PEGylation of graphene oxide nanosheets modulate cancer cell motility and proliferative ability

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Abstract. Recently, graphene oxide (GO) has been increasingly investigated for its biomedical and biological applications, including cancer research. The interest is set on GO chemical modifications and their implications in the development of therapeutic approaches for various diseases. Recent data have demonstrated that PEGylation of nanoparticles (NPs) improves NPs solubility and stability in physiological solutions and alters their reactivity toward cancer cells. In this work, we have evaluated the effect of PEGylated GO nanosheets on the migratory and proliferation ability of A375 melanoma cells, used as a cancer cell model and have compared it to normal kidney MDCK cells. Both types of GOs, pristine and PEGylated, demonstrated an inhibitory effect on the cancer cells proliferation and mobility while on normal MDCK cells the effect of GO was significantly weaker at 48 hours of exposure suggesting that cancer A375 cells were more sensitive to GO and GO-PEG treatment. In general, PEGylation mitigates the inhibitory effect of GO on the growth and migratory ability of melanoma cells. Our results prove that the effects of both GOs NPs on cancer cells proliferation and mobility are dose-, NPs- and cell-type-dependent, hence providing a rationale for future design and use of graphene-based nanomaterials for cancer research.

1. Introduction
With about 9.6 million death cases in 2018 cancer is the leading cause of death globally [1]. The ability of cancer cells to spread in the body makes difficult their surgical treatment thus the main treatment options remain chemo- and radiotherapy [2]. This makes metastases major reasons for death from cancer. Chemo- and radiotherapy have many disadvantages like poor efficiency, drug resistance of cancer cells, side effects and body toxicity. Searching new approaches for cancer treatment is a constantly evolving field and every year several new approaches are proposed. In particular, promising results were obtained with nanomaterials used as drug delivery systems [3-6]. Nanoparticles (NPs) may facilitate tumour-targeting, increase drug loading, and allow controlled drug release which improves the
effectiveness of the treatments and consequently lead to significant improvement in survival and quality of life of cancer patients [7,8].

Among different types of NPs graphene oxide (GO) is the most widely used carbon derivative in the field of biomedicine for the past few years. GO nanosheets possess a large and specific surface area that yield excellent adsorption properties for drug delivery. GO is suitable to carry different agents, such as small drugs, DNA and antibodies, that bind to the surface of nano-GO by π–π stacking and hydrophobic interactions [9,10]. Drug-loading capacities of multilayered GO is higher than monolayer shapes and increases with the increasing of the number of layers. However, a disadvantage of NPs including GO is their aggregation and instability in biological fluids. One approach to tackle this challenge is the surface functionalization with polymeric ligands [11]. The last provides the surface with a physical barrier preventing the NPs from coming into contact and thus stabilize NPs in aqueous dispersions.

Polyethylene glycol (PEG) is a hydrophilic biocompatible polymer extensively applied to functionalize various nanomaterials to improve their solubility and stability in physiological solutions, as well as their biocompatibility [12]. Coating of nanoparticles with PEG or the so-called “PEGylation” passivates the surface of the material by preventing nonspecific protein adsorption from the biological milieu. Besides, PEGylation has shown success in the protection of nanoparticles from aggregation, opsonisation, and phagocytosis, as well as in prolonging systemic circulation time of NPs in blood [13]. Thus, the surface functionalization of GO with PEG can be used to design graphene-based nanocarrier systems with high solubility in the physiological fluids and acceptable biocompatibility for biomedical application in physiological environments.

In this work, we have developed PEGylated nanosized GO NPs (nGO-PEG) using a simple one-step method of reduction and functionalization of GO with mPEG-NH2 and have studied the effects of nGO and nGO-PEG on melanoma cells used as a model of melanoma cancer - an aggressive skin cancer with a steadily increasing incidence over the last 50 years. The results were compared to those of non-tumour kidney cells. Our attention was on the effect of GO-PEG on cell growth and motility because the main characteristics of cancer are the uncontrolled growth and spread and little is known about the effect of NPs on metastasis.

2. Materials and Methods

2.1. PEGylation of graphene oxide

PEGylation of nGO was performed using the method of Chen et al with some modifications [14]. In brief, pristine GOs (4 mg/ml, Graphenea, Spain) were diluted in deionized water to a concentration of 2 mg/ml and sonicated for 1 hour at 500W, then mPEG-NH2 was added and the mixture was sonicated for 5 min. The suspension was allowed to react overnight at 80°C on magnetic stirrer then was centrifuged at 12500 g to remove particles aggregates. The final product was kept at 4°C until further use.

