Effect of Graphene Oxide and Ammonia-modified Graphene Oxide Particles on ATPase Activity of Rat Liver Mitochondria

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Abstract: *Graphene and its derivatives have become promising materials for biomedical applications in the last decade. Before their widespread application, however, evaluating their toxicity and mechanisms underlying interactions with cellular components is imperative. Aims: Assessment of the effect of two graphene derivatives, pristine graphene oxide (GO) and ammonia-modified GO (GO-NH_2) particles, on the ATPase activity of rat liver mitochondria and ROS production. Methods: Liver mitochondria were isolated from male albino rats and treated with different concentrations of GO and GO-NH_2 particles (4, 10, 25, and 50 μg/ml). ATPase activity of both, intact and uncoupled by freezing/thawing mitochondria was determined by the measurement of inorganic phosphate (Pi) released from ATP. The generation of hydrogen peroxide (H_2O_2) after exposure of mitochondria to GO and GO-NH_2 particles was determined by a DCFH-D assay. Results: GO and GO-NH_2 particles applied at concentrations of 4 and 50 μg/ml did not affect the ATPase activity of intact mitochondria. In contrast, in uncoupled mitochondria, they demonstrated a stimulating effect on ATPase activity. The impact of GO-NH_2 was more substantial and concentration-dependent. ROS production was also higher in GO-NH_2-treated mitochondria. Conclusion: The present study demonstrated that GO and GO-NH_2 particles can exert a cytotoxic effect on mitochondria even after a short-time of exposure to both types of particles.*

Keywords: *Graphene-based flakes, Cytotoxicity, ATP production, Nanoparticle-induced mitochondrial toxicity.*

Introduction

In the last decade, graphene and its derivates have been under the scientific spotlight due to their exceptional physicochemical properties, such as extreme strength, remarkable light weight, large surface-to-volume ratio, chemical stability, and excellent thermal and electrical conductivity [10, 37]. Graphene is a two-dimensional material made of sp^2 hybridized carbon atoms, forming a hexagonal arrangement with a structure similar to honeycombs [14]. Recent progress has shown that graphene-based materials have a broad range of applications

which is expected to increase significantly [7, 16, 26, 28, 45, 47, 51, 52]. Graphene oxide (GO) is one of the most important graphene derivatives with unique properties such as low toxicity, irregular shape and geometry, high loading capacity, and simple and low-cost synthesis [1]. GO is a highly oxidized form of graphene with carboxylate groups on the periphery, providing a pH-dependent negative charge and colloidal stability, and hydroxyl and epoxide groups on the basal plane which are polar and hydrophilic. The remaining regions of the basal plane are unmodified, hydrophobic, and capable of binding biomolecules or drugs via π–π interactions [11]. Oxygen groups allow easy modification of GO, either covalently or non-covalently, with different molecules, including DNA, proteins, or drugs, allowing a wide range of biological applications [41, 43]. However, before the widespread application of GO, it is imperative to evaluate its potential risk to environmental and human health and establish an approach for the safe design of these materials.

The safety and biocompatibility of GO have been the subject of intense research in the scientific community [12]. The data in the literature, however, are very controversial. Some authors reported that GO is nontoxic for cells, even in a high dose of 200 μg/ml, while others have demonstrated that a low amount of 10 μg/ml GO interacted with DNA and induced cell apoptosis [2, 3, 30]. Others have shown that intravenous GO injections at 4 mg/kg for five days caused mutagenesis in mice [25]. The toxicity and biocompatibility of GO are highly dependent not only on concentration and exposure time but also on its intrinsic physicochemical properties such as morphology, size (lateral dimension, thickness, and number of layers), surface chemistry, and functional groups [6, 23, 24, 38]. The surface modification affects the physicochemical properties of nanomaterials, their processability and performance, toxicity, and impact on human health and the environment. Functionalization with different functional groups, for example, changes the degree of ionization, surface charge, and hydrophobicity, which consequently influence biological effects [40, 42]. Due to the abundance of carboxyl groups (-COOH), GO's surface is negatively charged. Through coupling reactions, the COOH groups can be functionalized with amino groups (e.g., NH_2-PEG-NH_2), which renders the surface positive, while functionalized with poly-*m*-aminobenzene sulfonic acid- (-NH_2/-SO_3H) or methoxyl- (-OCH_3) terminated functional groups makes the surface neutral [39]. It is generally believed that cationic particles are more toxic than anionic particles, whereas neutral particles are more biocompatible, mainly due to different affinities for negative phospholipids or proteins [39].

The toxicity of aminated GO nanoparticles is not well studied. Xu et al. have shown that aminated GO induces less toxicity in macrophages than pristine GO, while Singh et al. [35] have found that GO-NH_2 did not cause thrombogenicity in Swiss male mice under intravenous administration [44]. In our previous experiments, we observed that commercially available ammonia-modified GO with an approximate size of 560 nm in diameter induced apoptosis in lung cancer cells but did not influence the viability of noncancer embryonic stem cells [21].

