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Minimizing Oxidation and Stable Nanoscale Dispersion Improves the Biocompatibility of Graphene in the Lung

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Abstract

To facilitate the proposed use of graphene and its derivative graphene oxide (GO) in widespread applications, we explored strategies that improve the biocompatibility of graphene nanomaterials in the lung. In particular, solutions of aggregated graphene, Pluronic dispersed graphene, and GO were administered directly into the lungs of mice. The introduction of GO resulted in severe and persistent lung injury. Furthermore, in cells, GO increased the rate of mitochondrial respiration and the generation of reactive oxygen species, activating inflammatory and apoptotic pathways. In contrast, this toxicity was significantly reduced in the case of pristine graphene after liquid phase exfoliation, and was further minimized when the unoxidized graphene was well-dispersed with the block copolymer Pluronic. Our results demonstrate that the covalent oxidation of graphene is a major contributor to its pulmonary toxicity and suggest that dispersion of pristine graphene in Pluronic provides a pathway for the safe handling and potential biomedical application of two-dimensional carbon nanomaterials.

Keywords

graphene; graphene oxide; biocompatibility; pluronic; poloxamer

Graphene is a flat monolayer of carbon atoms tightly packed in a two-dimensional honeycomb lattice. Both graphene and its oxidized derivative graphene oxide (GO) exhibit remarkable electronic, chemical and mechanical properties that suggest their use in a variety of applications ranging from electronics to therapeutics.^{1–6} A GO flake is a layered graphene sheet with epoxide, carboxyl and hydroxyl groups on its basal planes and edges.^{2, 7, 8} PEGylated GO has shown promise in cancer therapy^{4, 5} and several studies support its biocompatibility.^{3–6, 9} However, the results of studies examining the biologic effects of GO itself are inconclusive.^{10–12} Unlike GO, which is highly dispersed in aqueous solutions, the hydrophobic nature of pristine graphene results in large aggregates, and it

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Supporting Information Available: Graphene and GO synthesis, graphene and GO optical absorbance and Raman spectra, atomic force microscopy images of graphene and GO, mitochondrial ROS generation and apoptosis results. Detailed methods are also provided. This material is available free of charge via the Internet at <http://pubs.acs.org>.

must be stabilized via a surfactant to remain highly dispersed. We sought to elucidate processes to enhance the biocompatibility of graphene preparations to facilitate their proposed use in large-scale biomedical, electronic, and energy technologies.

Here, we find that GO is highly toxic when administered directly to the lungs of mice, causing severe and persistent lung injury. In cells, GO donated electrons to site I/II of the mitochondrial electron transport chain, increasing mitochondrial respiration and the generation of reactive oxygen species, thereby activating inflammatory and apoptotic pathways. We find that the toxicity was significantly reduced by the generation of pristine graphene via liquid phase exfoliation, and was further minimized when dispersed with the block copolymer Pluronic. As the toxicity of both aggregated and dispersed graphene is minimal, our findings indicate a major contributor to the pulmonary toxicity of GO is the functional groups introduced during the oxidative process. These results further suggest that the generation of stable nanoscale dispersed aqueous/Pluronic preparations of graphene enhance the biocompatibility and improve the delivery of graphene in the lung.

Materials and Methods

Detailed methods for the generation of the graphene and graphene oxides preparations and their characterization by optical absorbance spectroscopy, Raman Spectroscopy, atomic force microscopy, and X-ray photoelectron spectroscopy are supplied in the Supporting Information. Briefly, dispersed graphene flakes were produced by ultrasonication of natural graphite flakes (3061 grade material from Asbury Graphite Mills) in 2% w/v aqueous solution of Pluronic F 108NF (BASF Corporation) followed by centrifugation at ~4620 g for 30 min to remove large flakes.^{13, 14} This procedure was followed by ultracentrifugation at 288,000 g for 12 hours to concentrate and further purify the solution. Aggregated graphene was produced by flocculation through the addition of 4 parts isopropyl alcohol to one part dispersed graphene followed by 4 wash steps in deionized water. GO was produced by a modified Hummer's method from the same natural graphite flakes used for graphene production. This oxidation process was followed by ultrasonication to achieve dispersed material and centrifugation at 15,000 g for 5 minutes to remove any remaining large flakes. Photographs of the resulting preparations are shown in Figure S1. The resulting GO contains significantly higher levels of hydroxyl and carbonyl functional groups compared to dispersed graphene (Supporting Information Figure S2).

