Dose-dependent genotoxicity of ammonia-modified graphene oxide particles in lung cancer cells

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Abstract. Graphene oxide (GO), the water soluble form of 2D graphene, has received much attention because of its attractive properties for a wide range of applications and products. Surface modification with different functional groups can improve GO biocompatibility for further biomedical applications. In the present study we have evaluated genotoxicity of pristine and ammonia-modified graphene oxide (GO-NH2) nanoparticles (NPs) in a human lung epithelial cell line, A549, exposed for 24 h to different concentrations of NPs (0.1, 1, 10, 20 and 50 μg/ml). Quantification of reactive oxygen species (ROS) indicated that exposure to higher concentrations of both types of NPs resulted in enhanced ROS generation. The observed comet tail migration in the method of Single Cell Gel Electrophoresis in the cells treated with 20 and 50 μg/ml GO and GO-NH2 indicated presence of damages in DNA. Cell cycle analysis showed that after treatment of A549 cells with increasing concentrations of NPs for 24h the percentage of cells in G0/G1 phase of the cell cycle decreased while the percentage of cells in G2/M increased. The presented results suggest that ammonia-modified GO NPs applied at concentrations higher than 20 μg/ml induced stronger toxicity effect in A549 cells compared to pristine GO and that the use of low concentrations of GO and GO-NH2 NPs is important to avoid adverse biological effects.

1. Introduction
Graphene-based nanomaterials possess remarkable physiochemical properties suitable for diverse applications in electronics, telecommunications, energy and healthcare [1-3]. The human and environmental exposure to nanomaterials is increasing due to progress in the synthesis, characterization and large-scale production of graphene as well as the subsequent development of graphene-based biomedical and consumer products [1, 4, 5]. Recently, there is an increasing awareness of nanoparticles pollution as an environmental problem [6]. A large number of in vitro and in vivo toxicological studies have evaluated the interactions of graphene-based nanomaterials with various living systems such as microbes, mammalian cells and animal models [1, 7]. A significant number of studies have examined the short- and long-term toxicity of nanomaterials at the cellular level.
Graphene oxide (GO) is one of the most important graphene derivatives known for its exceptional electrical, chemical and mechanical properties [8]. Due to its attractive physicochemical properties for biomedical applications toxicity of GO is subject of multiple in vitro and in vivo research [9, 10]. Some papers have demonstrated that GO has low cell toxicity but results achieved so far are inconsistent. [11, 12]. Ideally, therapeutic nanoparticles should be nontoxic and invisible to the immune system. Hence, much efforts have been devoted to improve biocompatibility of GO-based nanomaterials. It is proposed that surface modification could be helpful to decrease the toxicity of GO as a component of nanomedicine because GO contains many oxygen containing groups (hydroxyl, carbonyl groups, carboxylic and epoxy group) making it easy for functionalization [13].

In this study we compare the toxicity of pristine GO and ammonia-modified GO at molecular level in a human alveolar cell line, A549 after exposure to the increasing concentrations of NPs. We used lung cells as a cell model for our toxicological evaluation because many biodistribution studies have shown that GO accumulates predominantly in the lungs [14, 15]. The aim was to understand if ammonia modification of GO would improve its toxicity and if the concentration of NPs in the range of 0.1–50 μg/ml is toxic in a dose-dependent manner. Only few studies report the toxicity response of pristine and functionalized graphene at molecular level. This sort of information is still largely lacking in the literature, though the health effects of NPs have attracted considerable attention.

2. Materials and Methods

2.1. Characterization of GO and GO-NH2 particles
The GO samples were characterized by X-ray photoelectron spectroscopy (XPS, Axis DLD Ultra, Kratos, Manchester, UK), Atomic Force Microscopy (AFM, Bruker Inc.) and Nanosizer (Zetatrac instrument, S3500; Microtrac, Largo, FL) in order to determine chemical composition, particle size and thickness, particle size distribution and zeta potential. For cellular experiments stock solution of 1.0 mg/ml of GO (Graphenea, Spain) and GO-NH2 (Sigma-Aldrich, Germany) particles in distilled water was prepared. Just prior to the cell exposure the stock solutions were sonicated for 1 h then NPs were added to the cells in different concentrations.