Studies exploring nanoparticle toxicity have shown that nanoparticles can specifically target the mitochondria and exert toxic effects. The most probable mechanism of nanoparticle-induced mitochondrial toxicity is by generation of ROS. ROS are highly reactive chemical agents that can cause various harmful damage to cellular macromolecules. The overproduction of ROS following nanoparticle exposure can occur through direct damage to the mitochondria or following a reduction in the activity of the mitochondrial respiratory chain complexes, which can lead to the generation of ROS. Consequently, mitochondrial dysfunction may lead to apoptosis and inflammation [34]. There is a direct connection

between mitochondrial dysfunction and the toxic indices of nanoparticles. In addition to the inherent adverse effects associated with high levels of ROS, the ability of nanomaterials to alter mitochondrial dynamics can lead to changes in adenosine triphosphate (ATP) production levels. Mitochondria are the main site where ATP is synthesized by ATP synthase [17]. ATP synthase is the enzyme that uses adenosine diphosphate, inorganic phosphate, and an electrochemical gradient of protons through the internal mitochondrial membrane as substrates. The enzyme can function as ATP synthase (ATP synthesis) or ATPase (ATP hydrolysis). ROS inhibits ATP synthase activity, and oxidative stress is another factor that could contribute to the increase of ATPase under pathological conditions [5, 53].

Reduced levels and activities of respiration chain-related proteins and suppressed ATP production caused by graphene have been observed [29, 54]. However, there is a lack of data about graphene's direct or indirect effects and its derivatives on mitochondrial ATPase activity. Therefore, in the present work, we aimed to study the effects of two graphene derivatives – pristine GO and ammonia-modified GO (GO-NH_2) particles in different concentrations (4, 10, 25 and 50 μg/ml), on ATPase activity of rat liver mitochondria on both, intact mitochondria (coupled mitochondria), and freezing/thawing disrupted mitochondria. Additionally, we have measured the ROS production after 5 min exposure to freezing/thawing disrupted mitochondria to reveal if the direct damage of the mitochondria by nanoparticles results in the generation of ROS.

Materials and methods

Graphene oxide particles

Graphene oxide (C1576m Graphenea, Spain) and ammonia-modified graphene oxide (791520, Sigma-Aldrich, Germany) are commercially available products, purchased as water suspensions with concentrations of 4 mg/ml and 1 mg/ml, respectively. A stock solution of 1.0 mg/ml of GO and GO-NH_2 particles in distilled water was prepared for experiments. Just before the mitochondria exposure, the stock solutions were sonicated for 1 hour (40 kHz, 50 W) then mitochondria were treated with particles in different concentrations.

Physico-chemical characterization of GO and GO-NH_2 particles

Scanning electron microscopy characterized the morphology of GO sheets (SEM, JEOL, JSM-7100F, Japan). Images were acquired at an average working distance of 8 mm with an accelerating voltage of 15 kV. Before SEM imaging, GO and GO-NH_2 suspensions were sonicated for 30 min, deposited on a Si-wafer, and evaporated by a stream of nitrogen. UV-Vis absorption spectra were analyzed by Specord 210 Plus spectrophotometer (Edition 2010, Analytik Jena AG, Jena, Germany). The hydrodynamic diameters (HD) and the zeta (ζ-) potential of both particles in water were measured in triplicate by photon correlation spectroscopy (Zetatrac Instrument, S3500; Microtrac, Largo, FL).

Measurement of mitochondrial ATPase activity

Liver mitochondria were isolated from male albino rats (Wister strain, 50-60 days of age, 120-150 g, supplied by the Breeding base of the Faculty of Biology, Sofia University) as described in [4].

The ATPase activity of mitochondria was determined by the measurement of inorganic phosphate (Pi) released from ATP. The reaction with intact mitochondria was carried out at room temperature and continuous stirring in a 5 ml assay medium consisting of 200 mM sucrose, 10 mM KCl, 50 mM Tris-HCl, 100 μM EDTA-KOH, and 1 mM ATP (pH 7.5) and 50 μM 2,4-dinitrophenol (DNP) was included wherever indicated. Aliquots of particle stock

solution were added to reach final concentrations of 4 and 50 μg/ml, respectively. The reaction was started by adding mitochondrial suspension. Samples were taken after 15, 30, 60, 120, 180, and 300 s incubation and added to 3 M perchloric acid for termination of the reaction.

In a second set of experiments, ATPase activity of mitochondria uncoupled by freezing/thawing was assayed in 1 ml medium consisting of 200 mM sucrose, 10 mM KCl, 50 mM Tris-HCl, and 100 μM EDTA-KOH (pH 7.5). Aliquots of particle stock solution were added to reach final concentrations of 4, 10, 25, and 50 μg/ml, respectively. After pre-incubating the mitochondria in the assay medium for 30 min at 37 °C, the reaction was initiated by adding 1 mM ATP, continued for 5 min at 37 °C, and terminated by adding 3 M perchloric acid.

In all cases, the protein precipitates and particles were removed by centrifugation at 8800 g for 30 min. The concentration of Pi in the supernatant was determined spectrophotometrically at 750 nm (Spectrophotometer S-22UV/Vis, Boeco, Germany). Blanks in which the reaction was blocked by the addition of perchloric acid before ATP addition was carried out in parallel to determine the background Pi amount as a result of non-enzymatic hydrolysis. The activity of mitochondrial ATPase was expressed as μmol Pi/mg protein^{-}/min or μmol Pi/mg protein.