The protocol for the use of mice was approved by the Animal Care and Use Committee at Northwestern University. We treated 8–12 week old, 20–25 g, male, C57BL/6 mice intratracheally with equal weights and volumes of nanomaterials or control vehicles (50 µg/mouse in a total volume of 50 µL/mouse) as previously described (details in the Supporting Information).¹⁵ The nanomaterials included: (1) aggregated graphene (graphene in water), (2) dispersed graphene (graphene in 2% Pluronic) and (3) GO (in water) delivered in two equal aliquots, 3 minutes apart.¹⁵ Dispersed graphene and GO solutions were vortexed immediately prior to instillation. Aggregated graphene in water was vortexed and sonicated for 10 minutes prior to instillation. After each aliquot, the mice were placed in the right and then the left lateral decubitus position for 10–15 seconds. A detailed assessment of lung injury and fibrosis was performed using histology, measurement of bronchoalveolar lavage leukocytes and pro-inflammatory cytokine levels, electron microscopy and measurement of total lung collagen using whole lung picrosirius red collagen precipitation and microscopic analysis of Trichrome and Sirius Red stained lung sections. Systemic inflammation was assessed by measuring thrombin-antithrombin (TAT) complex levels in citrated plasma. All of these procedures are described in the Supporting Information and in our previous publications.^{15–19}

Cellular assays were performed using mouse alveolar macrophages (MHS) and epithelial cells (MLE 12) (catalog no CRL-2019 and CRL-2110, respectively, ATCC, Manassas, VA). We used a lentiviral vector encoding a mitochondrially localized oxidant sensitive GFP probe to generate stable cell lines for the measurement of mitochondrial reactive oxygen species (ROS) by flow cytometry as we have previously described with further details in the Supporting Information.^{20, 21} Cell death was assessed using a commercially available photometric immunoassay that detects histone-associated DNA fragments (Roche Applied Science).²² Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining of murine lung sections was performed utilizing the TUNEL AP In Situ Cell Death Detection Kit (Roche Diagnostics Corp.) according to manufacturer's directions, as previously described.²³ For electron transport measurements, MHS cells were grown on a 96 well plate, and 24 hours later oxygen consumption was measured using a Seahorse XF analyzer as we have previously described.¹⁸ Mitochondrial respiration was determined by subtracting the respiration measured in the presence of rotenone (10 μ M) and antimycin A (4 μ M), which inhibit complexes I and III, respectively. Coupled respiration was determined as the total mitochondrial respiration less the respiration measured after administration of the ATP synthase inhibitor oligomycin (5 μ g/ml). Uncoupled respiration was determined as the mitochondrial respiration after inhibition with oligomycin.

Results and Discussion

The graphene and GO materials were characterized via optical absorbance spectroscopy, Raman spectroscopy and atomic force microscopy. Optical absorbance measurements demonstrated significantly decreased absorbance in the visible for GO (15 $\times$ less at 660 nm) as compared to Pluronic-dispersed graphene (Figure 1A), which is consistent with previously published results.²⁴ Raman spectra gathered at an excitation wavelength of 514 nm (Figure 1B) showed clear G, D, and 2D bands in locations consistent with published spectra for Pluronic-dispersed graphene²⁵ and GO²⁶. To determine the thickness and size of the flakes, the graphene and GO dispersions were deposited onto SiO₂ substrates and imaged by atomic force microscopy (Figure 1C–F). The dispersed graphene was found to have a thickness ranging from 1.2 to 5.0 nm with areas up to 40,000 nm². In contrast, GO had thicknesses ranging from 0.5 to 2.0 nm and areas up to 200,000 nm². However, in both cases, more than 90% of the flakes had areas less than 25,000 nm².

Gross examination of paraffin embedded lung blocks from mice treated 24 hours earlier showed a more homogenous distribution pattern in mice treated with the Pluronic dispersed graphene compared with the aggregated graphene. In lung sections from animals treated with Pluronic-dispersed graphene, we observed lung macrophages with a homogenous black cytoplasm throughout the lung, which were not observed in vehicle treated animals (Figure 2A–E). In mice treated with graphene aggregates suspended in saline, we observed the aggregates in medium sized airways surrounded by macrophages with a homogenous black cytoplasm (Figure 2A–E). To quantify the differences in the distribution of the dispersed and aggregated graphene in the lung, we counted the number of randomly chosen 600 X fields containing macrophages with black cytoplasm. In mice treated with nanoscale dispersed graphene, 91 $\pm$ 4% of the high powered (600X) fields contained macrophages with a black cytoplasm compared with 36 $\pm$ 1% in the mice treated with graphene aggregates. These results suggest that nanoscale dispersion of graphene improves its distribution in the lung after airway instillation.

