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Safety and tumor tissue accumulation of pegylated graphene oxide nanosheets for co-delivery of anticancer drug and photosensitizer

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ABSTRACT

Here, we report the safety, tumor accumulation and potential of polyethylene glycol-grafted graphene oxide (pGO) as a multimodal nanocarrier of photosensitizers and synergistic anticancer agents. First, both graphene oxide (GO) and pGO were synthesized, and their in vitro and in vivo toxicities were tested. When 80 mg/kg was injected intravenously into mice, there was 100% fatality in the GO-treated group, but 100% survival among mice treated with pGO nanosheets. Treatment of cells with a photosensitizer chlorin e6 (Ce6) in pGO nanophysisorplexes significantly enhanced cellular delivery compared to that seen with Ce6 alone. The combination and dose reduction indexes revealed that combining doxorubicin (Dox) with Ce6 with at a molar ratio of 1:2 provided the highest synergism. The Ce6- and Dox-loaded pGO nanophysisorplexes (Ce6/Dox/pGO) were 148.0 ± 18.0 nm in size. Molecular imaging of mice showed that Ce6/Dox/pGO could accumulate in tumor tissues over 3 days. Moreover, in SCC tumor-bearing mice, the photodynamic anticancer effects of Ce6/Dox/pGO were higher than those of Ce6/pGO or Dox/pGO. Moreover, tumor sections from illuminated mice treated with Ce6/Dox/pGO showed substantial disruption of tumor nuclei, whereas the other groups did not. Our results suggest that pGO nanosheets have superior in vivo safety relative to GO, and that it is possible to enhance the tumor tissue distribution and photodynamic anticancer effects of systemically administered Ce6 by forming multimodal nanophysisorplexes with pGO and synergistic anticancer chemotherapeutics such as Dox.

1. Introduction

Recently, graphene-based materials have drawn considerable attention for their potential in biomedical applications. Graphene, a single or few-layered two-dimensional sp^2-bonded carbon sheet, is a basic building block of other carbon allotropes [1,2]. Among the graphene-based materials, graphene oxide (GO) is a water-soluble derivative of graphene that has been studied as a potential material for nanomedicine, owing to its abundant functional groups (epoxide, hydroxyl and carboxyl groups) and the availability of its large planar surface area for efficient loading of aromatic drug molecules via π–π stacking. However, a lack of aqueous stability has limited the use of GO in biomedical applications to date.

To improve the aqueous stability of GO, polyethylene glycol (PEG)-grafted GO (pGO) nanosheets have been studied as a nanocarrier of anticancer drugs such as doxorubicin (Dox) and camptothecin derivatives [3,4]. Despite the potential of pGO for drug nanocarriers, the tolerable dose of pGO and its safety relative to GO have not been fully explored.

For photodynamic therapy, photosensitizers have been delivered using nanocarriers such as liposomes and solid lipid nanoparticles [5,6]. Only a few studies have examined the use of GO-based materials for the delivery of photosensitizers. For example, the photosensitizer, hypocrellin A, was loaded onto GO by physisorption, and the feasibility of this hybrid for photodynamic therapy was tested in vitro [7]. In addition, the photosensitizer, chlorin e6 (Ce6), was loaded onto pGO, and the photodynamic anticancer effects were tested in vitro [8]. However, further in vivo distribution and efficacy studies are needed to validate the potential of GO-based materials for the delivery of photosensitizers.

In this study, we synthesized GO and pGO nanosheets and compared their in vitro cytotoxicity and in vivo safety. Next, we

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tested whether a Ce6-pGO nanophysisorplex (defined as a nanocomplex between drugs and pGO nanosheets derived by physicochemical adsorption) could be delivered to tumor tissues, as assessed by in vivo imaging in mice. Moreover, we evaluated the synergistic photodynamic effects of Ce6- and Dox-dual loaded pGO nanophysisorplexes in tumor-bearing mice. Here, we report for the first time that pGO nanosheets have superior in vivo safety relative to GO nanosheets, and the formation of multimodal nanophysisorplexes with pGO nanosheets can enhance the tumor tissue distribution and photodynamic anticancer effects of Ce6 in vivo.