DCFA-DA analysis

Generation of hydrogen peroxide (H_2O_2) after exposure of mitochondria to GO and GO-NH_2 particles was determined by using 2',7'-dichlorofluorescein diacetate (DCFH-DA, Sigma-Aldrich). DCFH is a lipid-permeable non-fluorescent compound that oxidized by intracellular H_2O_2 forms the fluorescent compound 2',7'-dichlorofluorescein (DCF). Particles in different concentrations (4 and 50 μg/ml) were added to freezing-thawing disrupted mitochondria diluted with sucrose (250 mM) in a ratio of 1:4, and incubated for 5 min at room temperature (RT). Then the particles were removed by centrifugation for 15 s at 12 000 rpm. 10 μm DCFH-DA was added to each supernatant followed by 30 min incubation at RT and dark. The samples were washed by 5 min centrifugation at 14 500 rpm; the pellet was resuspended to 1 ml volume with sucrose, and fluorescence intensity of DCF was detected on a spectrofluorometer with excitation at 485 nm and emission at 530 nm. Untreated mitochondria were used as a control fluorescence was measured at 485 nm extension and 520 nm.

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM) of at least six independent experiments performed in triplicate unless otherwise indicated. When appropriate, the difference between GO- or GO-NH_2-treated samples and the untreated control was tested by independent samples Student's t-test. For some groups, the Shapiro-Wilk test showed that the data were not normally distributed and a non-parametric Mann-Whitney rank sum test was performed. A value of $p < 0.05$ was considered significant. All statistical analyses were computed using SigmaPlot version 11.0.

Results and discussion

Physicochemical characterization of GO and GO-NH_2 particles

Results from the physicochemical characterization of pristine and ammonia-modified GO particles showed that both GO particles differ significantly in their physicochemical

parameters. The morphology of GO and aminated GO sheets were investigated by SEM, and the images are presented in Fig. 1. Both, GO and GO-NH_2 have wrinkled and curled sheet-like structures with a rougher surface of GO-NH_2 than those of GO. The intensity of the backscattered electrons, resulting from the collision between the electron beam and the atoms within the specimen, depends on the nucleus size. Elements with a larger atomic nucleus appear brighter in the image. The higher degree of GO-NH_2 functionalization with amino-containing groups causes a much higher degree of brightness of the flakes than that of non-functionalized GO, as can be seen in Fig. 1.

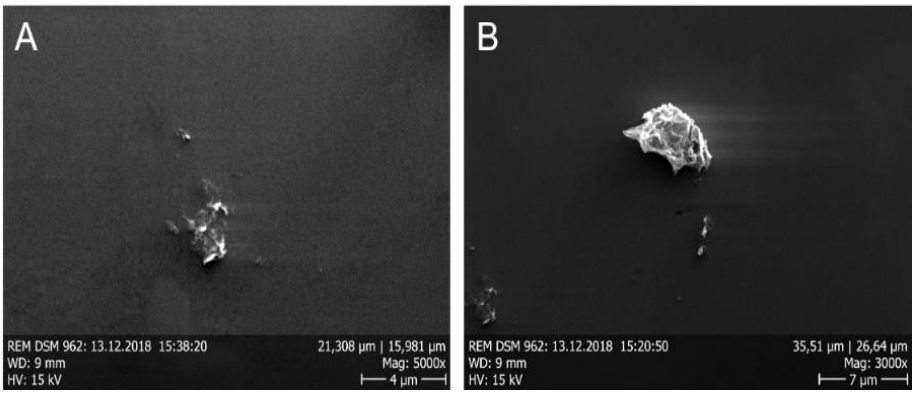

Fig. 1 SEM micrographs of GO (A) and ammonia-modified GO (GO-NH_2) (B) sheets

Further, the results from the measurements of hydrodynamic diameters (HD) showed that the GO flakes are more heterogeneous and their size is significantly larger than those of GO-NH_2 (Table 1). The pristine GO in the aqueous suspension consists of two fractions with very different sizes: a smaller fraction with an average particle size of 250 nm (9.7% of the particles) and a predominant fraction (90.3%) with an average particle size of 1.5 µm. GO-NH_2 fraction is more homogenous consisting of particles with an average size of 560 nm. The main fraction of GO had dimensions of a few microns which are approximately three times larger than the dimensions of GO-NH_2 flakes. Probably the reason for the smaller size of GO-NH_2 is that the amination of GO leads to the wrinkling and shrinking of GO and, consequently, to the reduction of the size of the sheets. In our previous studies, we have investigated the morphology of both, pristine GO and aminated GO sheets under TEM and found a similar, more shrunk morphology for GO-NH_2 with many folds and wrinkles, compared to the pristine GO [19, 21]. Moreover, [33] reported that the elastic corrugations and the scrolled or folded edges often result in different brightness on the surface of the GO which was observed during SEM investigation of GO's sheets. The surface charges represented as zeta (ζ-) potential measured in water were negative for GO with a value of -24.5 mV indicating the presence of carboxylic functional groups, and positive for GO-NH_2 with a value of 38.5 mV, pointing to the presence of amino groups (Table 1).