2.2. Spectroscopic Characterization

The absorption (UV-Vis) spectra of water solutions of nGO and nGO-PEG with concentration of 10 μg/ml were recorded on Specord 210 Plus spectrometer (Analytik Jena AG, Germany).

The IR spectra were recorded on Thermo Nicolet 6700 spectrometer (Thermo Fisher Scientific, USA) in the mid-infrared region (4000 – 400 cm⁻¹) at a resolution of 2 cm⁻¹. The spectra were performed on air-dried nGO, mPEG-NH2 and nGO-PEG using the standard KBr pellet method.

2.3. Preparation of the samples for biological studies

For cellular experiments stock solutions of 1.0 mg/mL of nGO and nGO-PEG particles in distilled water were prepared. Just before the cell exposure, the stock solutions were sonicated for 1 h in a water bath then NPs were added to the cells in different concentrations.

2.4. Cell lines and culture conditions
A375 (a human melanoma cell line) and MDCK (a canine kidney cell line) were purchased from American Tissue Culture Collection (ATCC). The cells were maintained in DMEM medium supplemented with 10% foetal bovine serum at 37°C in a humidified atmosphere and 5% CO2.

2.5. WST-1 Cell proliferation assay

The cell growth was assessed using a standard WST-1 assay (Sigma-Aldrich Co.) after 48 hours of exposure to nGO and nGO-PEG NPs following the manufacturers’ instructions. Cells were seeded in 96-well flat-bottomed microplates at a density of 1x10^4 cells per well and after 24 h incubation at 37°C, they were exposed to increasing concentrations of the tested NPs for 48 h. At the end of incubation with NPs WST-1 solution was added directly to the cells in ratio 1:10. After further 4 h incubation at 37°C, at dark, and the amount of the formazan produced was measured by absorbance at 450 nm using a standard microplate reader (Thermo Scientific Multiskan Spectrum). The cell proliferation data were normalized to the percentage of the untreated control. The corresponding half-maximal inhibitory concentration ($IC_{50}$) values was calculated using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). In order to assess the degree of selectivity in the anti-proliferative activity of pristine and PEGylated nGO selectivity index (SI) was calculated according to the following formula:

$$SI = \frac{IC_{50} \text{ non-cancerous cell line}}{IC_{50} \text{ cancer cells line}}$$

2.6. FDA staining

FDA staining was performed to visualize cell morphology after exposure to pristine and PEGylated nGO NPs. Cells were seeded in 24-well plates at a density of 2x10^4 cells per well and after 24 h incubation at 37°C, they were exposed to the tested NPs with concentrations of 100 µg/ml for 48 h. At the end of incubation, the cells were stained with fluorescein diacetate (FDA) as previously described [15]. The morphology of vital and attached cells was observed under fluorescent microscope Axiovert 25, Carl Zeiss, Germany.

2.7. Cell migration assay

The effect of nGO and nGO-PEG NPs on the migration of A375 and MDCK cells was studied by a wound-healing assay. Cells were seeded at a density of 4x10^5 cells in 6-well plates and cultivated in complete culture media to 90-95% confluence for 48 h. The cell monolayer was scratched with a 200 µl plastic pipette tip and cell debris were removed by repeated washing with phosphate buffer saline (PBS). Fresh medium, containing 1% foetal bovine serum and 50 and 100 µg/ml of both types of NPs were added and the cells were incubated for another 48 h. The cells, migrating in the wound zone were monitored and photographed under an inverted light microscope (Carl Zeiss, Jena, Germany) at the 0 h and after 48 h. Untreated cells were used as negative controls. The percentage of wound closure rate was estimated using the following formula:

$$\% \text{ wound closure} = \frac{(\text{width of scratch wound at 0 h} - \text{width of scratch wound at 48 h})}{\text{width of scratch wound at 0 h}} \times 100$$

2.8. Statistical Analysis

All data were expressed as means ± standard error of the mean (SEM). The statistical differences were determined by one-way analysis of variance (ANOVA) followed by Dunnett's post-hoc test and the Tukey's test and the results were considered significant if $p<0.05$.

