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Dose-dependent cytotoxicity of polyethylene glycol loaded nano-graphene oxide in cultured cerebral cortical cells

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Abstract

Cerebral cortex plays a crucial role in the functioning of the organism. This study aimed to examine the cerebral cortex cytotoxicity of polyethylene glycol (PEG)-loaded nano-graphene oxide (nGO), called PEGylated-nGO (PEG-nGO). Graphene oxide sheets were synthesised using an improved Hummer's method. The nGO was produced by ultrasonic cracking of these sheets. Biocompatibility of nGO was increased by PEG loading. Cortical cells were then treated with various concentrations of PEG-nGO for 24 h. Trypan blue exclusion, MTT, and LDH assays were used to determine cell viability and cytotoxicity respectively. Cellular uptake was examined by transmission electron microscopy. Together, our findings indicated dose-dependent effect of PEG-nGO. This study thus might contribute to conclusion on the clinical potentials of PEG-nGO.

1. Introduction

Multi-functional graphene oxide (GO) has been extensively used for biomedical applications including both diagnostic and therapeutic uses, due to its unique properties [1–4]. Since the last decade, GO [5] and functionalized GO [6] have been used in the field of drug delivery. GO has also been used for glucose detection [7]. Poly (acrylic acid)-functionalized GO interaction with an anticancer drug (1, 3-bis (2-chloroethyl)-1-nitrosourea) has been studied by Lu *et al* [8]. Depan *et al* [9] and Yang *et al* [10] revealed a strong bonding between GO and the anticancer agent doxorubicin (DOX). Moreover, GO based sensors have been used for electrochemical detection of tyrosine [11], 4-nitrophenol [12], neonicotinoids [13], ascorbic acid, dopamine, and uric acid [14]. Also, GO has been used for intracellular sensing [15–17]. GO has further been used to target stem cells of various tumors [18]. A different study indicated good biocompatibility of GO with red blood cells [19].

PEGylated nano-graphene oxide (PEG-nGO) has been extensively studied. Xiong *et al* used PEG-nGO and PEG-nGO conjugates for chemo-photothermal therapy [20]. Chang *et al* treated A549 cells with PEG-nGO [21]. Increased cancer cell uptake of Chlorin e6 loaded on functionalized PEG has been observed by Tian *et al* [22]. Zhang *et al* used PEG-GO for siRNA delivery [23].

GO electrolyte films have been used to control neuronal gain and for construction of brain-inspired cognitive systems [24]. Lv, Min *et al* used GO to enhance the differentiation of SH-SY5Y cell lines by evaluating the expression of the neuronal marker MPA2 and neurite length [25]. Rolled graphene oxide has shown to be efficient for regeneration of the nervous system by Akhavan *et al* [26].

The previous studies suggested no significant alteration in the neuronal and glial cell viability upon graphene exposure [27, 28]. However, the clear change in the neuronal viability due to the alteration of physiological pathways, such as synaptic connectivity and plasticity, calcium and lipid homeostasis upon GO nano-sheets exposure has been observed [29, 30]. However, cerebral cytotoxicity of PEG-nGO has not yet been sufficiently

studied. The purpose of the present study was to uncover the cerebral cytotoxicity and was to expand the understanding of PEGylated nGO-cerebral cells interactions *in vitro*, promoting safe and responsible use of PEG-nGO for future therapeutic application studies.

2. Materials and methods

2.1. Materials

All reagents used were purchased from Sigma-Aldrich Co. (St Louis, MO, USA) and were used without any further purification.

2.2. Preparation of graphene oxide & PEGylated nano-graphene oxide

The synthesis and characterization of graphene oxide (GO), nano-graphene oxide (nGO), and polyethylene glycol loaded nGO (PEG-nGO) is described in our previous study [31].

2.3. Isolation of cerebral cortical cells

Institute of Laboratory Animal Resources (ILAR) approved protocol which was used for isolation of cerebral cortical cells [32]. Four albino mice of 8–10 weeks with a body weight of 22-24 g were obtained from the Experimental Surgery and Animal (ES&A) Laboratory of the King Saud University. The mice were sacrificed by cervical dislocation and the brains were removed immediately under a sterile condition under a laboratory fume hood (Ascent. Max). All materials were sterilized and were sprayed with ethanol before use. Each animal's skull was removed using a scalpel and iris scissors with care not to damage the cortex. Blood vessels around cortex were also removed carefully. Each cortex was washed with sterile phosphate-buffered saline (PBS), and then finely minced into small pieces using a scalpel. The minced tissues were then incubated in 15 ml of sterilized papain (2 mg ml^{-1}) solution for 20 min at 37 °C. After this, the cell suspension was filtered through a cell strainer, and cell debris and large tissue fragments were discarded. The cell suspension was centrifuged for 5 min at 1,000xg. The cell pellet was re-suspended in DMEM F12 medium and was washed twice with this medium.