Table 1. Average particle size and surface charge of the pristine GO and ammonia-modified GO (GO-NH_2) particles estimated by Zetasizer

Sample	Average particle size, (nm)	Surface charge, (mV)
GO	250.0 ± 68.0 1500.0 ± 0.7	-24.5 ± 0.4
GO-NH_2	560.0 ± 300.0	38.5 ± 2.8

The adsorption spectra of both particles measured in the range of 190-590 nm are given in Fig. 2. UV/Vis spectroscopy measurements showed that GO has a typical absorbance peak at ~230 nm due to $\pi \rightarrow \pi^*$ transition of the aromatic C–C ring. A weak shoulder peak at ~300 nm was ascribed to the $n \rightarrow \pi^*$ transitions of C=O bonds, confirming the presence of oxygen-containing functional groups [46]. However, this shoulder peak was not observed in the UV-Vis spectrum of GO-NH_2. In ammonia-modified GO, the absorbance peak was red-shifted at ~250 nm and GO-NH_2 exhibited higher absorbance at a long wavelength (more than 300 nm) (Fig. 2), pointing out that the ammonia strengthened the reduction extent of GO during the process of amination. Additionally, a colour change of GO suspension from light brown to black was observed during ammonia modification (Fig. 2, the built-in photo) verifying the reduction of GO under amination. The dark colour is typical for reduced graphene oxide and has been reported previously in many works [46, 48].

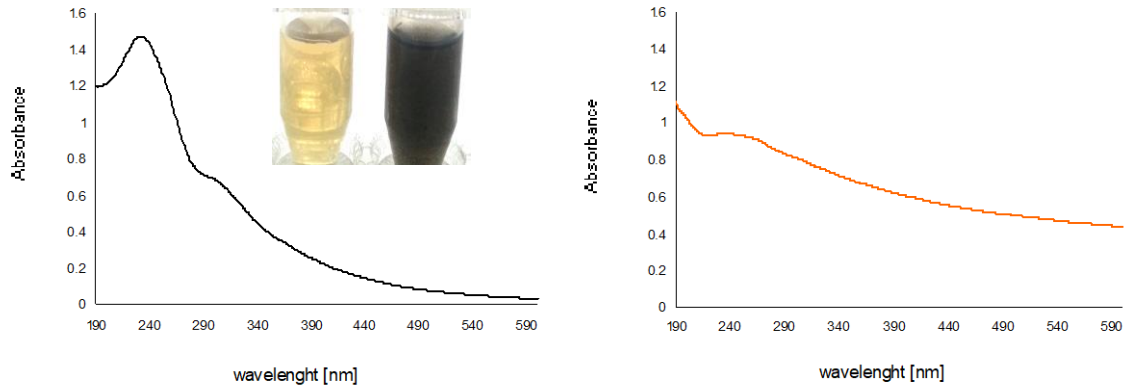

Fig. 2 UV-Vis-absorption spectra and photographs of GO and GO-NH_2 particles.

All registered differences between pristine and ammonia-modified GO flakes can be due to the differences in their synthesis. The method of synthesis and the source of carbon need to be considered since it has a significant impact on the structure and chemical composition of obtained GO [36]. The most commonly used method for GO synthesis is the Hummers method [15]. GO produced by Hummers's approach is relatively rich in oxygen groups compared to GO produced by other methods, and this is essential for further attempts at GO surface modifications. The ammonia-modified GO particles which are the subject of our study are also produced by Hummer's method according to the manufacturer datasheet and modified further by treating GO with NH_3. This tunes the electronic structure by introducing amino and amide groups to its surface and results in a positively charged surface, wrinkled and curled sheet-like structure, and smaller size when compared to pristine GO [18].

Effects of GO and GO-NH_2 particles on intact mitochondria

GO particles were initially tested for possible uncoupling action on intact liver mitochondria. Fig. 3 shows the results from one representative experiment with GO (Fig. 3A) and GO-NH_2 (Fig. 3B) applied in concentrations of 4 and 50 μg/ml respectively. ATPase activity remained low during the registration under control conditions. The addition of the uncoupler DNP powerfully stimulated ATP hydrolysis which indicated a normal functional state of mitochondria and a low permeability of their inner membrane. GO and GO-NH_2 particles in both tested concentrations did not show an uncoupling effect on intact mitochondria.

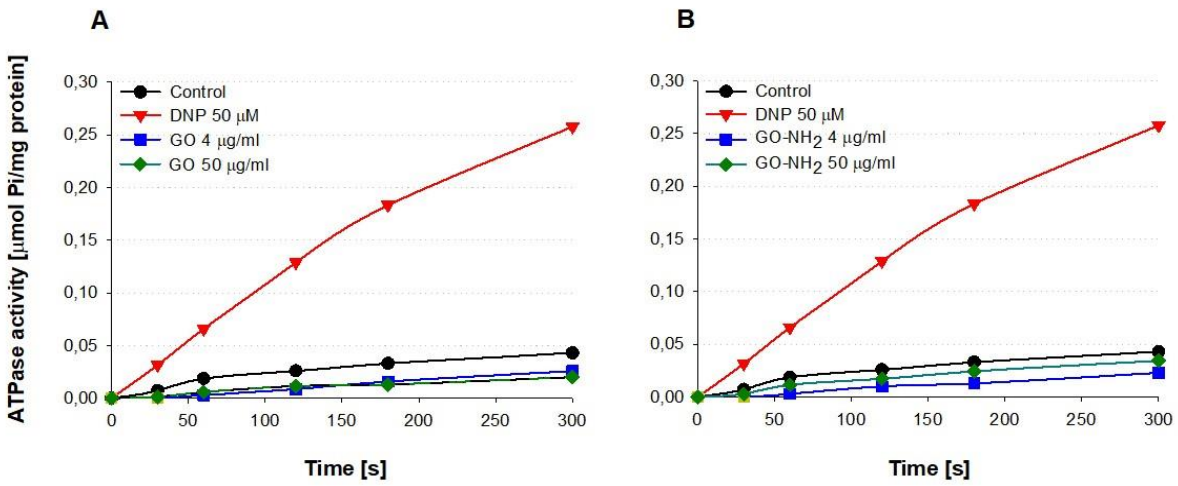

Fig. 3 Effects of GO (A) and GO-NH_2 (B) particles on ATPase activity of intact mitochondria. The reactions were started by adding 2.75 mg of mitochondrial protein and carried out for 5 min as described in Materials and methods. Data are from one representative experiment.

