

Supporting Information

Design of peptide-grafted graphene oxide targeting the actin cytoskeleton for efficient cancer therapy

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Contents

Experimental Section	S1-S3
Table S1. XPS analysis of the un-grafted and grafted graphene nanosheets	S4
Table S2. IC ₅₀ of the tested GO nanosheets and related agents	S4
Fig. S1 Characterization of the synthesized GO	S5
Fig. S2 Mass spectrum of ABP	S6
Fig. S3 HPLC analysis of ABP	S6
Fig. S4 Mass spectrum of CPP	S7
Fig. S5 HPLC analysis of CPP	S7
Fig. S6 Stability of the synthesized GO nanosheets	S8
Fig. S7 Effect of GO-TF-ABP-CPP on actin organization	S8
Fig. S8 Effect of the GO nanosheets on the actin cytoskeleton in A549 cells	S9
Fig. S9 ABP alone has no impact on actin organization	S9
Fig. S10 Effect of the grafted GO nanosheets on apoptosis in tumor and normal cells	S10
Fig. S11 Distribution of the GO nanosheets in the S180 tumors	S10
Fig. S12 Blood circulation of GO-TF-ABP-CPP in S180 tumor-bearing mice	S11
Fig. S13 Effect of the GO nanosheets on the weight of the body and the organs in the S180 tumor-bearing mice	S11
Fig. S14 H&E staining of the RES organs in the GO nanosheet-treated and S180 tumor-bearing mice	S12

Experimental section

Chemicals

Aminopropyltriethoxysilane (APTES), glutaraldehyde and human transferrin (TF, MW=77,035) were purchased from Sigma (USA). Other reagents were purchased from Aladdin (China).

Synthesis and characterization of the graphene oxide (GO) precursor

The GO precursor used for further modification was synthesized using the modified Hummer's method. The obtained GO was characterized by atomic force microscopy (AFM, NT-MDT, NTEGRA Prima, Russia), Raman spectroscopy (Renishaw, inVia, UK), X-ray photoelectron spectroscopy (XPS, Kratos Analytical Ltd., Axis Ultra DLD, UK) and Fourier transformed infrared spectra (FT-IR, Bio-rad, FTS6000, USA).

Fluorescein isothiocyanate (FITC) tagging of transferrin (TF)

To tag the tumor targeting protein TF with FITC, 10 mg of TF were dissolved in 10 mL of NaHCO₃ buffer (25 mM, pH 9.5), and then 20 μ L of FITC (5 mg mL⁻¹, dissolved in ethanol) were added into the transferrin solution. The mixture was magnetically stirred at 4 °C for 12 h, dialyzed twice in PBS (pH 7.4) using dialysis bags (MW 8,000~10,000, Dingguo Ltd., China) to remove unconjugated FITC, and lyophilized in a freezing vacuum dryer, obtaining FITC-TF for further use.

Synthesis of ABP and CPP

The ABP (MGVADLIKKFESISKEEC, MW=2,027) and CPP (YGRKKRRQRRRC, MW=1,663) were synthesized using the solid phase method, and prepared by Beijing Protein Innovation Ltd., China. The obtained peptides were characterized by mass spectra (LC-MS2010, Shimadzu, Japan) and HPLC (LC3000, Constant innovation, China).

Synthesis and characterization of GO nanosheets grafted by TF and peptides

For modification of GO with TF and peptides, 40 mg of the prepared GO nanosheets were suspended in 40 mL of ethanol, and then 1 mL of APTES was added. The mixture was magnetically stirred at 80 °C for 2.5 h. The products were harvested by centrifugation, and then washed 3 times by ethanol and twice by distilled water, obtaining the silanized GO nanosheets with free amino groups (GO-NH₂). Ninhydrin assay indicated that there was \sim 1 μ mol of free amino groups per mg GO-NH₂. The obtained nanosheets were suspended in 5 mL of PBS buffer (pH 7.4), and then 1 mL of 8% glutaraldehyde was added. The mixture was gently shaken for 6 h at the room temperature and centrifuged to pellet GO-NH-glutaraldehyde. The pellets were washed 3 times by PBS and 2 times by distilled water, and lyophilized in a freezing vacuum dryer for further use.

