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Ocular biocompatibility evaluation of hydroxyl-functionalized graphene

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ABSTRACT

We have presented our recent efforts on genotoxicity and intraocular biocompatibility of hydroxylated graphene (G-OH) prepared by ball milling. We have previously demonstrated that the as-synthesized G-OH could be considered as an excellent alternative for graphene oxide which had been applied widely. Following our last report on G-OH, we carried out detailed studies on genotoxicity and in vivo biocompatibility of G-OH in this work. Less than 5% enhanced caspase-3 level was observed for cells exposed to more than 50 μg/mL G-OH over 72 h, suggesting G-OH caused cell apoptosis was slight. The G-OH induced DNA damage was also found to be mild since expression of p53 and ROS regeneration level was quite low even at high concentration of G-OH over a long time. Cell viability was found to be higher than 90% with 50 μg/mL G-OH and 80% with 100 μg/mL G-OH using flow cytometry. Comet results suggested that less than 5% tail could be found with 100 μg/mL G-OH. TEM results confirmed that G-OH could penetrate into and out of the cytoplasm by means of endocytosis and exocytosis without causing damage on cell membranes. In vivo biocompatibility of G-OH was studied by intravitreal injection of G-OH into rabbits. The ocular fundus photography results showed that G-OH could be diffused in the vitreous body gradually without any damage caused. Injection of G-OH had caused few damages on eyesight related functions such as intraocular pressure, electroretinogram and histological structures of the retina.

1. Introduction

Graphene and its derivatives have attracted intensive attention since its discovery in 2004 [1] due to their unique excellent physical and chemical properties [1–5]. Particularly, graphene oxide (GO), due to its high solubility in water and many other common polar solvents, and rich oxygen-containing functional groups which are ready for device assembly, has been considered as the most exciting graphene based candidate for biological and biomedical applications [6–10]. It is necessary to evaluate biocompatibility of graphene and its derivatives both in vitro and in vivo for their potential applications.

Furthermore, chemical functional groups modified on graphene have showed much influence on its interaction with biomolecules. For instance, Lee et al. have demonstrated promising applications of graphene and graphene oxide to be biocompatible, transferable, and implantable platforms for stem cell growth and differentiation [11]. Insulin was found to be denatured on graphene via strong π–π interaction while no insulin denaturation was observed on graphene oxide (due to the presence of H-bond and electrostatic interaction which facilitated its binding capacity for insulin and enhanced adipogenic differentiation). Another type of graphene derivative, graphene nanogrids prepared from oxidative unzipping of multi-walled carbon nanotubes were found to be excellent capacity in the adsorption of the chemical inducers which accelerated differentiation of human mesenchymal stem cells [12]. In another study, Akhaven and co-worker found out that UV-assisted photocatalytically reduced GO/TiO_2 hybrid accelerated differentiation of human neural stem cells into neurons, associating with electron injection from the photoexcited TiO2 into the cells on the reduced GO via Ti–C and Ti–O–C bonds [13].

Numerous scientists have reported the cytotoxicity of both graphene and GO [14–22], more recent studies are focused on genotoxicity of graphene based materials particularly the effects of chemical modification of graphene on genotoxicity. For example, Akhavan's group measured various genotoxicities of GO, hydrazine reduced GO (N_2H_4-rGO), hydrothermally reduced GO (HT-rGO), and green tea polyphenols reduced GO (GTP-rGO) with spermatozoa [23]. The highest genotoxicity was observed at the N_2H_4-rGO due to its ultra sharp edge and high mobility which facilitated the penetration of nanomaterials into spermatozoa and interaction with the cell nuclei, while the lowest genotoxicity

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was observed at GTP-rGO since steric effect was induced by the GTP attached on the rGO. Size- and concentration-dependent cytotoxicity and genotoxicity of reduced graphene oxide nanoplatelets with human mesenchymal stem cells were also reported [24]. Reduced graphene oxide nanoribbons (prepared from oxidative unzipping of multi-walled carbon nanotubes) were found to penetrate into the human mesenchymal stem cells and cause DNA fragmentation and chromosomal aberrations even at low concentration (e.g. 1.0 μg/mL within 1 h) while much lower genotoxicity was observed at the reduced graphene nanosheets, suggesting that different inner structures of graphene derivatives played an essential role in their interaction with cells and corresponding biocompatibility [25].

In our previous work, we have demonstrated the preparation of the large-scaled graphene via a novel modified ball milling technique [26,27]. The as-prepared hydroxylated graphene (G-OH) exhibited much better properties than GO in many aspects including electroactivity and biocompatibility with human retinal pigment epithelium (RPE) cells (the cell viability with G-OH was higher than 80% after 4 days' culturing [27] compared to the 60% cell viability with GO after 3 days' culturing [28]), while remaining many advantages of GO such as water solubility and processability [27]. Moreover, the reported ball milling technique is a simple but efficient, environmentally-friendly graphene preparation method without the use of any toxic chemicals. G-OH is thus considered as an excellent candidate for various application areas where GO is utilized. Though we have carried out some preliminary cytotoxicity studies (such as CCK-8 for cell viability, LDH for cell membrane integrity, and fluorescent micrography for cell apoptosis) on G-OH in our previous work [27], it is necessary to perform further biocompatibility studies such as genotoxicity and in vivo evaluation on G-OH for applications especially in biology and biomedicine. Due to unique properties and toxicity mechanism of nanomaterials particularly carbon nanomaterials [29,30], genotoxicity studies on G-OH is essential for its further large-scaled applications and commercialization. Particularly eye contact is inevitable for many areas involving G-OH e.g. research, industry production, transportation and other related areas. Detailed in vivo ocular biocompatibility evaluation on G-OH is thus necessary. In this work, we have carried out detailed genotoxicity evaluation on G-OH including caspase 3 activity, western blots, flow cytometry, comet assay, ROS generation, and transmission electronic microscopy. Furthermore, the influence of G-OH on eyesight related functions such as intraocular pressure, electroretinogram and histological structures of retina has been discussed.