Changes in the mitochondrial structure and function with a decreased membrane potential, impaired ATP production and ROS generation have been demonstrated in studies with various cell lines treated with GO and GO-related nanomaterials [9, 32, 49, 50]. However, in all these experiments the duration of the treatment varied from 4 h to 96 h. In the present study, intact mitochondria were exposed to GO and GO-NH_2 during the registration of ATPase reaction for 5 min. To keep the freshly isolated mitochondria intact, pre-incubating them with the nanoparticles was not appropriate, therefore we followed GO and GO-NH_2 effects only during the registration in the course of the ATPase reaction. Furthermore, this allows studying nanoparticles' effects on the enzyme activity during the first minutes after the reaction initiation, when its rate is the highest. The short time of exposure could explain the lack of an uncoupling effect.

Effects of GO and GO-NH_2 particles on freeze-thawed mitochondria

Freezing/thawing disrupts the mitochondrial inner membrane leading to uncoupling and stimulation of initial ATPase activity. In parallel with this, mitochondria preserve their structure and the membrane-spanning F_0 sector of the ATPase.

ATPase activity values were calculated as percentages of the activity (expressed as µmol Pi/mg protein/min) measured under control conditions (particle-free assay medium). GO in concentrations of 4, 10, 25, and 50 µg/ml stimulated ATPase activity of freeze-thawed mitochondria to 142.36 ± 18.43% of the control (in a concentration of 10 µg/ml), but the differences between the groups were not statistically significant (Fig. 4).

The effect of GO-NH_2 was more pronounced and concentration-dependent (Fig. 4). ATPase activity was stimulated to 251.11 ± 100.27% of the control (in a concentration of 50 µg/ml).

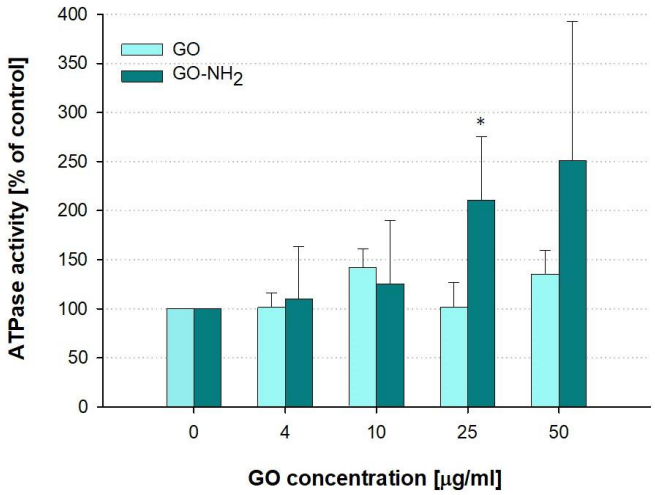

Fig. 4 Effect of GO and GO-NH_2 particles on ATPase activity of freeze-thawed mitochondria. ATPase activity values were calculated as percentages of the activity measured under control conditions (particles-free assay media). Data are plotted as mean ± SEM of six independent experiments (three parallel samples per group per experiment).
The asterisk indicates a significant difference (*p = 0.04).

Effects of GO and GO-NH_2 particles on ROS generation

The measurement of ROS production and more correctly, of hydrogen peroxide (H_2O_2) after 5 min treatment of freezing-thawing disrupted mitochondria with GO and GO-NH_2 particles showing that only the higher concentrations of GO's NPs resulted in a generation of ROS in the disturbed mitochondria. Among both types of particles aminated GO produced more ROS suggesting a higher toxicity (Fig. 5).

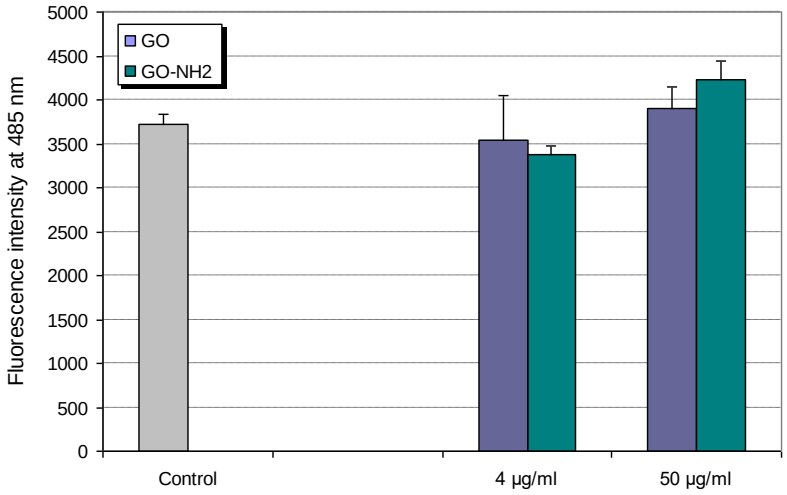

Fig. 5 Effect of GO and GO-NH_2 particles on ROS production of freeze-thawed mitochondria