2.4. Characterization of primary cell culture

Transmission electron microscopy (TEM) was used for the identification of mixed cell culture. The mixed population of about 50% neurons and rest 50% of glial cells and astrocytes were observed under TEM.

2.5. Cerebral cortical cells culture

Cells were counted using a TC20^{TM} automated cell counter (Bio-Rad) and were plated in 24-well cell culture plates (Millipore, Merck, KGaA) at a density of 5×10^6 cells ml^{-1}. After plating, the cells were treated with different concentrations of the PEG-nGO PBS suspension. Treated cells along with control cells (which received no treatment) were incubated under 5% CO_2 at 37 °C in a humidified environment (95%) for 24 h.

2.6. Trypan blue exclusion assay

Cell viability was assessed by a trypan blue exclusion assay. Grown cells (treated cells and controls) were carefully collected, mixed separately with 0.4% trypan blue dye in a 1:1 (v/v) ratio, and applied to a counting slide to determine viable and dead cells using a Bio-Rad cell counter. Each sample was counted in three replicates.

2.7. MTT assay

Cell viability was additionally calculated by measuring the activity of viable mitochondrial enzymes. For MTT assays, cells were plated on a 96-well plate (Millipore, Merck, KGaA) at a density of 5×10^3 cells/μL, and treated with various concentrations of the PEG-nGO PBS suspension. Control cells (without any treatment) and treated cells were incubated at 37 °C under 5% CO_2 in a 95% humidified environment for 24 h. Incubated (control and treated) cells were further treated with 10 μl of MTT solution (5 mg ml^{-1} of the total volume) for 3 h under same conditions. After that, 50 μl of isopropanol with 0.04 N HCl was added into each well to (producing a homogenous pink solution), and the solution was further incubated for 30 min. Then, absorbance of each well was measured at 570 nm with a reference at 630 nm, using an ELISA plate reader. PEG-nGO treated cell viability was expressed as percentage of control and calculated as follows:

$$\% \text{ cell viability} = \text{PEG} - \text{nGO treated cells/control cells} \times 100\%.$$

2.8. Lactate dehydrogenase assay

Cytotoxicity of PEG-nGO was calculated by determining LDH enzyme activity of dead cells. One millilitre of each cell suspension (control and treated) was centrifuged for 1 min at 3000xg. Then, the supernatant and the

pellet were separated. Ten μl of cell lysis buffer was added to the pellet and to the supernatant to release the LDH enzyme into the extracellular environment. To measure LDH enzyme activity, 2.7 ml of tris was placed in a cuvette, and 10 μl of each, sodium pyruvate, Nicotinamide adenine dinucleotide (NAD), and sample solution were added. The LDH enzyme released from the damaged cells converts the lactate to pyruvate by oxidation, generation NADH from NAD. The LDH enzyme activity was measured by the NADH decomposed per minute at 340 nm and is calculated by the product of NADH decomposed per minute and molar extinction coefficient ($6.22 \times 10^3\ M^{-1}\ cm^{-1}$) as fellow:

$$\text{LDH activity Units}/1 \times 10^6 \text{ cells} = \Delta A/\text{ min} \times 6.22 \times 10^3\ M^{-1}\ cm^{-1}$$

2.9. Analysis of cellular uptake

Collected grown cells were centrifuged at 1,000xg for 5 min. Glutaraldehyde solution (2.5%) was used to fix cell pellets. For this, the cell pellets were placed in glutaraldehyde solution for 24 h. The fixed cells pellet was then washed with PBS and was fixed into melted agarose gel (2%). The agarose gel was centrifuged at 4 °C for 5 min. Then, cells embedded in agarose gel were oxidized in a 2% osmium tetroxide solution for 24 h. Uranyl acetate (1%) was used for sample staining. The slides were dehydrated in an acetone series, and an epoxy resin was added, which was then polymerized. Ultrathin films were cut with an ultramicrotome and transferred to a TEM grid.

2.9.1. Data analyses

Each analysis of control and treated cells was performed in three replicates. Results are presented as mean $\pm$ standard deviation.

3. Results

3.1. Cell viability

Cell viability was observed using two methods, including trypan blue exclusion and MTT assays. The percentage of cell viability measured by trypan blue exclusion assay is shown in figure 1(a), and the percentage of cell viability according to the MTT assay is shown in figure 1(b).

Increasing concentrations of PEG-nGO caused a decrease in cell viability. This indicates a dose-dependent cytotoxicity of PEG-nGO (figure 1(c)).

3.2. Lactate dehydrogenase enzyme activity

Cytotoxicity of PEG-nGO was determined by LDH enzyme activity in the supernatant and in pelleted cells. LDH activity of control and treated cells is shown in figure 2. LDH activity corresponded to cell viability of cells treated with PEG-nGO.

