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Multi-functional graphene as an *in vitro* and *in vivo* imaging probe

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ABSTRACT

A strategy has been developed for the synthesis of multi-functional graphene (MFG) using green synthetic approach and explored its biomedical application as a promising fluorescent marker for *in vitro* and *in vivo* imaging. *In-situ* microwave-assisted reduction and magnetization process was adopted to convert the graphene oxide into magnetic graphene within 1 min, which was further covalently modified to build a polyacrylic acid (PAA) bridge for linking the fluorescein o-methacrylate (FMA) to yield MFG with water-dispersibility (~2.5 g/l) and fluorescence property (emission maximum at 526 nm). The PAA bridges also functions to prevent graphene-induced fluorescence quenching of conjugated FMA. The extent of reduction, magnetization, and functionalization was confirmed with TEM, AFM, Raman, XPS, FT-IR, TGA, and SQUID measurements. *In vitro* cytotoxicity study of HeLa cells reveal that MFG could stand as a biocompatible imaging probe with an IC_{50} value of ~100 μg/ml; whereas *in vivo* zebrafish study does not induce any significant abnormalities nor affects the survival rate after microinjection of MFG. Confocal laser scanning microscopy images reveals that MFG locates only in the cytoplasm region and exhibits excellent co-localization and biodistribution from the head to tail in the zebrafish. Our results demonstrate the applicability of graphene based fluorescence marker for intracellular imaging and, more significantly, as well as whole-animal imaging. Hence, MFG could preferentially serve as a dual functional probe in biomedical diagnostics.

1. Introduction

The focus of nanotechnology research has gradually shifted from the development of high quality nanomaterials (NMs) and investigation of their physical properties to the application prospects. Production of multi-functional NMs with emerging properties is a challenging task. There is every concern to employ NMs for the potential breakthrough in biomedical applications such as cellular imaging, diagnosis, and therapeutics [1,2]. Modern therapeutics would preferentially require some specialized delivery systems to maximize their therapeutic efficacy of water insoluble drugs, imaging them at cellular level and also to monitor or manipulate with an external magnetic force [3]. Recently, several NMs such as quantum dots (QDs), photonic crystals and metallic nanoparticles (NPs) [4], silica [5], polymeric [6] and magnetic NPs [7], fullerenes [8], nanodiamonds [9–11], single and multi walled carbon nanotubes [12] have been extensively studied their fortune in biological systems for imaging and drug targeting purposes.

Graphene, a fascinating material has recently emerged with many intriguing properties including electrical, thermal, optical, sensing [13,14], high surface area and biocompatibility [15]. The single or fewer layered structure of graphene provides richness for diversified surface chemistry on both sides of the sheet including edges. The fate of graphene and graphene oxide (GO) in the biomedical field is still ongoing. Recently, there has been an increasing effort in exploiting graphene and GO as fluorescent markers for intracellular imaging studies. For example, nanoscale polyethylene glycol (PEG)-grafted GO has been reported exhibiting intrinsic photoluminescence and provides a useful vehicle for several biomedical applications such as cellular imaging, drug delivery and photothermal therapy [16–18]. GO QDs and covalent approach of fluorescein-functionalized GO via PEG linker was successfully demonstrated as an intracellular fluorescent probe in HeLa cells [19–21], GO and graphene based magnetic NP hybrid materials have also been successfully employed as an anticancer drug carrier and magnetic resonance imaging (MRI) applications [22–24]. *In vivo* study of nano-GO in mice with ultrahigh tumor uptake found it as a potential photothermal agent [25,26]. Nano-sized reduced graphene oxide was also utilized for photothermal therapy owing to its affordable 6-fold increase in the near infra-red absorbance [27]. As the graphene based nanomedicine is becoming widespread extensively, the development of facile process to prepare multi-functional graphene (MFG) with water-dispersibility, fluorescence, and magnetic properties for *in vitro* and *in vivo* biomedical applications is warranted. Current strategies

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of preparing magnetic or fluorescent graphene are time-consuming and use large amounts of potentially hazardous chemicals. To the best of our knowledge, there was no report to prepare MFG using a facile and green process as well as explored its biomedical applicability as a fluorescent marker in both *in vitro* and, *in vivo* zebrafish imaging.