These findings demonstrate a stronger effect of GO-NH_2 as compared to those of GO. This is in agreement with the results from our previous study which showed that ammonia-modified GO particles were more toxic than pristine GO in both, tumour lung A549 cells and non-tumour, embryonic Lep3 cells [19]. The stronger effects of GO-NH_2 than that of the pristine GO could be due to their different chemical and physical properties. Particles' parameters such as size, zeta-potential, and ability to aggregate in the medium used can influence their interactions with cell and intracellular organelles. Greater damage to the

cell membrane caused by the larger-sized GO as compared to the smaller-sized GOs was observed [49]. Other authors reported stronger cytotoxicity induced by nanoparticles with smaller dimensions [8, 27]. Smaller silver nanoparticles cause more significant cytotoxicity than larger silver NPs because of their larger surface area available for interaction with cellular organelles [20].

Our previous study on silver nanoparticles [4] also demonstrated more significant effects of starch-stabilized silver nanoparticles on ATPase activity of rat liver mitochondria compared to those of raffinose-stabilized silver nanoparticles, which could be assigned mainly to their smaller size (15.4 ± 3.9 nm and 24.8 ± 6.8 nm, respectively). In the present study, the stronger effect of GO-NH_2 than that of GO could also be referred to the smaller size of the former. The GO-NH_2 fraction was more homogenous with an average size of the particles of 560 ± 300 nm whereas the main fraction of GO consisted of particles with an average size of 1.5 ± 0.7 μm. In addition, the positive zeta-potential of GO-NH_2 (38.5 ± 2.8 mV) can contribute to their increased reactivity compared to that of the negatively charged GO particles (zeta-potential of -24.5 ± 0.4 mV). Positively charged nanoparticles are considered to possess higher reactivity towards the negative cell surface [13] and, hence, they can be more toxic due to a destructive effect on the membranes [22, 31].

Conclusions

It was demonstrated that pristine GO and ammonia-modified GO particles do not possess an uncoupling effect on intact liver mitochondria but stimulate the ATPase activity of mitochondria uncoupled by freezing/thawing with a more pronounced and concentration-dependent effect of GO-NH_2. The stronger effect of GO-NH_2 compared to those of GO could be explained by their physicochemical properties – increased reactivity of GO-NH_2 particles due to their smaller size (560 ± 300 nm vs. 1.5 ± 0.7 μm for GO-NH_2 and GO, respectively) and positive zeta-potential (38.5 ± 2.8 mV vs. -24.5 ± 0.4 mV for GO-NH_2 and GO, respectively).

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References

1. Banerjee A. N. (2018). Graphene and Its Derivatives as Biomedical Materials: Future Prospects and Challenges, Interface Focus, 8(3), 20170056.
2. Bengtson S., K. Kling, A. M. Madsen, A. W. Noergaard, et al. (2016). No Cytotoxicity or Genotoxicity of Graphene and Graphene Oxide in Murine Lung Epithelial FE1 Cells *in vitro*, Environ Mol Mutagen, 57(6), 469-482.
3. Chang Y., S. T. Yang, J. H. Liu, E. Dong, et al. (2011). *In vitro* Toxicity Evaluation of Graphene Oxide on A549 Cells, Toxicol Lett, 200(3), 201-210.

