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Raman spectroscopy for the detection of organ distribution and clearance of PEGylated reduced graphene oxide and biological consequences

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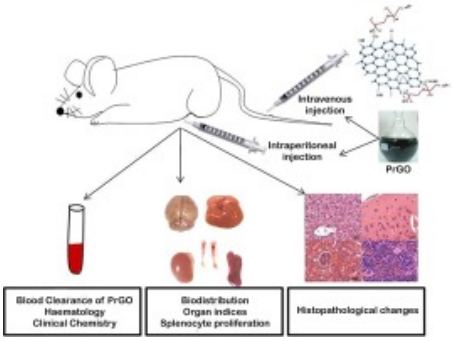
Abstract

Graphene, a 2D carbon material has found vast application in biomedical field because of its exciting physico-chemical properties. The large planar sheet like structure helps graphene to act as an effective carrier of drug or biomolecules in enormous amount. However, limited data available on the biocompatibility of graphene upon interaction with the biological system prompts us to evaluate their toxicity in animal model. In this study organ distribution, clearance and toxicity of PEGylated reduced nanographene (PrGO) on *Swiss Albino* mice was investigated after intraperitoneal and intravenous administration. Biodistribution and blood clearance was monitored using confocal Raman mapping and indicated that PrGO was distributed on major organs such as brain, liver, kidney, spleen and bone marrow. Presence of PrGO in brain tissue suggests that it has the potential to cross blood brain barrier. Small amount of injected PrGO was found to excrete via urine. Repeated administration of PrGO induced acute liver injury, congestion in kidney and increased splenocytes proliferation in days following exposure. Hence the result of the study recommended that PrGO should undergo intensive safety assessment before clinical application or validated to be safe for medical use.

Graphical abstract



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Introduction

Graphene tagged as the “strongest material” is a single layer of one atom thick 2D carbon sheets [1] that forms a honey comb lattice structure. Graphene has raised the global importance and created intense interest in the scientific community because of its versatile properties such as high electrical conductivity, mechanical strength, thermal stability and large surface area. Natural graphite is oxidized and exfoliated to obtain hydrophilic graphene oxide (GO) that contains chemically reactive functional groups on the edges and basal plane. GO can be further reduced to form hydrophobic reduced graphene oxide (rGO) using reducing agents such as hydrazine, sodium borohydride, sodium hydroxide, hydroquinone and vitamin C [2]. rGO looks similar to pristine graphene but differs from GO in terms of electrical, thermal and mechanical properties. Graphene is going to revolutionize the modern scientific era with potential applications in energy technology, electronic devices, material sciences and biomedical field [3].

Biomedical application of graphene is a flourishing area still in its infant stage. The large planar sheet like structure helps graphene to act as an effective carrier of drug or biomolecules in enormous amount. PEG functionalized nano graphene oxide (NGO-PEG) carrying anticancer drugs such as SN38 [4], paclitaxol [5] showed high efficacy against cancer cells proliferation. In addition, NGO-PEG has been used as an efficient protein delivery vector to control cell fate [3]. Similarly, polyethyleneimine functionalized GO (PEI-GO) also act as an efficient gene delivery vector with increased transfection efficiency and localization [6]. Beside this, graphene is also used for bioimaging, photothermal therapy, bacterial inhibition and biosensors development [3], [7]. Increase in production and usage, concomitantly increases the safety and toxicity concern of nanoparticles since they are generally considered to be non-biodegradable. Hence it is crucial to understand the interaction of these nanoparticles with the biological system before biomedical application. Nonetheless the research on toxicity and harmful effects imposed by graphene towards the living system remains obscure and the outcomes of the studies were conflicting.

