. In these assays, the basal green fluorescence of FITC-GOs associated to cells in the absence of DCFH/DA, was subtracted from the total fluorescence values (FITC-GOs plus DCF), in order to measure only the DCF fluorescence due to the DCFH oxidation by ROS. Cell viability was determined by addition of propidium iodide (PI; 0.005% in PBS). The PI exclusion indicates the plasma membrane integrity.

2.4. Cell-cycle analysis and apoptosis detection

After culture with GO material and detachment, cells were incubated for 30 min with Hoechst 33258. Hoechst fluorescence was excited at 350 nm and measured at 450 nm in a LSR Becton Dickinson flow cytometer. The fluorescence of Hoechst was excited at 350 nm and the emission was measured at 450 nm in a LSR Becton Dickinson flow cytometer. The cell percentage in each cycle phase: G_0/G_1, S and G_2/M was calculated with the CellQuest Program of Becton Dickinson and the SubG_1 fraction was used as indicative of apoptosis.

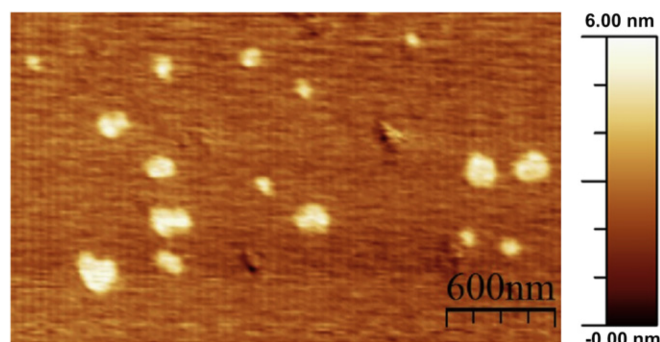

Fig. 1. AFM topographic image of GOs.

2.5. Cell size and complexity

Forward angle (FSC) and side angle (SSC) scatters were indicative of cell size and complexity respectively using a FACScalibur Becton Dickinson flow cytometer.

2.6. Confocal microscopy studies

Cells were seeded on glass coverslips and cultured in the presence of FITC-GO material for 24 h, fixed with 3.7% paraformaldehyde in PBS, permeabilizated with 0.1% Triton X-100 and preincubated with PBS containing 1% BSA. Then, cells were incubated for 20 min with rhodamine-phalloidin (1:40), stained with 4′-6-diamidino-2′-phenylindole (DAPI, 3×10^{-6} M in PBS) and examined using a Leica SP2 Confocal Laser Scanning Microscope. Rhodamine fluorescence was excited at 540 nm and measured at 565 nm. DAPI fluorescence was excited at 405 nm and measured at 420–480 nm. FITC fluorescence was excited at 488 nm and measured at 491–586 nm.

2.7. Inflammatory cytokine detection

IL-1β, TNF-α, and IL-6 cytokines released by cells to the culture medium were quantified by ELISA with a Gen-Probe Diaclone kit.

2.8. Statistics

Data are expressed as means + standard deviations of a representative of three experiments carried out in triplicate. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 19 software. Statistical comparisons were made by analysis of variance (ANOVA). Scheffé test was used for *post hoc* evaluations of differences among groups. In all of the statistical evaluations, $p < 0.05$ was considered as statistically significant.

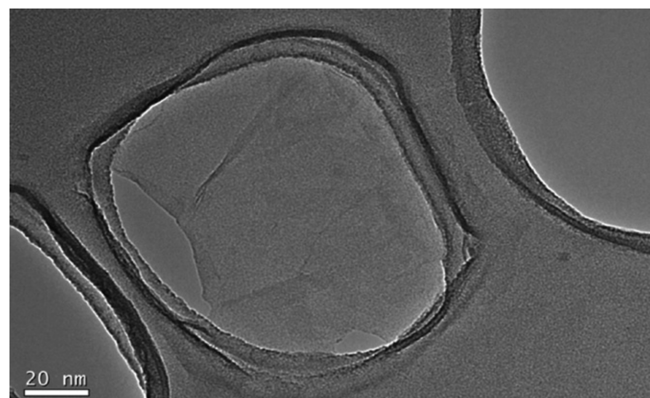

Fig. 2. Transmission electron micrograph of activated GOs.

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