To synthesize the grafted GO nanosheets, GO-NH-glutaraldehyde, FITC-TF, ABP and CPP were dissolved in PBS to the indicated concentrations, respectively. GO-TF was synthesized by mixing 10 mL of GO-NH-glutaraldehyde (1 mg mL⁻¹) and 100 μ L of FITC-TF (50 μ M) for reaction. GO-TF-ABP were synthesized by mixing 10 mL of GO-NH-glutaraldehyde (1 mg mL⁻¹), 100 μ L of FITC-TF (50 μ M) and 100 μ L of ABP (1 mM). GO-TF-CPP were synthesized by mixing 10 mL of GO-NH-glutaraldehyde (1 mg mL⁻¹), 100 μ L of FITC-TF (50 μ M) and 100 μ L of CPP (1 mM). GO-TF-ABP-CPP were synthesized by mixing 10 mL of GO-NH-glutaraldehyde (1 mg mL⁻¹), 100 μ L of FITC-TF (50

μM), 100 μL of ABP (1 mM) and 100 μL of CPP (1 mM). The mixtures were gently agitated in 4 °C for 20 h. The grafted GO nanosheets were harvested by centrifugation, washed 3 times by distilled water and lyophilized in a freezing vacuum dryer. During the reactions, the free amino groups ($\sim 10 \mu\text{mol}$ of free amino groups per 10 mg GO-NH₂) were much excessive as compared to FITC-TF (5 nmol for 10 mg GO-NH₂), ABP (100 nmol for 10 mg GO-NH₂) and CPP (100 nmol for 10 mg GO-NH₂). After the reactions, no free TF or peptides were detected in the supernatants, indicating that the added TF or peptides were thoroughly grafted into GO-NH₂. Therefore, the corresponding GO nanosheets were successfully obtained: GO-TF (containing 5 nmol FITC-TF per 10 mg GO), GO-TF-ABP (containing 5 nmol FITC-TF and 100 nmol ABP per 10 mg GO), GO-TF-CPP (containing 5 nmol FITC-TF and 100 nmol CPP per 10 mg GO) and GO-TF-ABP-CPP (containing 5 nmol FITC-TF, 100 nmol ABP and 100 nmol CPP per 10 mg GO).

To examine the stability of the synthesized GO nanosheets, the nanosheets were suspended in PBS buffer (pH 7.4) or serum (FBS, Hyclone) at the concentration of 20 $\mu\text{g mL}^{-1}$. 800 μL of the suspensions were then added into Eppendorf tubes (1.5 mL). The tubes were horizontally positioned for 24 h and photographed.

Actin staining and imaging

The mammalian cell lines, including the tumor cell lines (the human lung tumor cell line A549, the mouse sarcoma cell line S180) and the normal cells (the human lung fibroblast line MRC-5, the mouse embryo fibroblast line NIH3T3), were purchased from the Cell Resource Center, China Academy of Medical Science, Beijing, China. To observe actin distribution in the GO- or ABP-treated A549 cells, the cells were cultured in F12 medium supplemented with 10% FBS (Hyclone) in the CO₂ incubator at 37 °C for 24 h. The solutions of GO nanosheets (1,000 $\mu\text{g mL}^{-1}$, prepared in PBS buffer, pH 7.4) or ABP (1,000 $\mu\text{g mL}^{-1}$, prepared in PBS buffer, pH 7.4) were then added into the cell cultures to a final concentration of 20 $\mu\text{g mL}^{-1}$. The cells were further cultured for 24 h, washed twice with PBS, and fixed by methanol for 30 min. After treated by 0.5% Triton X-100 for 10 min, the cells were stained by rhodamine B-tagged phalloidin (final concentration of 50 nM) and DAPI (final concentration of 5 $\mu\text{g mL}^{-1}$), washed 3 times by PBS, and observed using a confocal microscope (Olympus, FV1000, Japan).

***In vitro* cytotoxicity assays of GO nanosheets, TF and peptides**

To tested the *in vitro* cytotoxicity of GO nanosheets, TF or peptides, these materials (1,000 $\mu\text{g mL}^{-1}$, prepared in PBS buffer) were added into the 96-well microplates (containing pre-cultured cells) to a final concentration of 20 $\mu\text{g mL}^{-1}$ in most of the cytotoxicity experiments or to the following final concentrations for evaluation of IC₅₀, 0, 10, 20, 40, 80 and 160 $\mu\text{g mL}^{-1}$, respectively. The cells were cultured for 24 h and washed twice by PBS buffer. 100 μL of MTT (500 $\mu\text{g mL}^{-1}$) were then added into each well, and the microplates were incubated at 37 °C for 2 h. The solutions were then removed, and the wells were washed twice by PBS. 100 μL of DMSO were added into each well to dissolve the produced purple formazan. The extraction solutions were then transferred into a new microplate and used for determining OD₄₉₀. The percent of viability is calculated by OD₄₉₀ of each group divided by OD₄₉₀ of the control (without treatment of the materials) $\times 100$. To detect apoptosis and cell cycle, A549 cells were treated by 20 $\mu\text{g mL}^{-1}$ of the nanosheets for 24 h as described above and harvested. Apoptosis and cell cycle were then examined by the FITC-Annexin V/PI apoptosis assay kit and the cell cycle assay kit (Beyotime

Biotech., China), respectively.