2. Materials and methods

2.1. Materials and reagents

Graphite flakes were obtained from Qingdao Haida Corporation. 12 Zelanian white rabbits as experimental animals were provided by Experimental Animal Center of Wenzhou Medical University. TobraDex eye ointment and Proxymetacaine eye drops were from Alcon Corporation. Balance salty solution (BSS) was purchased from Alcon Corporation. All other chemicals were obtained from Sigma-Aldrich.

2.2. Preparation of hydroxyl-graphene (G-OH)

G-OH was prepared by the ball milling technique as we reported elsewhere [27]. Briefly, graphite and potassium hydroxide were mixed at the mass ratio of 1:10–1:30 and vigorously shaken at the speed of 200–400 rpm for 8–12 h by ball milling. The resulting material was then removed by deionized (DI) water and further centrifuged at 2000 rpm for 20 min. The residual impurities were removed using a dialysis bag prior to biocompatibility measurements on the final products.

2.3. G-OH induced ARPE-19 cell apoptosis analysis

ARPE-19 cell is a cell line derived from human RPE. ARPE-19 cells seeded in 6-well plates at a density of 3×10^5 cells/well were incubated with G-OH at different concentrations of 5, 10, 50 and 100 μg/mL for 24, 48 and 72 h respectively. Cells were harvested and lysed in protein buffer. BCA (bicinchoninic acid) method was used to calculate the extracted protein concentrations. The protein samples were detected with expression of caspase-3 and p53 respectively.

2.4. Flow cytometry analysis

ARPE-19 cells were incubated with G-OH at different concentrations of 5, 10, 50 and 100 μg/mL for 24, 48 and 72 h and were subsequently collected into centrifuge tubes, respectively. The cells incubated with 0.5 M H_2O_2 for 20 min at 4 °C were used as the positive control while the cells incubated without the addition of nanomaterials were employed as the negative control. The cells were washed twice using cold phosphate buffer solution (PBS pH 7.4) and re-suspended in binding buffer at a density of 106/mL. 100 μL cell suspension was transferred to a 1.5 mL EP tube, with the addition of 5 μL Fluorescein Isothiocyanate (FITC) Annexin and 5 μL Propidium Iodide (PI). The cells were vortexed gently and incubated for 15 min at room temperature in the dark. 400 μL of binding buffer was then added to each tube prior to be analyzed using flow cytometry.

2.5. Comet assay

ARPE-19 cells were incubated with G-OH at different concentrations ranging from 5 to 100 μg/mL over 24, 48 and 72 h, respectively. The cells incubated with 0.5 M H_2O_2 for 10 min at 37 °C were used as the positive control while cells without addition of nanomaterials were used as the negative control. After being washed twice by cold PBS, the cells were resuspended by cold PBS at a density of 10^{5-6}/mL. 1 mL solution of 1% NMA (Normal Melting-point Agarose) dissolved in double-distilled water (ddH_2O) was spread on the glass side and then stored at 4 °C for 30 min. 5 μL 1% LMA (Low Melting-point Agarose) mixed with 25 μL cell suspension was dropped onto the first layer of the NMA gel and remained at 4 °C for 30 min. Coverslips were subsequently removed carefully and the slide with the gel was soaked in cell lysis buffer at 4 °C for 2 h. After being washed gently by ddH_2O twice, the slide was soaked in electrophoresis buffer (pH = 13) for 40 min, followed by electrophoresis at 25 V for 25 min. After being washed by ddH_2O twice and PBS once more, the resulting gel was dehydrated by ethanol overnight and dyed with PI at concentration of 30 μg/mL for 15 min in the dark. Images were taken using a fluorescent microscope at 550 nm. 50–100 comets were measured for each sample to obtain the percentage of tail DNA in cells.

2.6. Reactive oxygen species (ROS) generation

Possible DNA damage with G-OH was measured using ROS generation level. ARPE-19 cells were cultured in 6-well plate at the density of 10^4 cells/well and allowed to adhere to the well bottom for 24 h. The G-OH with concentrations varied from 5 μg/mL to 100 μg/mL was introduced into the culture media and co-cultured with cells over 24 h, 48 h, and 72 h, respectively. A 10 μM DCFH-DA was then introduced into the culture media and incubated for 20 min. The resulting media was subsequently removed and the well was rinsed using PBS three times to remove extra DCFH-DA, followed by the addition of a fresh culture media. Fluorescence intensity was determined using SpectraMas M5 Microplate reader on 488 nm excitation wavelength and 525 emission wavelength.