2.2. Cell culture
A549 cells were purchased from American Tissue Culture Collection (ATCC) and maintained in DMEM medium supplemented with 10% foetal calf serum and antibiotic/antimicotic solution at 37°C in a humidified atmosphere of 5% CO2. For genotoxicity evaluation experiments, the confluent cells were harvested and seeded in a concentration of 1x10⁵ cells/well in 6-well plates then cells were incubated 24h before to be exposed to the increasing concentrations of NPs.

2.3. Cell morphology
Overall cell morphology was observed under phase contrast microscope (Axiovert 25, Carl Zeiss, Germany) and phase contrast pictures were done at the 24 h after exposure of the cells to the NPs using a digital camera.

2.4. Neutral Comet Assay (nCA)
Briefly, 1x10⁵ cells were mixed with 0.7% (f.c.) of low-gelling agarose and were layered as microgels on microscopic slides. Slides were then lysed in 146 mM NaCl, 30 mM EDTA, pH7, 10 mM Tris-HCl, pH7 and 0.1% N-lauroylsarcosine at 10 °C for 20 min and we electrophoresed for 20 min at 0.46 V/cm. Results were visualized under a fluorescent microscope after staining of gels with SYBR green. Results are quantified by Comet Assay specialized software CometScore.

2.5. Cell cycle analysis
FACS was performed on A549 cells fixed with 76% of cold ethanol immediately after incubation and left at -20 °C for a night. Cells were pelleted by centrifugation, washed in PBS buffer and treated with 100 μg/ml RNAse A for 30 min at 37 °C followed by staining with 50 μg/ml of propidium iodide for 30 min in the dark. 50 000 cells were counted through flow cytometry, detecting red fluorescence at
excitation wavelength of 488 nm. The light scattering was detected as well. The results were quantified by FlowJo V10.

2.6. Determination of reactive oxygen spices (ROS)
Intracellular ROS was measured based on the intracellular peroxide-dependent oxidation of 2,7-dichlorofluorescein diacetate (DCFA-DA, Sigma-Aldrich) to form the fluorescent compound 2,7-dichlorofluorescein. The cells were seeded onto 24-well plates at a density of 3 x 10^4 cells per well and cultured for 24h. After washing twice with phosphate-buffered saline (PBS) fresh media containing different concentration of GO and GO-NH$_2$ particles were added and incubated for another 24h. Then the cells were washed intensively with PBS and fresh serum-free medium containing 20 μM DCFA-DA was added to each well. After 30 min incubation at 37°C, the samples were rinsed with PBS and 1 ml of PBS was added to the wells. Then the cells were scraped and the fluorescence intensity was determined using a spectrofluorometer with excitation at 485 nm and emission at 530 nm.

3. Results

3.1. Physico-chemical characterization of GO and GO-NH$_2$ NPs
As can be seen from figure 1 pristine and ammonia-modified GO differ in their size and chemical composition. XPS analysis (figure 1 C) revealed the same major characteristic peaks of C1s spectra, including C–C (284.7 eV), C–O (286.2 eV), C=O (287.8 eV) and O–C=O (289.1 eV) in both GO NPs but in GO-NH$_2$ the peak intensities were much weaker than those in GO, pointing a reduction in the oxygen content as a result of ammonia modification. The existence of nitrogen element in GO-NH$_2$ was confirmed by the appearance of N1s peak. All this indicates both, the de-oxygenation of GO and the incorporation of nitrogen in the ammonia-modified GO. AFM images of sonicated GO-NH$_2$ particles showed that GO-NH$_2$ flakes had an irregular shape and lateral dimensions of few microns (figure 1 A). The thickness of the single GO layer is around 0.9 nm and most of GO sheets exist as single or few layers according to the datasheet. DLS measurements demonstrated that the size of GO sheets was larger than that of GO-NH$_2$ sheets. The pristine GO particles consist of two fractions with quite different sizes: 250 ± 68 nm (9.7% of particles) and 1.5 ± 0.7 µm while the average particle size of GO-NH$_2$ NPs was 560 ±300 nm (figure 1 B). Zeta potentials of GO and GO-NH$_2$ particles measured in aqueous solutions showed negative values for GO (−24.5 ± 0.4 mV) and positive for GO-NH$_2$ NPs (38.5± 2.8 mV) (data not shown).
3.2. Cell morphology

The morphology is one important indicator of the status of cells. The cell morphological changes after GO exposure were recorded to demonstrate the effect of both types of GO NPs on A549 cells directly (figure 2). There was no obvious difference between morphology of cells treated with GO and GO-
NH$_2$ and the control cells. Most cells were tightly adhered to the substrate and had normal spindle shape. However, in the cells exposed to GO-NH$_2$ NPs the number of the attached cells seems to be smaller (Fig.2, lower panel). Also, at the highest concentrations of GO (20 and 50 µg/ml) and GO-NH$_2$ treated cells (50 µg/ml) less attached cells were observed suggesting a detachment of cells as a result of NPs exposure.