Animal model

The S180 tumor cells were suspended in distilled saline with the concentration of 10^7 cells mL^{-1} . 100 μL of S180 cell suspensions were subcutaneously inoculated into each 4 week-old female BALB/c mice (Huafukang, China). After inoculation for 7 days, the suspensions of GO nanosheets were intravenously injected into the mice (20 mg kg^{-1} , once per 2 days), and the tumor volumes were monitored for further 12 days. The mice were weighed, anaesthetized and sacrificed. The tumors and reticulo-endothelial system (RES) organs (e.g., liver, spleen and kidney) were sampled, weighed, fixed by 4% formaldehyde solution, embedded with paraffin and sectioned into slices. The slices were then stained by the H&E kit and observed by a light microscope (DM3000, Leica, Germany).

To detect the half-life time of GO nanosheets, the suspensions of GO nanosheets were intravenously injected into the mice (20 mg kg^{-1}). Approximately 10 μL of blood were sampled from the tail vein of the mice at different time points, and the fluorescence intensity (FLU, excitation wave 488 nm, emission wave 520 nm) of the blood samples was detected by a fluorescent microplate reader (Perkin Elmer, USA).

The *in vivo* biodistribution and tumor targeting efficiency of GO nanosheets were evaluated in the S180 tumor-bearing nude mice (Huafukang, China). 100 μL of S180 cell suspensions (1×10^7 cells mL^{-1}) were subcutaneously inoculated into the 4 week-old female BALB/c nude mice. After 7 days of inoculation, the GO nanosheets were intravenously injected into the mice (20 mg kg^{-1}). The biodistribution of the nanosheets were detected at 0, 1 and 24 h post injection using the In-Vivo Imaging System (Xenogen, IVIS Lumina II, USA). The mice were then sacrificed, and the organs and tumors were also sampled for detection of fluorescence using the same imaging system.

Ethical statement

All of the biological experiments, including the animal experiments and the cell culturing experiments, were performed in compliance with the guidelines of the Animal Care and Use Committee at Nankai University and the experiment guidelines of the College of Life Science at Nankai University. The Committee approved all of the experiments.

Statistical analysis

Each experiment was performed with three replicates under the tested conditions, and the values represent the means + (or \pm) standard deviations (SD) of three experiments. Differences in the strains were compared by one-way ANOVA test ($p < 0.05$). All statistical tests were performed using the SPSS Statistics Software (V20, IBM, USA).

Table S1. XPS analysis of the un-grafted and grafted GO

	Atomic %				
	GO	GO-TF	GO-TF-ABP	GO-TF-CPP	GO-TF-ABP-CPP
C 1s	69.28	64.02	65.38	61.23	66.71
O 1s	30.15	28.62	27.16	31.64	21.92
N 1s	0.00	4.63	4.26	4.75	8.67
Si 2p	0.00	2.32	2.86	2.00	2.32
S 2p	0.56	0.41	0.33	0.38	0.38

Table S2. IC₅₀ of the tested GO nanosheets and related agents

	IC ₅₀ (μg mL ⁻¹)			
	A549	S180	NIH3T3	MRC-5
GO	>160	>160	>160	>160
GO-NH ₂	>160	>160	>160	>160
TF	>160	>160	>160	>160
ABP	>160	>160	>160	>160
CPP	>160	>160	>160	>160
GO-TF	>160	>160	>160	>160
GO-TF-ABP	73.97±10.45	67.57±7.92	>160	>160
GO-TF-CPP	93.35±13.62	93.26±11.50	>160	>160
GO-TF-ABP-CPP	13.87±2.01	9.83±2.64	>160	>160