2. Materials and methods

2.1. Preparation of GO and pGO nanosheets

GO nanosheets were prepared from graphite using Hummers' method with slight modification [9]. Briefly, graphite powder (0.5 g, Sigma–Aldrich, St. Louis, MO, USA) was added to cold H_2SO_4 (23 ml), and then gradually stirred with $KMnO_4$ (3 g) and $NaNO_3$ (0.5 g) on ice. The resulting mixture was further stirred at 35 °C for 1 h. Subsequently, 46 ml of distilled water was added and the temperature was increased to 90 °C and maintained for 1 h. The reaction was stopped with 140 ml of distilled water and 10 ml of 30% H_2O_2. The reaction products were washed by repeated centrifugation, first with an aqueous 5% HCl solution, and then with distilled water. Finally, the products (200 mg) were dispersed in 40 ml of distilled water and sonicated at 400 W (Sonic VCX 500, Sonics & Materials, Inc, Newtown, CT, USA) for 2 h to exfoliate the GO layers and form GO nanosheets. The unexfoliated GO was removed by centrifugation at 1600× *g* for 10 min. The supernatant containing GO nanosheets was collected and an extruder (Northern Lipid, British Columbia, Canada) was used to filter it through 0.2-μm polycarbonate membrane filters (Millipore Corp., Billerica, MA, USA).

To generate pGO nanosheets, the surfaces of the GO nanosheets were modified with PEG to increase their aqueous stability. In brief, N-(3-dimethylaminopropyl-N′-ethylcarbodiimide) hydrochloride (5 mM, Sigma–Aldrich) and N-hydroxysulfosuccinimide sodium (5 mM, Sigma–Aldrich) were added to the GO nanosheets (1 mg/ml) in distilled water to activate the carboxyl groups in GO, and stirred for 24 h at room temperature. Then, 5 mM of NH_2-PEG (Mw ~ 2000; NOF, Tokyo, Japan) was added and the mixture was stirred for 24 h at room temperature. Unreacted NH_2-PEG and excess salts were removed by dialysis (MWCO 100K; Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA) against distilled water for 2 days with 4 changes of distilled water at 12 h interval. The presence of functional groups in GO and pGO nanosheets was confirmed by FT-IR spectrometry (Nicolet 6700, Thermo Fisher Scientific, Inc., Waltham, MA, USA). The topography and thickness of pGO nanosheets were measured using atomic force microscopy (AFM, XE-150, PSIA Inc., Santa Clara, CA, USA) in non-contact mode. The AFM sample was prepared by dropping 20 μl of pGO (10 μg/ml) in distilled water onto Si substrate covered with SiO_2, and left at room temperature for 1 h to allow the adhesion of pGO onto substrate surface. The sample was then dried under N_2 gas fluid, and observed under AFM.

2.2. Loading of photosensitizer and anticancer drugs onto pGO nanosheets

Photosensitizers and anticancer drugs were sequentially loaded onto pGO nanosheets. The photosensitizer, Ce6 (Frontier Scientific Inc., West Logan, UT, USA) was loaded onto pGO nanosheets by adding 0.1 ml of Ce6 (10 mM in dimethyl sulfoxide) to 2 ml of pGO nanosheets dispersed in distilled water at a concentration of 1 mg/ml. The mixture was stirred overnight, and then the dimethyl sulfoxide was eliminated by dialysis against distilled water. After dialysis, the unloaded Ce6, which precipitated in the distilled water, was removed by centrifugation at 1600× *g* for 10 min. Next, 0.2 mg of Dox (Sigma–Aldrich) was added to either 1 ml of pGO (1 mg/ml) to produce doxorubicin-loaded pGO (Dox/pGO), or 1 ml of Ce6-complexed pGO nanosheets (Ce6/pGO, 1 mg/ml) to produce Dox and Ce6 dual-loaded pGO (Ce6/Dox/pGO) nanosheets. Free Dox was removed with a Sephadex G-25M column (PD-10 Column; GE Healthcare, Buckinghamshire, UK). The contents of Ce6 and Dox on the pGO nanosheets were determined by UV/Vis spectrometry at 662 nm and 400 nm, respectively.

2.3. Characterization of drug-loaded pGO nanosheets

The sizes of pGO nanosheets with or without drugs were determined using dynamic light scattering (DLS). The samples were diluted with distilled water and placed in an ELS-8000 instrument (Photal, Osaka, Japan). The hydrodynamic diameters were measured by He–Ne laser (10 mW) light scattering at an angle of 90° at 24.1 °C. Zeta potential values of GO or pGO with or without drug loading were determined by laser Doppler microelectrophoresis at an angle of 22° using an ELS-8000 instrument (Photal, Osaka, Japan). The fluorescent spectra of free Ce6, Dox, Ce6/pGO, Dox/pGO and Ce6/Dox/pGO were recorded by fluorometry under 400 nm excitation for Ce6 and 485 nm excitation for Dox.