4. Chichova M., M. Shkodrova, P. Vasileva, K. Kirilova, et al. (2014). Influence of Silver Nanoparticles on the Activity of Rat Liver Mitochondrial ATPase, J Nanopart Res, 16, 2243.
5. Chinopoulos C., V. Adam-Vizi (2010). Mitochondria as ATP Consumers in Cellular Pathology, Biochim Biophys Acta (BBA) – Mol Basis Dis, 1802, 221-227.
6. Chng E. L., C. K. Chua, M. Pumera (2014). Graphene Oxide Nanoribbons Exhibit Significantly Greater Toxicity than Graphene Oxide Nanoplatelets, Nanoscale, 6(18), 10792-10797.
7. Dikin D. A., S. Stankovich, E. J. Zimney, R. D. Piner, et al. (2007). Preparation and Characterization of Graphene Oxide Paper, Nature, 448(7152), 457-460.
8. Ema M., M. Gamo, K. Honda (2017). A Review of Toxicity Studies on Graphene Based Nanomaterials in Laboratory Animals, Regul Toxicol Pharmacol, 85, 7-24.
9. Feng X., Y. Zhang, R. Luo, X. Lai, et al. (2021). Graphene Oxide Disrupted Mitochondrial Homeostasis through Inducing Intracellular Redox Deviation and Autophagy-lysosomal Network Dysfunction in SH-SY5Y Cells, J Hazardous Materials 416, 126158.
10. Geim A. K., K. S. Novoselov (2007). The Rise of Graphene, Nature Materials, 6(3), 183-191.
11. Georgakilas V., M. Otyepka, A. B. Bourlinos, V. Chandra, et al. (2012). Functionalization of Graphene: Covalent and Non-covalent Approaches, Derivatives and Applications, Chem Rev, 112(11), 6156-214.
12. Guo Z., S. Chakraborty, F. A. Monikh, D. D. Varsou, et al. (2021) Surface Functionalization of Graphene-based Materials: Biological Behavior, Toxicology, and Safe-by-design Aspects, Adv Biology, 5(9), e2100637.
13. Honary S., F. Zahir (2013). Effect of Zeta Potential on the Properties of Nano-Drug Delivery Systems – A Review (Part 2), Tropical Journal of Pharmaceutical Research, 12(2), 265-273.
14. Huang X., Z. Yin, S. Wu, X. Qi, et al. (2011). Graphene-based Materials: Synthesis, Characterization, Properties, and Applications, Small, 7(14), 1876-902.
15. Hummers W. S., R. E. Offeman (1958). Preparation of Graphitic Oxide, J Am Chem Soc, 80(6), 1339-1339.
16. Ibrahim A., A. Klopocinska, K. Horvat, Z. A. Hamid (2021). Graphene-based Nanocomposites: Synthesis, Mechanical Properties, and Characterizations, Polymers (Basel), 13(17), 2869.
17. Juménez C. S., F. P. P. Moisés, M. E. Cano, K. N. Andrade, et al. (2020). Folate- and Glucoronate-functionalization of Layered Double Hydroxides Containing Dysprosium and Gadolinium and the Effect on Oxidative Stress in Rat Liver Mitochondria, Heliyon, 6(1), e03111.
18. Kasztelan M., A. Słoniewska, M. Gorzkowski, A. Lewera, et al. (2021). Ammonia Modified Graphene Oxide-gold Nanoparticles Composite as a Substrate for Surface Enhanced Raman Spectroscopy, Applied Surface Science, 554, 149060.
19. Keremidarska-Markova M., K. Hristova-Panusheva, T. Andreeva, G. Speranza, et al. (2018). Cytotoxicity Evaluation of Ammonia-modified Graphene Oxide Particles in Lung Cancer Cells and Embryonic Stem Cells, Advances in Condensed Matter Physics, 1-11.
20. Kim S., D.-Y. Ryu (2013). Silver Nanoparticle-induced Oxidative Stress, Genotoxicity and Apoptosis in Cultured Cells and Animal Tissues, J Appl Toxicol, 33, 78-89.
21. Krasteva, N., M. Keremidarska-Markova, K. Hristova-Panusheva, T. D. Andreeva, et al. (2019). Aminated Graphene Oxide as a Potential New Therapy for Colorectal Cancer, Oxidative Medicine and Cellular Longevity, 2019, 3738980, https://doi.org/10.1155/2019/3738980.

22. Lee J.-K., H.-S. Lim, J.-H. Kim (2002). Cytotoxic Activity of Amino Derivatized Cationic Chitosan Derivatives, Bioorganic & Medicinal Chemistry Letters, 12, 2949-2951.
23. Liu J. H., S. T. Yang, H. Wang, Y. Chang, et al. (2012). Effect of Size and Dose on the Biodistribution of Graphene Oxide in Mice, Nanomedicine, 7(12), 1801-1812.
24. Liu S., M. Hu, T. H. Zeng, R. Wu, et al. (2012). Lateral Dimension-dependent Antibacterial Activity of Graphene Oxide Sheets, Langmuir, 28(33), 12364-12372.
25. Liu Y., Y. Luo, J. Wu, Y. Wang, et al. (2013). Graphene Oxide Can induce *in vitro* and *in vivo* Mutagenesis, Sci Rep, 3, 3469.
26. Magne T. M., T. de Oliveira Vieira, L. M. R. Alencar, F. F. M. Junior, et al. (2022). Graphene and Its Derivatives: Understanding the Main Chemical and Medicinal Chemistry Roles for Biomedical Applications, J Nanostruct Chem, 12, 693-727.
27. Noga M., J. Milan, A. Frydrych, K. Jurowski (2023). Toxicological Aspects, Safety Assessment, and Green Toxicology of Silver Nanoparticles (AgNPs) – Critical Review: State of the Art, Int J Mol Sci, 24(6), 5133.
28. Paek S. M., E. Yoo (2009). Honma Enhanced Cyclic Performance and Lithium Storage Capacity of SnO2/graphene Nanoporous Electrodes with Three-dimensionally Delaminated Flexible Structure, Nano Lett, 9(1), 72-75.
29. Park E.-J., G.-H. Lee, B. S. Han, B.-S. Lee, et al. (2015). Toxic Response of Graphene Nanoplatelets *in vivo* and *in vitro*, Arch Toxicol, 89(9), 1557-68.
30. Pelin M., L. Fusco, V. Leyn, C. Martín, et al. (2017). Differential Cytotoxic Effects of Graphene and Graphene Oxide on Skin Keratinocytes, Sci Rep, 7, 40572.
31. Qi L. Z. Xu, X. Jiang, Y. Li, et al. (2005). Cytotoxic Activities of Chitosan Nanoparticles and Copper-loaded Nanoparticles, Bioorg Med Chem Lett, 15, 1397-1399.
32. Rahimi S., Y. Chen, M. Zareian, S. Pandit, et al. (2022). Cellular and Subcellular Interactions of Graphene-based Materials with Cancerous and Non-cancerous Cells, Adv Drug Del Rev, 189, 114467.
33. Senapati S., A. K. Mahanta, S. Kumar, M. Maiti (2018). Controlled Drug Delivery Vehicles for Cancer Treatment and Their Performance, Signal Transduction and Targeted Therapy, 3-7.
34. Simon H. U., A. Haj-Yehia, F. Levi-Schaffer (2000). Role of Reactive Oxygen Species (ROS) in Apoptosis Induction, Apoptosis, 5, 415-418.
35. Singh S. K., M. K. Singh, P. P. Kulkarni, V. K. Sonkar, et al. (2012). Amine-modified Graphene: Thrombo-protective Safer Alternative to Graphene Oxide for Biomedical Applications, ACS Nano, 6(3), 2731-2740.
36. Smith A. T., A. M. LaChance, S. Zeng, B. Liu, et al. (2019). Synthesis, Properties, and Applications of Graphene Oxide/reduced Graphene Oxide and Their Nanocomposites, Nano Mater Sci, 1, 31-47.
37. Stankovich S., D. A. Dikin, G. H. B. Dommett, K. M. Kohlhaas, et al. (2006). Graphene-based Composite Materials, Nature, 442(7100), 282-286.
38. Talukdar Y., J. Rashkow, G. Lalwani, S. Kanakia, et al. (2014). The Effects of Graphene Nanostructures on Mesenchymal Stem Cells, Biomaterials, 35(18), 4863-4877.
39. Tu Q., L. Pang, Y. Chen, Y. Zhang, et al. (2014). Effect of Surface Charges of Graphene Oxide on Neuronal Outgrowth and Branching, Analyst, 139(1), 105-115.
40. Vallabani N. V., S. Mittal, R. K. Shukla, A. K. Pandey, et al. (2011). Toxicity of Graphene in Normal Human Lung Cells (BEAS-2B), J Biomed Nanotechnol, 7(1), 106-107.
41. Wan C., B. Chen (2011). Poly (E-caprolactone)/graphene Oxide Biocomposites: Mechanical Properties and Bioactivity, Biomed Mater, 6, 055010.