In mice treated 24 hours earlier with nanoscale dispersed or aggregated graphene, we found minimal histologic evidence of lung inflammation (Figure 2B–F). In marked contrast, we observed severe lung inflammation with alveolar exudates and hyaline membrane formation in mice treated with GO (Figure 2A–E). The lung injury induced by GO treated mice was

evident in low power electron micrographs (Figure 2F) and was accompanied by a leakage of protein into the alveolar space, BAL fluid pleiocytosis, and elevated BAL levels of pro-inflammatory cytokines (Figure 2G–K). We have reported that lung inflammation following particle exposure is sufficient to induce a prothrombotic state in mice, which may contribute to the increased risk of heart attack and stroke observed in humans exposed to particulate matter air pollution.¹⁶ Significant increases in plasma thrombin antithrombin complexes were observed only in GO treated animals (Figure 2L). The severity of the lung injury in GO-treated animals prompted us to examine the number of apoptotic cells in the lung, which we and others have shown can contribute to the development of acute lung injury.^{23, 27} There was a significant increase in the number of TUNEL positive nuclei in the lungs of mice treated with GO (Figure 2M). These results suggest that pristine graphene induces minimal inflammation in the lung and systemically whether it is administered as an aggregate or as a Pluronic dispersed material. By contrast, graphene oxide causes disruption of the alveolar-capillary barrier allowing the exudation of a protein rich fluid into the airspaces (acute lung injury) accompanied by an infiltration of inflammatory cells into the lung and the release of pro-inflammatory cytokines.

We used electron microscopy to examine the cellular fate of the graphene preparations in the lung. We observed membrane bound vesicles containing a black material in the alveolar macrophages of mice treated 24 hours earlier with Pluronic-dispersed or aggregated graphene. These vesicles were less numerous and less dense in macrophages from mice treated with GO (Figure 3A). Our observations are consistent with those of other investigators who have shown that iridium labeled carbon black nanoparticles administered to rats intratracheally are taken up and cleared by alveolar macrophages.²⁸ Preparations of GO have been reported to support redox cycling of cytochrome c and electron transport proteins in bacteria, suggesting that the oxygen moieties on the GO may accept electrons from cellular redox proteins, in the process forming highly reactive oxygen radicals.^{29, 30} In this case, GO, but not graphene, should result in the generation of intracellular reactive oxygen species after their uptake into macrophages.

To test this hypothesis, we generated a line of murine alveolar macrophages (MHS) stably expressing an oxidant sensitive GFP probe localized to the mitochondrial matrix.^{21, 22} These cells showed significant oxidation of the mitochondrially localized probe 4 hours after treatment with GO, while even at the highest doses (50 $\mu\text{g}/\text{cm}^2$), we did not observe significant oxidation of the probe in cells treated with aggregated or Pluronic-dispersed graphene (Figure 3B). Although we observed an increase in proinflammatory cytokines in the lungs of animals 24 hours after the administration of graphene, the administration of graphene did not cause MHS cells to release IL-6 into the media (Figure S3). However, consistent with our *in vivo* findings, treatment with GO but not aggregated or dispersed graphene induced high levels of apoptosis (assessed using a DNA fragmentation ELISA) in MHS cells 24 hours after exposure (Figure 3C). Similar results were observed in a murine lung alveolar epithelial cell line (MLE-12) (Supporting Information Figure S4).