3. Results and Discussion

3.1. Synthesis and Spectroscopic Characterization of PEGylated nGO

In the present work GO nanosheets were PEGylated to improve their biocompatibility based on literature data demonstrating that PEG molecules decreased the interactions of GO nanosheets with cells and cell mechanical damage and thus induced less cell death and caused less cytotoxicity effect [16]. Our
previous research carried out on pristine and aminated graphene oxide nanosheets have showed that these few-layered structures were capable of inducing adverse effects in cancer cells [17] by generating ROS and as a result of their aggregation causing physical blockages [15]. However, for drug delivery application GO NPs should have an adequate safety profile which could be achieved by PEGylation. The synthesis approach performed here is a simple one-step method of simultaneous reduction and functionalization of nGO by mPEG-NH₂. This approach lacks the conventional use of chemical agents needed for conversion of OH groups of GO to COOH to activate the PEG molecules and to accelerate the reaction process [18]. Thus, the new NPs are free of chemical waste and impurities that could support cross-chemical effects. Fourier transform infrared spectroscopy (FT-IR) is a potent technique to characterize the presence of the functional group upon functionalization. Figure 1A presents the FT-IR spectra of nGO-PEG compared to nGO and mPEG-NH₂. The nGO spectrum showed bands belonging to the O-containing functional groups, such as OH, COOH, epoxy groups, adsorbed water molecules and also low intensive bands of in-plane vibrations of the skeletal –C=C- bonds of the hexagonal aromatic rings from graphene sheets. In the IR spectrum of pure mPEG-NH₂ (figure 1) the weakly intense broad bands at 3265 and 3110 cm⁻¹ and the peak at 1641 cm⁻¹ were assigned to the asymmetric and symmetric, and deformation vibrations of NH₂-functional groups. The bands around 2880 cm⁻¹ are due to the stretching vibrations of CH₂ and CH₃ (from methoxy groups) and these at 1473, 1410, 1365 and 1350 cm⁻¹ are assigned to their deformation modes. The intensive triplet with the maximum at 1114 cm⁻¹ (with an overtone at 1970 cm⁻¹) together with the bands at 964 and 850 cm⁻¹ were assigned –C-O-C- functional groups of the polymeric structure. The infrared spectrum of nGO-PEG contains the characteristic bands of mPEG. The lack of a band at 1720 cm⁻¹ belonging to the IR spectrum of pristine nGO, suggested the removal of carbonyl functions (of COOH) most likely due to the formation of an amide bond (-CO-NH-) during the incorporation of mPEG-NH₂ on the nGO surface. Following this assumption, the low intensive peak at ~2950 cm⁻¹ and the intensive peak at 1635 cm⁻¹ can be assigned to the formed amide linkage (-CO-NH-) of the PEGylated nGO. Additional confirmatory results of GO-PEG structural features can be obtained through UV-Vis absorption analysis (figure 2). Typically, GO exhibits an absorption peak at around 235 nm with a weak shoulder at around 305 nm due to -C = C- and - C = O bonds, respectively [18, 19]. The observed absorption at ~230 nm in the spectrum of pure mPEG-NH₂ is connected with (n→σ*) transitions due to the polymeric structure (-C-O-)ₙ [20]. The nGO-PEG UV-Vis pattern revealed a bathochromic shift of the GO-peak into ~260 nm because of the process of PEGylation.

3.2. Cellular morphology, proliferation, IC₅₀, selectivity index and motility
Melanoma cancer was adopted in this work to elucidate the effect of PEGylation of nGO mainly on the proliferation ability and motility of cancer cells because both are important processes during melanoma progression, leading to invasion and metastases. Additionally, cellular morphological changes, IC₅₀ and selectivity indices were determined. Parallel assessment of nGO-PEG NPs and pristine nGO on a normal cell line was conducted for more accurate probing the safety profile.
To evaluate cell proliferation ability, WST-1 assay was performed using various concentrations at 48 h after exposure to pristine GO and GO-PEG NPs. Results as presented in figure 3, show similar growth trends for both NPs but differential effects between both cell types.

**Figure 3.** Cell proliferation rates of A375 cells (upper panel) and MDCK cells (lower panel) 48 hours post-incubation with nGO and nGO-PEG in different concentrations.

In the melanoma A375 cells, the exposure to the increasing concentrations from 5 to 20 μg/mL of GO NPs resulted in a slight increase (from 10 to 16%) in cell proliferation rates. In contrast, further increasing of GO NPs concentration to 200 μg/mL induced a dramatic decrease in cell proliferation rates to 55.5, 43.27 and 4.45% for A375 cells treated with 50, 100 and 200 μg/mL (p< 0.001). In samples treated with the highest nanoparticles concentration of 500 μg/mL no signal was detected suggesting a strong anti-proliferative effect of those concentrations on A375 cells (figure 3).

Similarly, in nGO-PEG-treated A375 cells, cell proliferation rates increased with increasing of NPs concentrations to 20 μg/mL nGO-PEG NPs and decreased with further increasing of nGO-PEG NPs to 200 μg/mL. In comparison to nGO-treated samples, the decrease in cell proliferation was not so sharp suggesting that PEGylation mitigates the effect of nGO on melanoma cell growth (figure 3).