3.3. Cellular uptake

Transmission electron microscopy was used to study cellular uptake of PEG-nGO. PEG-nGO was clearly visible in the cerebral cortical cells (figure 3).

PEG-nGO uptake was observed in all parts of the nerve cells (dendrite, cell body, and axon), as shown in figure 3(a). PEG-nGO uptake into the glial cells (which support neural growth) was also observed (figure 3(b)). Moreover, Embedded PEG-nGO in the membrane of a glial cell is visualized in figure 3(c), indicating poor cell penetration of PEG-nGO.

4. Discussion

Nanoparticles (NPs) have been used as carriers in the field of drug delivery [33–36]. It has been proven that NPs can cross the blood-brain barrier easily due to their small size [37, 38]. However, cerebral cortical cytotoxicity of NPs has remained a serious issue for their practical use in drug delivery. An organic compound, graphene oxide (GO), has previously been studied extensively due to its potential for biomedical applications and its low manufacturing cost, compared to other materials such as gold, silver, or titanium.

However, toxic effects of graphene and single-walled carbon nanotubes on neural pheochromocytoma-derived PC12 cells have been described by Zhang *et al* [39], but so far, the potential cerebral cortical cytotoxicity of PEGylated GO (PEG-nGO) has not yet been sufficiently investigated. This study aimed to examine the toxic effects of PEG-nGO on cortical cells. For this, cortical cells were treated with different concentration of synthesised PEG-nGO. Trypan blue exclusion and MTT assays were used to determine the percentage of viable cells and LDH enzyme activity assay was used to determine cytotoxicity.

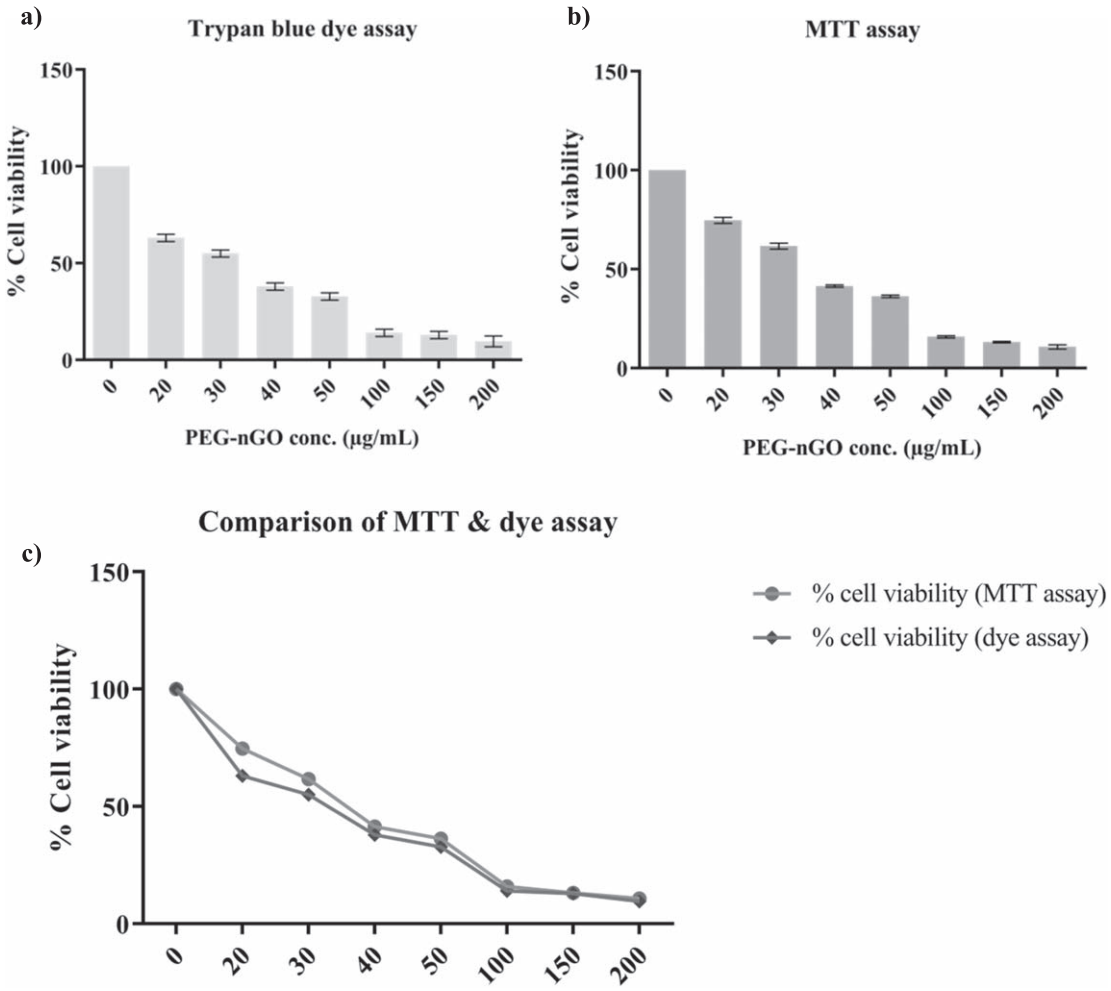

Figure 1. Percentage of viable cells in cerebral cells treated with PEGylated nGO measured by trypan blue exclusion (a) and MTT assay (b). (c): Percentage of viable cells of cerebral cells treated with PEG-nGO.