We then sought to determine the mechanism by which GO induces ROS generation in macrophages. To determine whether GO might participate in redox cycling with proteins in the electron transport chain, we measured the oxygen consumption rate in MHS cells treated with GO. The administration of GO resulted in a significant increase in coupled (oligomycin sensitive) and uncoupled (oligomycin insensitive) mitochondrial (inhibitable by the combination of rotenone and antimycin A) oxygen consumption (Figure 3D). Inhibition of mitochondrial electron transport with the combination of rotenone and antimycin, which inhibit electron transport complexes I and III, respectively, reduced oxygen consumption to a similar level in GO treated and untreated cells suggesting that the excess oxygen consumption induced by GO was caused by an increase in electron transport. This result was

confirmed by the finding that oxygen consumption was reduced to a similar level in the presence or absence of GO by the administration of a succinate dehydrogenase inhibitor, 3-nitropropionic acid (3-NP), which prevents the provision of electron donors from the tricarboxylic acid cycle and inhibits complex II of the mitochondrial electron transport chain.³¹ We observed no change in coupled or uncoupled respiration in cells treated with dispersed graphene (GD) (Figure S5). To determine whether the increase in electron transport was required for the increased mitochondrial ROS generation induced by GO, we treated MHS cells with 3-NP and measured mitochondrial ROS generation in response to GO. 3-NP completely prevented the generation of mitochondrial ROS in response to GO (Figure 3E). These results suggest that GO acts as an electron donor, increasing the supply of electrons to site I/II of the electron transport chain, accelerating the generation of ROS as a byproduct of mitochondrial respiration. A similar increase in oxygen consumption was not induced by treatment with pristine graphene. Strategies that minimize oxidation of graphene are therefore predicted to enhance its biocompatibility.

Similar to other reports, our data do not allow us to definitively determine the mechanisms by which graphene oxide induces an increase in mitochondrial respiration or mitochondrial ROS generation.³² Nevertheless, a plausible explanation is that graphene oxide functions as a quinone and participates directly in redox cycling reactions with components of the mitochondrial electron transport chain.³³ Since our electron microscopy data suggest that the bulk of the graphene is localized in vesicular structures within the cytoplasm of the cell, we suspect that small amounts of GO escape these structures and gain direct access to the mitochondria or that GO at the membrane activates signaling pathways that increase mitochondrial ROS generation.

Aggregates of single-walled and multi-walled carbon nanotubes have been shown to induce peribronchiolar lung fibrosis 21 days or more after their administration.^{15, 34} We measured lung fibrosis in mice treated with GO, aggregated graphene or Pluronic-dispersed graphene and examined Trichrome stained lung sections for evidence of fibrosis 21 days later. Aggregates of graphene persisted in the medium or small airways of the lung and induced peribronchial inflammation and mild fibrosis (Figure 4A,B). In contrast, there was no evidence of fibrosis in mice treated with dispersed graphene (Figure 4A,B). In mice treated with GO, we observed persistent lung inflammation 21 days after their administration, however, there was little evidence of lung fibrosis (Figure 4A,B). These histologic findings were confirmed by analysis of whole lung collagen using picrosirius red collagen precipitation (Figure 4C). Interestingly, these findings are similar to those reported by our group and others 21 days after the intratracheal instillation or inhalation of aggregated single-walled carbon nanotubes in mice and rats.^{15, 34–37} While this toxicity has been attributed to the large aspect ratio of the individual nanotubes, this cannot apply to graphene flakes as the aspect ratio of this material is near 1. Furthermore, we and others have observed that nanoscale dispersion of both graphene and SWCNTs effectively prevents this toxicity.^{15, 38, 39} Collectively, these findings suggest that the peribronchiolar fibrosis observed after the administration of graphene and perhaps other carbon based nanomaterials results from a failure of macrophages to clear the large aggregates of the material lodged in the small airways rather than a direct toxicity of the nanomaterial itself. Consequently, strategies that provide stable nanoscale distribution of carbon based nanomaterials will improve their biocompatibility. Our data further suggest that nanoscale dispersion will dramatically improve the distribution of the nanomaterial in the lung and likely in other tissues.

The pulmonary toxicity of graphene differs dramatically as a function of its dispersion and oxidation of the carbon backbone. Highly dispersed preparations of graphene in the nonionic, amphiphilic block copolymer Pluronic induce modest acute lung inflammation and

are not fibrogenic, whereas aggregated graphene lodges in the airways and induces a local fibrotic response. Furthermore, graphene, either in a dispersed or aggregated form, does not increase mitochondrial oxidant generation or induce apoptosis in lung macrophages. On the other hand, GO induces severe lung injury that persists for more than 21 days after administration. In cultured alveolar macrophages and epithelial cells, GO increases the generation of mitochondrial ROS by participating in redox reactions with components of the mitochondrial electron transport chain. Our results suggest that processes that maintain the nanoscale dispersion of graphene and minimize contamination with GO will reduce the potential health consequences of workplace or environmental exposures and likely facilitate emerging graphene-based biomedical applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Properties of Pluronic-dispersed graphene (Dispersed) and graphene oxide (Oxide). (a) Optical absorbance spectra and (b) Raman spectra for Pluronic-dispersed graphene and graphene oxide samples. (c,d) Atomic force microscopy (AFM) histograms of lateral flake size (i. e., square root of flake area) and flake thickness for the Pluronic-dispersed graphene and graphene oxide samples. (e,f) Representative AFM images of Pluronic-dispersed graphene and graphene oxide on SiO₂. Scale bar = 250 nm.