2.4. Cellular uptake of Ce6 delivered by pGO nanosheets

Cellular uptake of Ce6 was studied using confocal microscopy. SCC7 cells were seeded onto cover glasses at a density of 8×10^4 cells/well (Fisher Scientific) in 24-well plates. When the cells reached 70% confluence, Ce6 in free form or loaded onto pGO nanosheets at a concentration of 20 μM were added to each well. After 1 h, the cells were fixed with 4% paraformaldehyde in phosphate buffered saline for 15 min, and then stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). The fixed cells were observed using a confocal laser-scanning microscope (LSM 5 Exciter; Carl Zeiss, Inc., Jena, Germany). For quantitation, the cells were harvested, washed three times with phosphate-buffered saline, and evaluated by flow cytometry using a BD FACSCalibur system and Cell Quest Pro analysis software (BD Bioscience, San Jose, CA, USA).

2.5. Evaluating the synergistic antitumor effects of Ce6 and Dox

The synergistic anticancer activity of dual drug-loaded pGO nanosheets was tested using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay. SCC7 cells were seeded to 48-well plates at a density of 8×10^4 cells/well. The following day (at ~70% confluence), each well was treated with various concentrations of Ce6 or Dox. For the dose-reduction index assay, wells were co-treated with Dox and Ce6 at molar ratios of 1:2, 1:4 or 1:8. After 24 h, the cell culture medium was replaced with fresh medium to eliminate potential artifacts associated with the presence of residual Ce6. For drug-loaded pGO, the cells were treated for 0.5 h, and culture medium was replaced with fresh medium to eliminate the excess amounts of drug-loaded pGO. The cells were then exposed to a 660 nm LED (Mikwang Electronics, Busan, South Korea) with a luminous intensity of 8000 mCd for 30 min. For MTT assays, 50 μl of MTT solution (5 mg/ml) was added to each well. After 2 h incubation, the culture medium was removed and 200 μl of a 0.04 N HCl/isopropanol solution was added. The reduction of MTT to its insoluble formazan product (indicating viable cells) was measured colorimetrically at 570 nm using an enzyme-linked immunosorbent assay reader (Sunrise-Basic TECAN, Männedorf, Switzerland). The cell viability in each group was expressed as a percentage of that in control cells. The synergism of Ce6 and Dox together was evaluated using the Calcusyn software (Biosoft, Cambridge, UK) [10].

2.6. Cytotoxicity of GO and pGO nanosheets

The cytotoxicities of GO and pGO nanosheets were tested using the MTT assay. Murine SCC7 squamous carcinoma cells were purchased from the American Type Culture Collection (Rockville, MD, USA) and cultured in Dulbecco's modified Eagle medium (Gibco BRL Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and 100 units/ml penicillin plus 100 μg/ml streptomycin in 5% CO_2 at 37 °C in a humidified incubator. For cell viability measurement, SCC7 cells were seed to 48-well plates at a density of 6×10^4 cells/well. After overnight incubation, cells were exposed to various concentrations of GO or pGO nanosheets for 1, 2, and 3 days. The viabilities of the treated cells were measured by MTT assay, as described above.

2.7. Animals

For the various experiments, 5-week-old female Balb/c and athymic nude mice, as well as 7-week-old female C3H/HeN mice, were obtained from Orient Bio, Inc. (Seongnam, South Korea). All animals were maintained and used in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, Seoul National University.

2.8. In vivo acute toxicity tests of GO and pGO nanosheets

For in vivo acute toxicity evaluation, GO or pGO nanosheets were dispersed in 5% glucose solution and intravenously injected into the tail veins of healthy female Balb/c mice (Orient Bio, Inc.). Their survival at 24 h post-injection was recorded ($n = 10$).

2.9. In vivo molecular imaging

The in vivo biodistribution of Ce6 to tumor tissues was tested by molecular imaging. Five-week-old athymic nude mice (Orient Bio, Inc.) were subcutaneously inoculated in the dorsal left side with 1×10^6 SCC7 cells, and tumors were allowed to become established over time. When the tumor volume reached 100 mm^3, Ce6 (10 mg/kg) was intravenously administered in free form or on pGO nanosheets. After 1, 24, and 48 h, the delivery efficiency of Ce6 was assessed using a Xenogen IVIS-200 system (Perkin Elmer Inc., Waltham, MA, USA) with the built-in Cy5.5 filter set.

2.10. In vivo photodynamic anticancer activity test

The photodynamic anticancer effects of Ce6 alone or combination with Dox on pGO nanosheets were tested using SCC7-bearing mice. C3H/HeN mice (7-week-old, male; Orient Bio, Inc.) were subcutaneously injected in the dorsal left side with