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# Functionalized graphene oxide as a drug carrier for loading pirfenidone in treatment of subarachnoid hemorrhage

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ABSTRACT

Subarachnoid hemorrhage (SAH) is a life-threatening disease that causes high morbidity and mortality. Pirfenidone is a SAH drug that prevents secondary bleeding and cerebral infarction. To improve its therapeutic efficacy, this study aimed to employ a functionalized graphene oxide nanosheet (FGO) as a drug carrier loading pirfenidone to treat SAH. The graphene oxide nanosheet was introduced with transcription activator peptide (Tat), followed by functionalization with methoxy polyethylene glycol (mPEG) and loading with pirfenidone. The pirfenidone-loaded FGO (pirfenidone-FGO) exhibits better treatment efficacy than the single pirfenidone due to more effective loading and controlled release of the drug in tissue. The introduction of Tat and mPEG onto GO nanosheet contributes to the ability to cross the blood–brain barrier and the stability in blood circulation of the drug. At lower pH values, the highly efficient release of the drug from the pirfenidone-FGO exerts effective treatment to acidic inflammatory lesion after severe SAH. Besides its treatment function, FGO is also shown as a strong near infrared absorbing material which can be applied in photoacoustic imaging, allowing rapid real-time monitoring with deep resolution of brain tissues after SAH. The treatment efficacy of pirfenidone-FGO for central nervous system injuries is further demonstrated by hematoxylin and eosin staining of coronal brain slices, as well as measurements of brain water content and blood–brain barrier permeability. Our study supports the potential of FGO in clinical application in treatment of SAH.



## 1. Introduction

Subarachnoid hemorrhage (SAH) is a common and frequently neurologic and neurosurgical disease that has high morbidity and mortality rate [1]. Cerebral vasospasm is the major cause of death and disability following SAH, which causes arteries constriction at the base of the brain and leads to delayed cerebral ischemia and other serious complications. Cerebral ischemia is defined as potential development into ischemic neurologic deficits and brain injury after SAH. Current treatments of SAH, including pirfenidone and curcumin, aim to prevent re-bleeding and protect against the induction of cerebral vasospasm and secondary cerebral infarction after SAH. However, due to the limited ability of SAH drug to cross the blood–brain barrier, the behavioral outcome of patients receiving SAH treatment remains poor [2–4]. In order to be more effective, the drug must cross the blood–brain barrier to reach the cerebral arterial smooth-muscle cells to inhibit the contractions of these cells [5].

Due to the advantages in drug loading, delivery and release, nanomaterial-based drug carriers have shown highly promising prospect in various biomedical applications [6–10]. Graphene is a novel two-dimensional carbon nanomaterial with unique physical and chemical properties. The highly specific surface area of graphene sheet allows its use in drug delivery system as a vehicle with high loading capacity [11]. Recent reports have demonstrated that functionalized nanoscale graphene oxide (GO) drug carrier with biocompatible coating such as polyethylene glycol (PEG) polymer leads to more efficient loading and higher stability of the drug, without induction of cellular toxicity to biological systems [12–14]. Therefore functional graphene oxide derivatives with good biocompatibility and physiological stability have great prospects in biomedical applications. Besides its function as nano-drug carrier, graphene-based nano-composites with strong optical near-infrared (NIR) absorbance have also exhibited clinical potential in photoacoustic imaging (PAI) and photo-thermal therapy for tumors [15,16], suggesting an efficient localized treatment in brain regions of SAH and real-time monitoring system against cerebral vasospasm.

Aimed to improve treatment efficacy to SAH, we introduced the functionalized GO nanosheet (FGO) as drug carrier, synthesized

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by conjugating transcription activator peptide (Tat) and methoxy polyethylene glycol (mPEG) onto graphene oxide nanosheet. Pirfenidone, a drug treating SAH, was loaded onto the carbon plane of FGO. As a strong NIR absorbing material, pirfenidone-FGO not only allowed rapid real-time monitoring with deep resolution of brain tissues after SAH in PAI, but also exhibited better treatment efficacy of central nervous system injuries than the drug pirfenidone alone, due to its blood–brain barrier crossing capability and long-circulation time *in vivo*. These results suggested potential clinical applications of pirfenidone-FGO for treatment of central nervous system injuries featured with high convenience and effectiveness.

## 2. Materials and methods

### 2.1. Materials

Graphite flake was purchased from Alfa Aesar; Methoxy polyethylene glycol amine ($NH_2$-mPEG, Mn 5000), Tat peptide and sodium monochloroacetate were purchased from Sigma. Pirfenidone was obtained from Aladdin. Other reagents were purchased from local suppliers and used as received. RPMI 1640 culture medium and fetal bovine serum (FBS) were purchased from Invitrogen.

### 2.2. Instrumentation

UV/vis spectral measurements were taken on a Perkin Elmer Lambda 25 spectrophotometer. Absorbance in the WST assay was read by a Biotek Elx 800 microplate reader. Morphological analyses were performed with a Veeco Dimension 3100 atomic force microscope. Cell lines were cultured with a water-jacketed $CO_2$ incubator (Thermo 3111).