Figure 2.

Graphene oxide induces acute lung injury in mice. Mice were treated intratracheally with three preparations of graphene: highly dispersed and purified in 2% Pluronic (Dispersed, D), aggregates suspended in water (Aggregated, A) or GO (Oxide, O) and the appropriate controls, water (-) or 2% Pluronic (-)^P. 24 hours later the mice were sacrificed for assessment of lung injury. (a) Photomicrographs of paraffin blocks of the lung after sectioning at approximately the same level demonstrating the distribution of particles, (b–e) Photomicrographs of lung sections at <1X (b), 50X (c) and 200X (d). (f) Representative electron micrographs from lung sections of mice treated 24 hours earlier with Pluronic-dispersed graphene (left) or GO (right), bar indicates 10 μ m. Bronchoalveolar lavage fluid (BALF) levels of (g) protein, (h) total cell count, (i) Differential cell count Macrophages (Mac), Lymphocytes (Lymph), Neutrophils (Neut) and levels of the pro-inflammatory cytokines (j) MCP-1 and (k) IL-6. In the same animals, (l) plasma levels of thrombin-antithrombin (TAT) levels and (m) the percentage of TUNEL positive nuclei in paraffin embedded lung sections were measured. (m) N = 3 for all measures, * indicates P < 0.05 for comparison with appropriate control.

Figure 3.

Graphene oxide, but not graphene, induces the mitochondrial generation of ROS and cell death in an alveolar macrophage cell line. (a) Electron micrographs of alveolar macrophages in lung sections from mice treated with highly purified and dispersed preparations of graphene in 2% Pluronic F 108NF (Dispersed), aggregates of graphene in water (Aggregated) or GO in water (Oxide) by intratracheal instillation 24 hours earlier. Arrows indicate vesicular structures containing an amorphous electron dense material. (b) Cells from an alveolar macrophage cell line (MHS) stably expressing a mitochondrially localized oxidant sensitive GFP probe (Mito-Ro-GFP) were exposed to increasing concentrations of dispersed graphene in 2% Pluronic F 108NF (GD), aggregates of graphene in water (GA), graphene oxide (GO), water (-) or water with 2% Pluronic (-)^P and oxidation of the probe was measured using flow cytometry 4 hours later. (c) MHS cells were exposed as in (b) and

cell death was measured 24 hours later using a DNA fragmentation ELISA. (d) Oxygen consumption was measured in MHS cells using a Seahorse XF analyzer. Coupled (oligomycin sensitive) and uncoupled (oligomycin insensitive) mitochondrial (rotenone/antimycin A inhibitable) respiration is shown. In addition, the cells were treated with 3-NP (2 mM) and oxygen consumption was measured. (e) Cells stably expressing Mito-Ro-GFP were treated with 3-NP (2 mM) before being exposed to GO for 4 hours for measurement of oxidation of the probe by flow cytometry. EM studies were performed in 2 animals per condition, N = 3 or 4 for all other conditions. * indicates Bonferroni corrected $P < 0.05$ for comparison with appropriate control (water or 2% Pluronic).

Figure 4.

Aggregated graphene induces patchy fibrosis in mice. Mice were treated with highly purified and dispersed preparations of graphene in 2% Pluronic F 108NF (Dispersed), aggregates of graphene in water (Aggregated) or GO in water (Oxide) by intratracheal instillation and 21 days later, the lungs were examined for markers of fibrosis. (a) Trichrome stained lung sections. (b) Sirius Red stained lung sections (bottom panels are photomicrographs obtained using a polarizing filter). (c) Total lung collagen determined by picrosirius red precipitation of whole lung homogenates (GD; dispersed graphene, GA; aggregated graphene, GO; graphene oxide). Representative images from 4 or more animals

per group are shown, N=8 for picosirius red precipitation, differences between groups are not significant.