42. Wang A., K. Pu, B. Dong, Y. Liu, et al. (2013). Role of Surface Charge and Oxidative Stress in Cytotoxicity and Genotoxicity of Graphene Oxide towards Human Lung Fibroblast Cells, J Appl Toxicol, 33(10), 1156-1164.
43. Wu S. Y., S. S. An, J. Hulme (2015). Current Applications of Graphene Oxide in Nanomedicine, Int J Nanomedicine, 10(Special issue), 9-24.
44. Xu M., J. Zhu, F. Wang, Y. Xiong, et al. (2016). Improved *in vitro* and *in vivo* Biocompatibility of Graphene Oxide through Surface Modification: Poly(AcrylicAcid)-Functionalization is Superior to PEGylation, ACS Nano, 10, 3267-3281.
45. Yang K., S. Zhang, G. Zhang, X. Sun, et al. (2010). Graphene in Mice: Ultrahigh *in vivo* Tumor Uptake and Efficient Photothermal Therapy, Nano Lett, 10(9), 3318-3323.
46. Yang S., Y. Wenbo, D. Huang, C. Chen, et al. (2012). A Facile Green Strategy for Rapid Reduction of Graphene Oxide by Metallic Zinc, RSC Adv, 2, 8827-8832.
47. Yang X. Y., X. Y. Zhang, Z. F. Liu, Y. F. Ma, et al. (2008). High Efficiency Loading and Controlled Release of Doxorubicin Hydrochloride on Graphene Oxide, J Phys Chem C, 112(45), 17554-17558.
48. Yeh T.-F., S.-J. Chen, C.-S. Yeh, H. Teng (2013). Tuning the Electronic Structure of Graphite Oxide through Ammonia Treatment for Photocatalytic Generation of H_2 and O_2 from Water Splitting, J Phys Chem C, 117(13), 6516-6524.
49. Zhang B., P. Wei, Z. Zhou, T. Wei (2016). Interactions of Graphene with Mammalian Cells: Molecular Mechanisms and Biomedical Insights, Adv Drug Del Rev, 105, 145-162.
50. Zhang H., C. Peng, J. Yang, M. Lv, et al. (2013). Uniform Ultrasmall Graphene Oxide Nanosheets with Low Cytotoxicity and High Cellular Uptake, ACS Appl Mater Interfaces, 5, 1761e7.
51. Zhang L. M., J. G. Xia, Q. H. Zhao, L. W. Liu, et al. (2010). Functional Graphene Oxide as a Nanocarrier for Controlled Loading and Targeted Delivery of Mixed Anticancer Drugs, Small, 6(4), 537-544.
52. Zhang X. Y., J. L. Yin, C. Peng, W. Q. Hu, et al. (2011). Distribution and Biocompatibility Studies of Graphene Oxide in Mice after Intravenous Administration, Carbon, 49(3), 986-995.
53. Zhang Y., O. Marcillat, C. Giulivi, L. Ernster, et al. (1990). The Oxidative Inactivation of Mitochondrial Electron Transport Chain Components and ATPase, J Biol Chem, 265, 16330-16336.
54. Zhou H., B. Zhang, J. Zheng, M. Yu, et al. (2014). The Inhibition of Migration and Invasion of Cancer Cells by Graphene via the Impairment of Mitochondrial Respiration, Biomaterials, 35, 1597-1607.

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