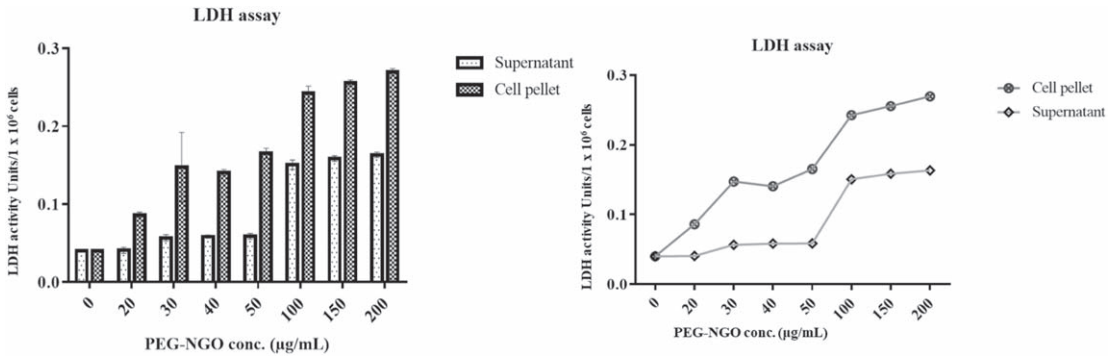

Figure 2. Lactate dehydrogenase activity of control and treated cerebral cells (supernatant and cell pellet).

Our results revealed dose-dependent cortical cytotoxicity of PEG-nGO, as described by Lammel *et al* [40], and are comparable to that of Robinson *et al* and Chang *et al* who demonstrated that cellular viability, morphology, mortality, uptake, and membrane integrity are not affected by GO in low concentrations [21, 41]. Cellular uptake of PEG-nGO was observed by TEM, demonstrating the presence of PEG-nGO in axons of consecutive neurons. This suggests a potential of PEG-nGO to cross synapse, supporting the work of Yang *et al* who used GO to promote brain chemicals [42]. Moreover, TEM indicated uptake of PEG-nGO into glial cells. Comparably, Tu *et al* used GO for neural growth [43]. However, PEG-nGO embedded in the outer membrane of glial cells indicated the poor penetration ability of PEG-nGO, as also described by Sun *et al* [44].

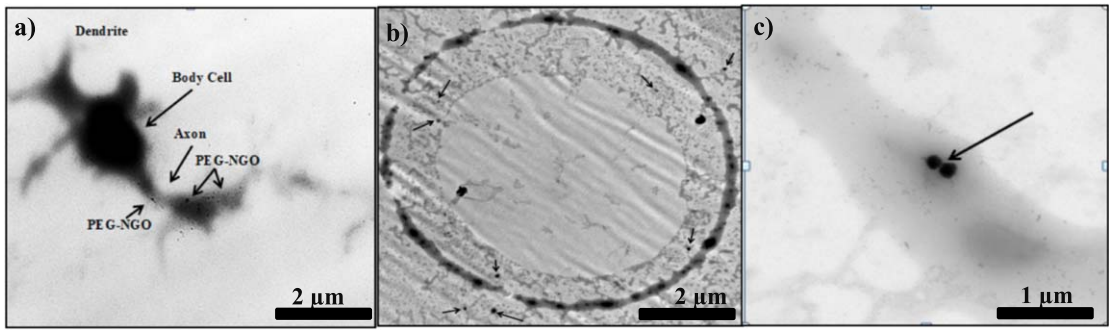

Figure 3. Transmission electron microscopy of PEG-nGO treated cerebral cortical cells.

5. Conclusion

In the present study, PEGylated nano-graphene oxide (PEG-nGO) was used to assess toxic effects on cerebral cortical cells. Trypan blue exclusion, MTT, and LDH assays data suggests a dose-dependent cerebral cortical cytotoxicity. Our results of dose-dependence cytotoxicity revealed similarities with previous studies. Taken together, our results indicated dose-dependent effect and that more studies are needed to reach more precise conclusions.

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Conflict of interest

All authors have no conflicts of interest or financial ties to disclose.

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