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Reduced graphene oxide nanosheets coated with an anti-angiogenic anticancer low-molecular-weight heparin derivative for delivery of anticancer drugs

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ABSTRACT

Here, we report reduced graphene oxide (rGO) nanosheets coated with an anti-angiogenic anticancer taurocholate derivative of low-molecular-weight heparin (LHT7) as a tumor-targeting nanodelivery platform for anticancer drugs. Surface coating of LHT7 onto rGO was confirmed using fluorescein isothiocyanate-labeled LHT7, monitored as fluorescence quenching due to associated rGO. Unlike plain rGO, LHT7-coated rGO (LHT-rGO) nanosheets maintained a stable dispersion under physiological conditions for at least 24 h. Moreover, LHT-rGO provided greater loading capacity for doxorubicin (Dox) compared with uncoated rGO nanosheets. Following intravenous administration into KB tumor-bearing mice, *in vivo* tumor accumulation of LHT-rGO/Dox was 7-fold higher than that of rGO/Dox 24 h post dosing. In tumor tissues, LHT-rGO/Dox was shown to localize not to the tumor vasculature, but rather to tumor cells. Intravenously administered LHT-rGO/Dox showed the greatest anti-tumor effect in KB-bearing mice, reducing tumor volume by 92.5% ± 3.1% compared to the untreated group 25 days after tumor inoculation. TUNEL assays revealed that the population of apoptotic cells was highest in the group treated with LHT-rGO/Dox. Taken together, our results demonstrate that LHT-rGO nanosheets confer improved dispersion stability, tumor distribution and *in vivo* antitumor effects, and may be further developed as a potential active nanoplatform of various anticancer drugs.

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1. Introduction

Graphene-based nanomaterials have been the focus of considerable research efforts by virtue of their unique features, including optical, mechanical, chemical and thermal properties, raising tremendous interest for their potential pharmaceutical applications [1–3]. However, because graphene-based nanosheets are derived from hydrophobic graphite through chemical or physical modification, their pharmaceutical applications have been hampered by their instability under physiological conditions [4–8].

Reduced graphene oxide (rGO) nanosheets that mimic single-layered graphene nanosheets have been generated by decreasing the polar groups on graphene oxide (GO). The planar structure of rGO serves a double-edged sword: it provides a high capacity for hydrophobic interactions among various functional molecules, but it leads to formation of aggregates with poor dispersion stability under physiological conditions [9,10].

Surface modification of rGO with synthetic polymers or biopolymers has been used to stabilize and improve the utility of rGO as nanocarriers for therapeutics [11–16]. Polyethylene glycol (PEG)-grafted rGO nanosheets have been shown to provide a high loading capacity and cellular-uptake efficiency for single-stranded RNA cargoes [12]. Functionalization of rGO with PEGylated, branched polyethylenimine has been reported to promote cytosolic drug delivery through photothermal disruption of endosomes [13]. Biopolymers, such as hyaluronic acid derivatives [14], dextran [15], and heparin [16], have been studied as surface coatings for rGO. However, most molecules used for surface coating do not possess intrinsic anticancer activity. If an rGO surface coating material were capable of providing both stabilization and anticancer effects, it might enhance the suitability of rGO for anticancer drug delivery applications.

Derivatives of low-molecular-weight heparin (LMWH), a highly sulfated polysaccharide, have been studied as potential anticancer agents [17]. Among LMWH derivatives, LMWH-taurocholate conjugate 7 (LHT7), in which LMWH is conjugated to taurocholic acid at a molar

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ratio of 1 to 7, has been suggested as a prominent anticancer drug with significantly enhanced anti-angiogenic activity and reduced anti-coagulant activity [18]. Recently, liposomal co-delivery of LHT7 and suberanilohydroxamic acid, a histone deacetylase inhibitor, was shown to provide synergistic anticancer activity [19].