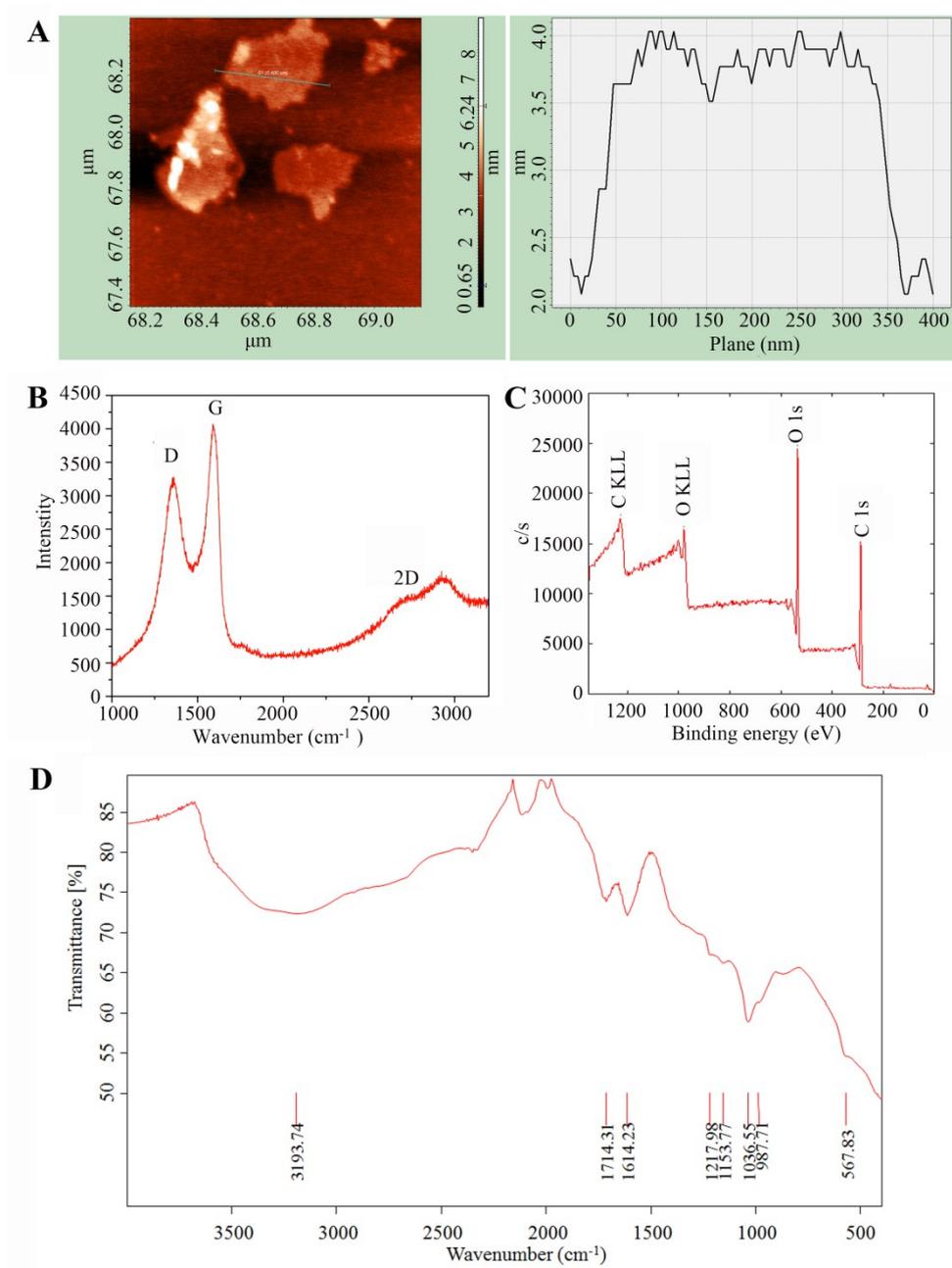


Fig. S1 Characterization of the synthesized GO. (A) AFM image. (B) Raman shift spectrum. (C) XPS spectrum. (D) FT-IR spectrum.

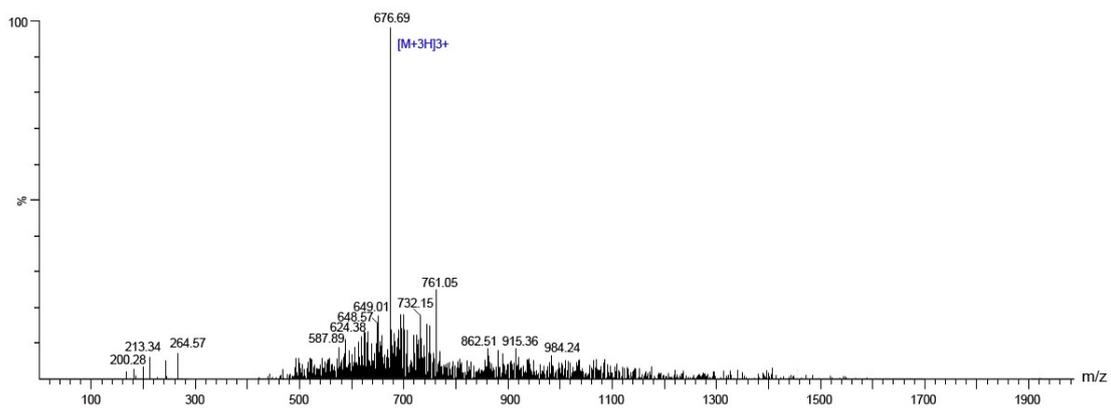


Fig. S2 Mass spectrum of ABP.