Herein, we synthesized MFG with magnetic, water-dispersibility, and fluorescent properties using microwave-heated and sonication-assisted process. MFG has been demonstrated as a potential fluorescent marker with lower cytotoxicity for both *in vitro* and *in vivo* imaging. The discovery of MFG provides new opportunities in the biomedical field with diversified potential applications such as in biomedical diagnostics, magnetically guided drug/gene delivery, and photothermal/photodynamic therapies etc. Research toward this end is currently ongoing.

2. Methods and materials

2.1. Synthesis of graphene oxide (GO)

GO was synthesized following the modified Hummers process by using the pristine graphite flakes as a starting material. In detail, graphite flakes (1 g) were grounded with NaCl (50 g) for 10 min. NaCl was dissolved in water and removed by filtration (~15% graphite was lost in this step). The remaining ground graphite flakes (0.85 g) were added to 98% H_2SO_4 (23 ml) and stirred for ~8 h. Potassium permanganate ($KMnO_4$, 3 g) was slowly added to the above mixture, while maintaining the temperature < 20 °C. The reaction mixture was stirred at 35–40 °C for ~30 min, and then 65–80 °C for ~45 min. Deionized water (46 ml) was added and the mixture was heated at 98–105 °C for ~30 min. Finally, the reaction was terminated by the addition of distilled water (140 ml) and 30% H_2O_2 solution (10 ml) which forms a eutectic mixture. The H_2O_2 reduced the $KMnO_4$ and MnO_2 residues into colorless $MnSO_4$. The final reaction mixture was washed with 5% HCl and distilled water by repeated centrifugation and filtrations to yield GO [17].

2.2. Synthesis of magnetic graphene (MG)

MG was obtained by adding as synthesized GO (50 mg) and ferrocene (100 mg) into a quartz tube containing 20 pieces of broken Si wafers, evacuated under vacuum for 30 min, irradiated inside a focus microwave oven (2.45 GHz, Discover system, CEM corporation, USA) under nitrogen atmosphere (1 atm) for ~1 min with 20 s intervals each. During irradiation, violent arcing occurred between the Si wafers and caused ferrocene decompose into Fe filled MG. The residual ferrocene was removed from free carbon sooth with toluene/acetone washings, and the product was collected by an external magnet. This process was repeated twice to remove the unreacted ferrocene completely. The intermediate products were washed with conc. HCl to etch away the free Fe and finally with one toluene/acetone washing to stop the etching process. The purified MG was dried under vacuum and subjected to various characterizations.

2.3. Synthesis of multi-functional graphene (MFG)

In order to achieve the water-dispersibility and fluorescent properties, MG was covalently modified with polyacrylic acid (PAA) and fluorescein o-methacrylate (FMA) via sonication followed by microwave irradiation method. In a typical experiment MG (50 mg) was placed into 20 ml water, followed by addition of 1.5 ml acrylic acid monomer. Upon addition of benzoyl peroxide (BPO, 100 mg/ml tetrahydrofuran (THF)); the mixture was subjected to sonication for 2 min which facilitates the dispersion of MG. After sonication, the mixture was immediately transferred to a domestic microwave oven (2.45 GHz, 600 W) and subjected to microwave irradiation for 10 s. The microwave energy helps to generate BPO radicals and initiates the polymerization process. This process of sequential addition of BPO –sonication – microwave irradiation was repeated twice to yield PAA-grafted MG (PMG). The fluorescence conjugate, FMA, was added to the PMG and the process of sequential addition was repeated for another two times. Finally, the surface-functionalized MG was collected and separated from free monomers and unbound polymers by repeated washings with THF followed by water, and centrifuged at 9000 rpm for two times to yield final product multi-function MG (MFG) [11]. The functionalization was characterized by FT-IR (Bomem model DA-83 FTS) and the amount determined by thermogravimetric analysis (Linseis Pt-1600) with an increase in the temperature at 10 °C/min under nitrogen atmosphere.

2.4. Cell cultures

HeLa (human cervical cancer cells) were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA, USA), 2 mM L-glutamine, 100 μg/ml penicillin and 100 U/ml streptomycin, placed and grown in a humidified incubator at 37 °C (95% humidity, 5% CO_2). Other chemicals were purchased from Sigma Aldrich (St. Louis, MO, USA).