### 2.3. Synthesis of GO

The yellow brown GO was prepared from natural graphite powder (300 mesh, Alfa Aesar) by a modified Hummers method [17,18]. Briefly, native graphite flake (2 g) was mixed with concentrated $H_2SO_4$ (5 mL), $K_2S_2O_8$ (2 g), and $P_2O_5$ (2 g), and then incubated at 80 °C for 6 h to preoxidize the graphite. The product was washed with distilled water until neutral, then filtered and dried in air at ambient temperature overnight. The preoxidized graphite powder (2 g) was placed in concentrated $H_2SO_4$ (50 mL) at 10 °C followed by addition of $KMnO_4$ (6 g) while keeping the temperature of the mixture below 10 °C. The mixture was then stirred at 30 °C for 10 h and the reaction was terminated by adding distilled water (200 mL) to the mixture. Subsequently, 30% $H_2O_2$ (5 mL) was added to the mixture which was then centrifuged and washed with 10% HCl solution (250 mL) to remove residual metal ions. The precipitate was centrifuged, washed with distilled water, and sonicated in a sonication bath (100 W) for 2 h repeatedly until the solution became neutral. The product obtained was steadily dispersed in water and would not precipitate for several months.

### 2.4. Carboxyl group modification of GO

GO (100 mg) was dispersed in distilled water (100 mL). The GO suspension was then mixed with NaOH (5 g) and $ClCH_2COONa$ (5 g), followed by sonication bath for 2 h to allow the conversion of the OH groups on the sheet into COONa groups. The resulting product was then neutralized using diluted hydrochloric acid and purified by repeated rinsing and centrifugation until the product was well dispersed in deionized water.

### 2.5. Synthesis of FGO

To improve the stability of the GO in blood circulation and its ability to cross the blood–brain barrier, Tat and PEG molecules were sequentially conjugated onto the carbon plane of GO by reaction between the COOH groups of the GO and $NH_2$ groups on the Tat and $NH_2$-mPEG. The GO (10 mg) suspension was dialyzed against distilled water for over 48 h to remove any ions. NHS (180 mg) and EDC (100 mg) were added to GO dispersion, and the mixture was treated by ultrasonication for 2 h. Then, 0.5% Tat (0.1 mL, adjusted to pH 8.0 using sodium bicarbonate solution) was added and the mixture was stirred overnight. The unreacted materials were separated out by centrifugation and rinsed with sodium bicarbonate solution (pH 8.0) for three times. After that, 40 mg of $NH_2$-mPEG was added into the GO-Tat aqueous dispersion and the mixture was diluted to 5 mL. The mixture was stirred for 24 h, followed by centrifugation and rinsing with PBS buffer for three times. The Tat content was measured with the BCA protein assay kit (Applygen, China). The content of mPEG chain was estimated by measuring the yield of FGO.

### 2.6. Pirfenidone-loaded FGO

Pirfenidone-loaded FGO (Pirfenidone-FGO) was fabricated by mixing FGO aqueous solution with pirfenidone solution (Aladdin) and vortexed for 24 h at room temperature in the dark [11,19]. The pirfenidone-FGO was harvested by rinsing the precipite (16,000 × *g*, 30 min) three times in water. The foams were obtained by freeze drying the aqueous solution of pirfenidone-FGO, and stored at 4 °C when not in use.

### 2.7. Culture of PC12 cells

PC12 cells were cultured in DMEM supplemented with 10% FBS, 100 μg/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere with 5% $CO_2$.

### 2.8. Cell viability assay

The cell viability assay was detected by employing the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Cell Counting Kit-8, Dojindo) test. Briefly, the PC12 cells were seeded in 96-well plates at a density of $1 \times 10^5$ cells per well in 200 μL culture medium and maintained for 24 h [19]. The cells were then incubated with pure GO and pirfenidone-FGO for 24 h. Subsequently, the media were replaced with 100 μL of serum-free media and 10 μL MTT stock solution (5 mg/mL) was added to each well. After incubation for another 4 h at 37 °C, the supernatant was removed, and cells were lysed with 100 μL of DMSO [19].

### 2.9. Lactase dehydrogenase release assay

LDH assay was performed to measure cell membrane integrity since the cell was targeted by various agents. LDH activity was measured using a cytotoxicity detection kit from Dingguo Bioreagent. A coupled enzymatic assay was carried out to measure released LDH in culture supernatants *via* detecting the conversion of a tetrazolium salt (INT) into a red formazan product. PC12 cells ($1 \times 10^5$ cells/mL) were incubated with GO or pirfenidone-FGO at different concentrations for 24 h. Subsequently, 50 μL of supernatant was removed to another plate, followed by addition of 50 μL of substrate mix. The plate was covered with foil to protect it from light and then incubated for 30 min at room temperature. 50 μL of stop solution was then added to each well to terminate the reaction.