Taking advantage of the anti-angiogenic anticancer activity of LHT7 and its highly sulfated polysaccharide and taurocholate moieties, we hypothesized that surface coating of rGO with LHT7 would improve the dispersion stability of these nanosheets and enhance the anticancer efficacy upon delivery with a chemical anticancer drug. In this study, we thus investigated whether LHT7 affected the dispersion stability of rGO and evaluated the *in vivo* distribution and antitumor effects of LHT7-coated rGO nanosheets loaded with the anticancer drug, doxorubicin (Dox).

2. Materials and methods

2.1. Synthesis of LHT7 and F-LHT7

LHT7 was synthesized according to a previously described method [19]. Briefly, ethylenediamine taurocholic acid (5 g) was dissolved in methanol (150 ml) in the presence of sodium hydroxide (5 g). After stirring for 2 h, the solution was precipitated with cold acetonitrile. The resulting precipitate, sodium ethylenediamine taurocholate, was washed with cold acetonitrile and freeze-dried. Five hundred milligrams of LMWH (Fraxiparin; Nanjing King-Friend Biochemical Pharmaceutical Company Ltd., Nanjing, China) was dissolved in distilled water in the presence of *N*-hydroxysuccinimide (126.6 mg). Then, *N*-ethyl-*N′*-(3-dimethylaminopropyl) carbodiimide hydrochloride (310 mg) and sodium ethylenediamine taurocholate (686 mg) was added consecutively into this solution. After an overnight reaction, the solution was precipitated, washed with cold methanol, and freeze-dried. The final product, LHT7, was obtained as a white powder.

For the synthesis of fluorescein isothiocyanate (FITC)-labeled LHT7 (F-LHT7), LHT7 (200 mg) was dissolved in 0.1 M borate buffer (pH 9.0). This solution was then mixed with 20 mg of FITC (Sigma-Aldrich Chemical Co., St. Louis, MO, USA), dissolved in anhydrous dimethyl sulfoxide (DMSO), and reacted for 5 h. The compound was washed twice with cold methanol and then dissolved in distilled water. The solution was purified by dialysis using a membrane with a molecular-weight cutoff of 2000 Da (Spectrum Laboratories Inc., Rancho Dominguez, CA, USA) and freeze-dried to yield the final product, F-LHT7.

2.2. Preparation of rGO nanosheets

rGO nanosheets were produced by reducing GO nanosheets. GO nanosheets were prepared from graphite using Hummer's method with slight modifications [14]. Briefly, graphite powder (0.5 g; Sigma-Aldrich), $KMnO_4$ (3 g; Sigma-Aldrich) and $NaNO_3$ (0.5 g; Sigma-Aldrich) were added to cold H_2SO_4 (23 ml), and the mixture was stirred initially on ice and then at 35 °C for an additional 1 h. After addition of 46 ml of triple-distilled water (TDW), the mixture was stirred at 90 °C for 1 h. The reaction was stopped by addition of 140 ml of TDW and 10 ml of 30% H_2O_2. After washing, the reaction products were dispersed in TDW and sonicated for 2 h to exfoliate the GO layers and form GO nanosheets. Un-exfoliated GO was removed by centrifugation at 1600 ×g for 10 min. The supernatant containing GO nanosheets was collected and filtered through 0.2-μm polycarbonate membrane filters (Millipore Corp., Billerica, MA, USA) using an extruder (Northern Lipid, British Columbia, Canada).

For preparation of rGO nanosheets, the resulting GO nanosheets were reduced by adding 2.0 ml of GO nanosheets (5 mg/ml) in TDW to 8.0 ml of TDW, 0.5 ml of ammonia solution (28 *w/w*% in water; Junsei Chemical, Tokyo, Japan), and 5.0 μl of hydrazine monohydrate (64 *w/w*% in water; Sigma-Aldrich). The resulting mixture was stirred at 80 °C for 10 min. After cooling to room temperature, excess hydrazine and ammonia were removed by dialysis (molecular weight cutoff, 100 kDa; Spectrum Laboratories, Inc.) against TDW. The obtained rGO nanosheets were stored at 4 °C until use.