Figure 2. Phase-contrast micrographs of A549 cells, incubated for 24 hours with pristine GO particles (upper panel) and ammonia-modified GO particles (lower panel); magnification 20x

3.3. ROS levels
Measuring the release of ROS produced by cells in response to particle treatment is a well-established method to evaluate toxicity. As shown in figure 3 both, GO and GO-NH$_2$ NPs applied at concentration of 20 and 50 µg/ml enhanced the formation of intracellular ROS in A549 cells. The mean fluorescence intensity of cells exposed to 50 µg/ml GO-NH$_2$ was 3.0 times higher than control and for cells exposed to pristine GO was 1.8 times higher pointing that ammonia-modified GO causes more serious oxidative stress at the highest concentrations. Exposure to lower doses of GO-NH$_2$ particles, up to 10 µg/ml did not induce generation of ROS. In cells treated with lower doses of GO (0.1-10 µg/ml) an increase in ROS production was observed but the values were not statistically significant (p>0.05).

Figure 3. ROS generation in A549 cells induced by GO and GO-NH$_2$ particles with different concentrations

3.4. Genotoxicity evaluation
Genotoxic potential of NPs was assessed by measuring DNA double strand DNA breaks first using the Single Cell Gel Electrophoresis, also called Comet assay at neutral pH. The results from the neutral CA (nCA) demonstrated that cells treated for 24 hours with different concentrations of GO
NPs showed presence of comets resembling cells undergoing apoptosis (figure 4). This means that GO and GO-NH$_2$ particles induced DNA damages and prove to have a genotoxic potential. The genotoxic potential of the NPs however did not increase proportionally to the concentrations. Although, the greatest genotoxic potential have been demonstrated in the cells treated with the highest particles concentrations of 50 µg/ml. Compared to the control, GO and GO-NH$_2$ particles demonstrated a genotoxic effect on A549 cells as among both types of GO particles ammonia-modified GO were more genotoxic.

![Neutral Comet Assay](image)

**Figure 4.** Neutral Comet Assay of A549 cells, treated for 24 hours with GO nanoparticles with different concentrations. A) Comet images of the A549 cells stained with SYBR green and observed under fluorescent microscope; upper panel – cells treated with pristine GO; lower panel – cells treated with ammonia-modified GO particles; magnification 250x B) Comet Assay data quantitation by specialized CA software CometScore – a graph representing the parameter Olive Moment (OM). This parameter essentially represents the product of the percentage of total DNA in the tail and the distance between the centres of the mass of head and tail regions [Olive moment = (tail mean-head mean) x % of DNA in the tail].

Further, we investigated whether GO particles can alter the cell cycle of human lung cells. FACS analysis of A549 cells exposed to GO NPs for 24h and the followed data analysis by FlowJo software showed that after treatment the percentage of viable cells decreased compared to the control cells in both, GO and GO-NH$_2$ NPs (figure 5A). The distribution of cells throughout different phases of the cell cycles showed an interesting correlation. At lower concentrations of the NPs there was a small percentage of cells blocked in S and G2/M phases of the cell cycle when treated with GO-NH$_2$ (compare GO 0.1 vs 0.1 µg/ml GO-NH$_2$ and GO 1 vs 1 µg/ml GO-NH$_2$). At higher GO concentrations 10-50 µg/ml this correlation was inverse with the GO-NH$_2$ (Figure 4B). At these high concentrations of GO and GO-NH$_2$ the percentage of cells in the whole population was smaller than in the control pointing to a cytotoxic and genotoxic effect.
Figure 5. FACS Cell cycle analysis of A549 cells treated with GO nanoparticles for 24 hours. A) Distribution of cells according to FACS data quantitation, assessed by FlowJo V10. B) Histograms of FACS analysis of each treated probe including the control cells (grey histogram).