2.5. MTT assay

One ml of HeLa cell-containing solution (~2.12×10^4 cells/ml) was added to each well of a 24-well plate and incubated 1 day to allow cells to stick on the surface of the plate. Aliquots of a PBS buffer solution containing different amounts of MG and MFG were added to the 24-well plate, and the cell solutions were incubated for another 2 days. A 50 μl aliquot of an MTT aqueous solution (0.5 mg/ml) was added to each well of the 24-well plate 4 h before termination of the 3-days incubation, and the cells were allowed to incubate for another 4 h. Then, the upper layer of the solutions in the 24-well plate was discarded, and 1 ml of DMSO was added to each well to lyse cell membrane followed by pipette stirring. The final solution in each well was centrifuged at 13,000 rpm to remove any solid residues before measurements of the optical absorbance at 570 nm. The optical absorbances were converted to cell viabilities based on a standard curve (absorbance vs. cell numbers) obtained from control experiments carried out under the same condition except that no NMs were added during cell culture processes.

2.6. Lactate dehydrogenase release (LDH) assay

2×10^4 cells/ml were loaded in a 24-well plate and incubated for 24 h. NMs were added and further allowed it to interact for another 24 h. The cells were washed with PBS, trypsinized and centrifuged at 13,000 rpm. 100 μL of the supernatant was transferred into another 96-well plate. To this, 100 μL of LDH reaction solution (Clontech Cytotoxicity Detection Kit, USA) was added and incubated for 30 min in the dark at room temperature. Before colorimetric detection at 490 nm, 1N HCl was added to stop the enzymatic reaction.

2.7. Reactive oxygen species (ROS) assay

HeLa cells were seeded into 6-well plates at the density of 2×10^5 cells per well. After 24 h, PMG stock solutions were added into cells and incubated for another 24 h under serum-free conditions. The cells were washed with PBS and replaced with serum containing medium along with 2,7-dichlorofluorescein diacetate (H_2DCF-DA) solution (5 μM in cell culture medium) were added and incubated for 30 min at 37 °C. Cells were then trypsinized and aspirated, followed by flow cytometry analysis (BD FACS Canto) equipped with 488 nm laser. Green fluorescence was monitored using FITC channel.

2.8. Annexin-V apoptosis assay

HeLa cells were seeded into 6-well plates at the density of 2×10^5 cells per well. PMG stock solutions were added into the cells under serum-free conditions and incubated for another 24 h. Cells were then trypsinized, aspirated, and suspended in 2 ml PBS. Cells were further stained with 5 μl FITC-Annexin-V and 5 μl 7-AAD from the BD Annexin-V apoptosis kit, and let it stand by for 15 min at room temperature in dark, followed by flow cytometry analysis (BD FACS Canto) equipped with 488 nm laser. Green and red fluorescence were monitored using FITC and PE channels respectively.

2.9. CLSM measurement

HeLa cells (2.0×10^5 cells per well in 6-well plate) cultured in DMEM supplemented with FBS (Fetal Bovine Serum, 10%) and penicillin-streptomycin (1%) were treated with different concentrations of MFG for 24 h. The cells were washed with a phosphate buffer solution (PBS, pH 7.4), further fixed onto a glass slide using paraformaldehyde solution (4%) in PBS for 5 min, and washed with PBST (5% Tween-20 in PBS) solution for three times. Further, cells were stained with DAPI (4′,6-diamidino-2- phenylindole, 1 ng/ml PBS, 30 min) and examined under a CLSM (Zeiss, LSM-700) equipped with an InGaN semiconductor laser (405 nm), an Ar laser (488 nm), and a He–Ne laser (543 nm). During measurements, a 63X objective lens was used with a spatial resolution of ~200 nm in x-y direction and ~100 nm in z-direction.

2.10. Microinjection of MFG into zebrafish embryos and microscopic measurements

Wild-type AB strains of *Danio rerio* (zebrafish) embryos obtained from zebrafish core facility center, National Tsing Hua University were used in all the experiments [28]. Fresh embryos were collected onto the microinjection embryo tray just before the experiment. MFG stock solutions were diluted to appropriate concentrations in double distilled water and sonicated until microinjection. Approximately 10 nl volume was microinjected into the zebrafish pole region of embryos between stages 1 (one cell embryo) and 3 (four cell embryo) using Drummond microinjector [29]. Each experiment was performed on 50 embryos per condition. Following microinjection, embryos were transferred onto the petri dish filled with the system water