2.3. Preparation and characterization of LHT-rGO nanosheets

For coating surfaces with LHT7, rGO nanosheets in TDW (1 mg/ml) were mixed with an equivalent volume of LHT7 solution (5 mg/ml) at a LHT7:rGO weight ratio of 5:1. Unloaded LHT7 was removed by gel filtration through a Sephadex G-25 M column (GE Healthcare, Piscataway, NJ, USA), yielding LHT-rGO nanosheets. The extent of LHT7 loading onto rGO was determined by measuring the decrease in F-LHT7 fluorescence at 525 nm caused by the quenching of adsorbed fluorophores by rGO using a fluorescence microplate reader (Gemini XS; Molecular Device, Sunnyvale, CA, USA). The size and morphology of LHT-rGO were examined by transmission electron microscopy (JEM1010; JEOL Ltd, Tokyo, Japan).

2.4. Stability test of LHT-rGO nanosheets

The stability of LHT-rGO nanosheets was tested in phosphate-beffered saline (PBS) (50 mM, pH 7.4) and fetal bovine serum (FBS; Gibco BRL Life Technologies, Carlsbad, CA, USA). An aliquot (0.1 ml) of rGO or LHT-rGO in TDW (rGO content, 0.5 mg/ml) was added to 0.9 ml of PBS, and the solutions were allowed to stand for 24 h. The stability of LHT-rGO nanosheets in PBS was evaluated by monitoring the mixtures for the appearance of precipitates using a digital camera (Canon PC1089; Canon Inc, Tokyo, Japan). For tests of LHT-rGO stability in serum, an aliquot (0.1 ml) of F-LHT-rGO in TDW (rGO content, 0.5 mg/ml) was added to 0.9 ml of RPMI-1640 media containing FBS (10%, 50%, or 90%), and the solutions were allowed to stand for 4 h. The stability of LHT-rGO complexes was determined by measuring the fluorescence of F-LHT7 released from LHT-rGO nanosheets using a fluorescence microplate reader (Gemini XS; Molecular Device).

2.5. Preparation of Dox-loaded nanosheets

Dox was loaded onto rGO and LHT-rGO nanosheets by physical adsorption. For drug loading, 1 ml of Dox solution (0.5 mg/ml; Sigma-Aldrich) was added to 1 ml of rGO or LHT-rGO nanosheets in TDW and stirred for 2 h at room temperature. Free Dox was then removed using a PD-10 desalting column (GE Healthcare), yielding Dox-loaded rGO (rGO/Dox) or Dox-loaded LHT-rGO (LHT-rGO/Dox). In some experiments, the resultant rGO/Dox or LHT-rGO/Dox were further eluted by the PD-10 desalting column, and the absorbance spectrum was measured using UV–vis spectrophotometry (UV-3100, Shimadzu Corp, Tokyo, Japan). The extent of Dox loading onto rGO and LHT-rGO nanosheets was determined by measuring the loss of Dox fluorescence at 485 nm caused by the quenching of adsorbed Dox by rGO or LHT-rGO using a fluorescence microplate reader (Gemini XS; Molecular Device).

2.6. Cellular uptake test of Dox delivered by LHT-rGO nanosheets

The cellular uptake of Dox was tested in human KB carcinoma cells using confocal microscopy. KB cells were seeded onto cover glasses at a density of 8×10^4 cells/well in 24-well plates. The next day, cells were treated with rGO/Dox or LHT-rGO/Dox at a concentration of 10 μM Dox. After incubating for 1 h, cells were washed and fixed with 4% paraformaldehyde in PBS for 15 min, and stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). The fluorescence of cellular Dox was observed using a confocal laser-scanning microscope (LSM 5 Exciter; Carl Zeiss, Inc., Jena, Germany).

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