4. Discussion
The aim of this study was to determine if ammonia-modified GO and pristine GO NPs are toxic at molecular level and if their effect on A549 cells is dose-dependent. The results collectively indicate that aminated GO exert slightly stronger genotoxic effect compared to pristine GO but only at higher concentrations (20 and 50μg/ml). When designing our experiments we supposed that ammonia modified GO would have an improved toxicity profile compared to pristine GO because NH₂ groups are known for their positive effect on cell adhesion, growth and functions of normal cells when have been used to modify biomaterials for cell culture substratum [16]. Besides, ammonia modification reduces oxygen-containing groups on the surface of the GO particles which groups are considered as a factor increasing nanomaterial toxicity. However, the reports on toxicity effect of reduced or modified with different functional groups GO are very controversial [17, 18, 12]. To date only limited studies have focused on the toxicity of aminated GO in cancer cells while the cytotoxicity and especially genotoxicity of aminated GO in normal cells still has not been investigated. Ming Xu et al. for example, have demonstrated a considerably reduced recognition of GO-NH₂ by macrophage sarcoma cells [13].

Based on physico-chemical characterization and data sheet of pristine GO we found that both particles, pristine and ammonia-modified differed not only in nitrogen content but also in size and zeta potential. GO are larger in size and negatively charged unlike GO-NH₂ particles which are smaller and positively charged. It is well known that particles size and surface charge are important factors for the toxicity effect of NPs because both regulate the uptake of nanomaterials from the cells. Positive zeta potential of NPs has been shown to increase the internalization of polymeric nanoparticles in the cells [19] suggesting that GO-NH₂ have been taken up more efficiently by cells than negatively charged pristine GO NPs. However, internalization of both NPs, GO and GO-NH₂ can be hampered from their size (560 nm for GO-NH₂ and 1.5μm for GO) probably due to the size limit of endosomal vesicles that restricts the size of NPs to be endocytosed. Moreover, GO and especially GO-NH₂ aggregate when being added into the culture medium and the aggregation may take the consequences too. Thus the size, charge and the aggregation of GO NPs could inhibit their endocytosis. The GO and GO-NH₂ NPs only partially can be taken up and induces toxicity in A549 cells. Considering that both GO NPs hardly enter into the A549 cells, GO more possibly interact with the cells on the cellular surface or via other pathway indirectly.

One possibility for indirect interaction is that GO and GO-NH₂ absorb the nutrients in culture medium and then the depletion of nutrients induces the oxidative stress and toxicity to A549 cells. Such toxicity mechanism has been reported in a study of CNTs [20]. Guo et al. reported that the depletion of nutrients by the absorption of amino acid and other biological molecules onto CNTs led to
severe toxicity to HepG2 cells. We found however increased ROS levels only in cells treated with the highest concentrations of GO and GO-NH$_2$ particles suggesting that this interaction is less likely. Moreover, Ming Xu et al. have measured the amount of proteins from the culture medium adsorbed on pristine GO and GO-NH$_2$ and have found that the pristine GO adsorbed higher amount of proteins than GO-NH$_2$ suggesting a higher cytotoxicity for GO which is inconsistent with our results.

Another possibility is that GO influences the cell adhesion ability of A549 cells. This mechanism is most probable because we found a decreased attachment of A549 cells exposed to GO-NH$_2$ particles as well as at the highest concentrations of both tested NPs. Although GO hardly enters A549 cells we found that the apoptosis of GO- and GO-NH$_2$ -treated A549 cells is much greater than that of the control cells. GO and GO-NH$_2$ induced statistically significant ROS generation only at high concentration. Oxidative stress is a well recognized toxicological mechanism of various nanoparticles [21,12]. At high dose, GO induces ROS generation, and this may contribute to the viability decrease of both GO particles at high concentration. Also, GO and GO-NH$_2$ induced DNA strand breaks at any of the studied doses but more pronounced at the highest doses. To make such a mechanism clear, more efforts are required.

5. Conclusions
In summary, the present study demonstrates that both tested NPs, pristine GO and GO-NH$_2$ exert a genotoxic effect at highest concentrations of 20 and 50 μg/ml pointing a dose-dependent genotoxicity effect in lung cancer A549 cells. Among both GO NPs ammonia-modified GO exert a more adverse effect on cell attachment and induced more intracellular ROS generation.

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