Properties including surface chemistry (O/C ratio), shape, surface area, layer number, size and lateral dimension are proposed to modulates the toxicity and cellular responses against graphene materials [8]. Several *in vitro* studies were available on the toxicity induced by pristine graphene and its derivatives towards mammalian cells [9], [10], [11]. Pristine graphene without any surface modification accumulates on the cell membrane and induce reactive oxygen species (ROS) mediated apoptosis whereas functionalized graphene is internalized by cells and are less toxic [11]. Researchers have explored the possibility of developing non-toxic and biocompatible graphene using various polymers and following different green synthesis. It is well known that graphene, GO and rGO can elicit toxic responses in bare form. Hence surface functionalization of graphene is recommended to reduce their toxicity. In addition, surface functionalization also improves the water solubility and stability of graphene in physiological environments. For biomedical

applications, graphene should be water soluble and biocompatible. To accomplish this and to reduce the toxicity of graphene, it is surface functionalized with biocompatible polymers such as polyethylene glycol (PEG), dextran, PEI and poly vinyl alcohol, chitosan and Pluronic [8]. The functionalization can be either covalent or non-covalent conjugation of polymers. PEG functionalization increases blood circulation time by decreasing macrophage uptake which is desirable for *invivo* application [12]. Most of the studies showed functionalized graphene are non-toxic and biocompatible. Unlike GO and rGO, amine-modified graphene fails to induce hemolysis or stimulatory effect on platelets [13]. GO can binds to several serum proteins and activates complementary pathway leading to local inflammatory responses whereas PEGylated GO reduces protein adsorption and complement activation [14].

Though *invivo* toxicity studies of graphene has been reported earlier, most of the studies focused mainly on pristine graphene and GO, the findings of which confirmed the toxicity of graphene in living system. For example, graphene when inhaled induced pulmonary inflammation [15], pulmonary edema and granuloma formation in mice when injected intravenously [16]. Increased expression of MIP-1R, MCP-1, MIP-2, IL-8, and IL-1β were noticed in the BAL and the pleural lavage [17], [18]. At high dose (0.4mg), GO induced chronic toxicity in mice lungs characterized by accumulation of neutrophils and macrophages and epithelioid granuloma formation [19]. Graphene nanosheets also influenced Th2 immune response and increased expression of IL-33 in mice after intravenous administration [20]. Prolonged exposure to 0.5–100μg/ml of GO via oral administration caused prolonged defecation behavior and organ damage in *Caenorhabditis elegans* [21]. At present very less reports has been documented on the toxicity of rGO or functionalized rGO to the best of our knowledge. In general, confound limited data available on the biocompatibility of graphene upon interaction with the biological system prompted us to evaluate their toxicity in animal model. Hence in the present study, biodistribution, blood clearance and systemic toxic responses induced by PEGylated reduced graphene oxide (PrGO) was evaluated after intraperitoneal (i.p.) and intravenous (i.v.) administration in mice (10mg/kg body weight).

Section snippets

Synthesis and characterization of PrGO

Graphene oxide (GO) was synthesized from graphite flakes using modified Hummer's method [23]. In short, graphite flakes were exfoliated in conc. Sulfuric acid (H_2SO_4) and potassium permanganate ($KMnO_4$) at<20°C. The reaction temperature was increased to 35°C and the reaction was continued for 30min. 46ml of de-ionized water was added and the temperature was further increased to 98°C. After 15min of incubation, the reaction was stopped by the addition of water and hydrogen peroxide (H_2O_2…

Synthesis and characterization of PrGO

GO was exfoliated from graphite under acidic condition. $KMnO_4$ was used as a strong oxidizing agent to oxidize exfoliated graphite. The aqueous stability of GO was further increased by conjugating it to O, O′- Bis (2-aminoethyl) polyethyleneglycol (PEG) under 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) catalyzed reaction forming amide bond. Nanosized GO-PEG was separated from larger sheets of GO using centrifugation and filtration. The engineered nanoGO-PEG was chemically reduced by…

Discussion

Despite the tremendous increase in the number of literature on graphene synthesis, the large scale industrial production of graphene is hard to achieve. Chemical reduction of exfoliated graphene oxide is the

widely accepted method for bulk production of graphene [22].GO can be reduced to rGO using reducing agents such as hydrazine hydrate, hydroxylamine, hydroquinone, sodium borohydride, ascorbic acid. In the present study, GO was successfully synthesized from graphite flakes using modified…

Conclusion

In conclusion, PrGO was found to be accumulated in major organs such as liver, spleen and kidney causing hepatotoxicity and immune responses during the initial days of exposure. The incomplete elimination of PrGO from the body urges the need for considering long term adverse health effects before these materials are designed for biomedical applications. The advantage of using confocal Raman mapping for quantifying the percent of graphene distribution in various organs eliminates the need of…

Conflict of interest

The authors declare that they have no conflict of interests.…

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