In MDCK cells only the highest concentration of 200 and 500 μg/mL of the NPs had an inhibitory effect on cell proliferation with a decline in cell growth to 82.51% and 41.13% for nGO-treated cells and to 66.16% and 42.14% for nGO-PEG-treated cells pointing that the nGO and nGO-PEG NPs affect in a greater degree the proliferation of melanoma cells (figure 3).

The IC$_{50}$ values, displayed in Table 1, demonstrate the anti-cancer effect of nGO and nGO-PEG NPs inhibiting 50% of cell populations at concentrations of approximately 73.03 and 131.5 μg/mL for nGO and 421.7 and 376.3 for nGO-PEG toward A375 cells and MDCK cell line, respectively. These results confirm that nGO and nGO-PEG inhibit melanoma cell proliferation much stronger than the proliferation of normal MDCK cells.

The normal kidney cell line (MDCK) demonstrated lower inhibition rates at all applied concentrations. The high IC$_{50}$ recorded for MDCK cells had proven the safety of nGO-PEG on normal tissue with SI exceeding 2.0 –the accepted threshold for selectivity of the compounds.

| NPs/Cell line          | nGO-A375 | nGO-MDCK | nGO-PEG-A375 | nGO-PEG-MDCK |
|------------------------|----------|----------|--------------|--------------|
| IC$_{50}$ [μg/ml]      | 73.03    | 421.7    | 131.5        | 376.3        |
| Selectivity index      | 5.77     |          | 2.86         |              |
We additionally evaluated the effects of NPs on the morphology of the vital cells and found that 48 h-exposure to nGO and nGO-PEG NPs with the concentration of 100 μg/mL did not lead to significant morphological changes in A375 and MDCK cells in comparison to the untreated group. Neither cellular shrinkage nor swollen which are signs of cell apoptosis and necrosis were detected in NPs-treated cells. Only mild morphological changes such as rounding of the cells were observed in GO-treated melanoma cells which are reported previously reflecting oxygen functionalities reactivity that may affect nGO biocompatibility [20]. As shown in figure 4, both cell types possess a spreading morphology with characteristic polygonal shape. Control cells formed a dense cell layer while in nGO-treated cells the cell layer was less dense suggesting a reduction of cell proliferative ability as a result of NPs exposure.

A wound-healing assay was applied to measure the rate of migration of melanoma A375 and normal MDCK cells after treatment with nGO and nGO-PEG NPs at the concentrations of 50 and 100 µg/ml for 48 h. As presented in figure 5, the pristine and PEGylated form of nGO significantly decreased the migratory ability of melanoma cells in a dose-dependent manner compared to the untreated control. The inhibitory effect of the pristine graphene oxide on the cancer cells mobility was insignificantly stronger concerning that of PEGylated nGO. Concerning MDCK normal cells, a reduction in cell motility was also observed after treatment but the suppressive effect was considerably weaker in comparison to the effect against A375 cells. PEGylated nGO decreased in a higher degree normal cells mobility than the pristine nGO.

Our results are in accordance to the results obtained by T. Zhou et al demonstrating that pristine GO and PEG-GO exhibited no apparent effects on the viability of cancer cells and non-cancerous cells but inhibited cancer cell migration in vitro and in vivo when applied with concentrations up to 80 mg/ml [21,22]. However, a more recent study of Zhu et al has shown the opposite effect of pristine GO which in low-dose could induce significant cellular membrane changes within cancer cells, suggesting enhanced invasion/migration [23]. These differences may be due to different concentrations used, cell types, target molecules, etc. Therefore, more efforts are needed to determine the pro-metastatic effects of pristine and PEGylated GO NPs. Our findings would help for the development of nanodrugs with better tumor-suppressing and targeting efficacies with minimal side effects.
Figure 5.

Representative images of the cell wound healing scratch assay for A375 and MDCK cells after nGO and nGO-PEG treatment with 50 and 100 μg/mL for 48 h. Quantified data are shown in the lower panel.

4. Conclusion
This study presented PEGylation of graphene oxide nanosheets by one step approach using mPEG-NH₂ and demonstrated its effectiveness to modulate proliferative and motility properties of melanoma cancer cells and of normal kidney cells. Results from biological evaluations revealed that PEGylation of nGO possesses lower inhibitory effect on proliferation and motility profile, with an increased IC₅₀ values and did not alter cell morphology of melanoma cancer cells after 48 h exposure in comparison to pristine nGO. However, on normal kidney cells PEGylation resulted in suppression of cell proliferation and migration while IC₅₀ values were higher for nGO-PEG than those of pristine nGO. Collectively, our findings enhance the understanding of the effects of GO nanosheets in the progression of metastatic tumors.

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