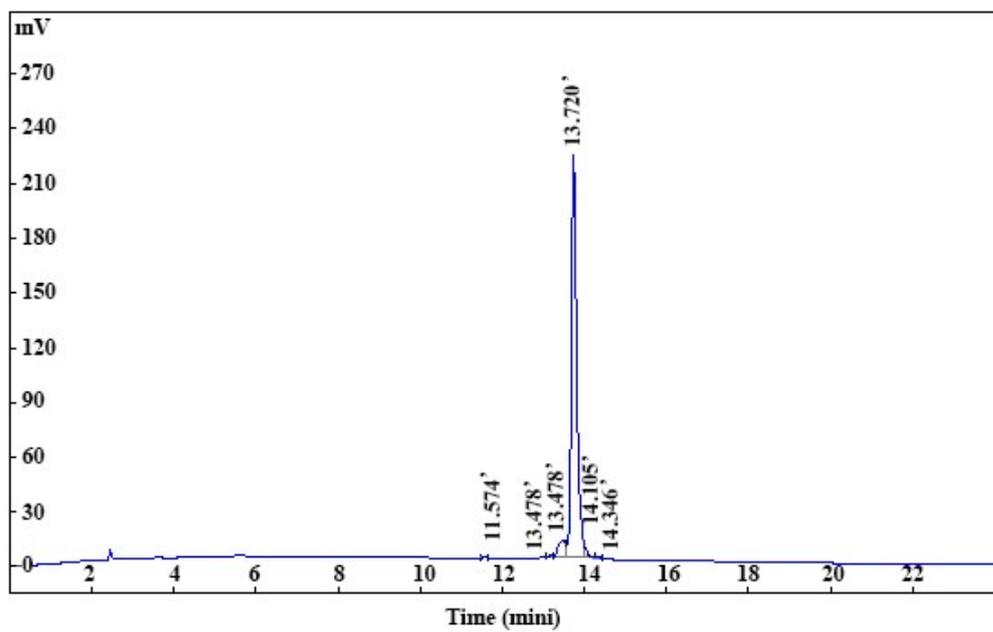


Fig. S3 HPLC analysis of ABP.

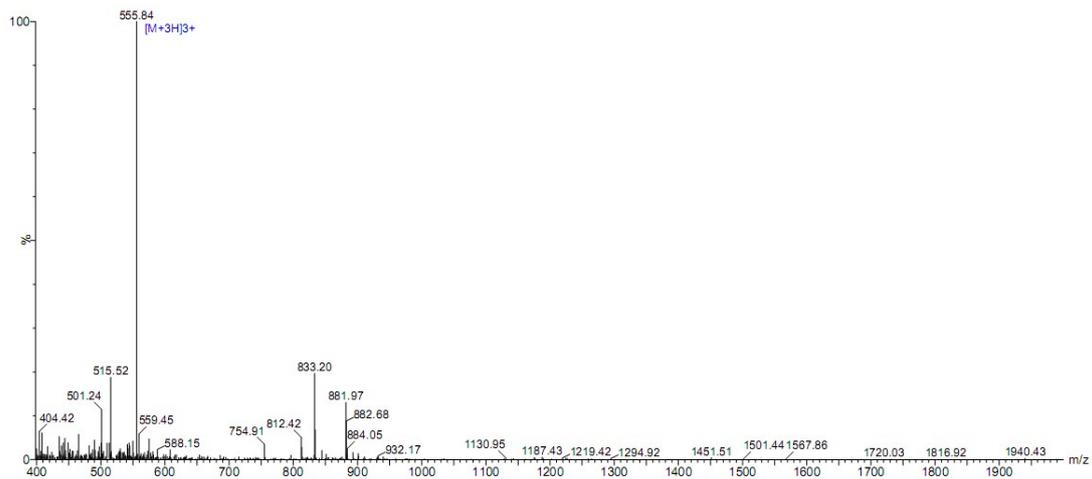


Fig. S4 Mass spectrum of CPP.

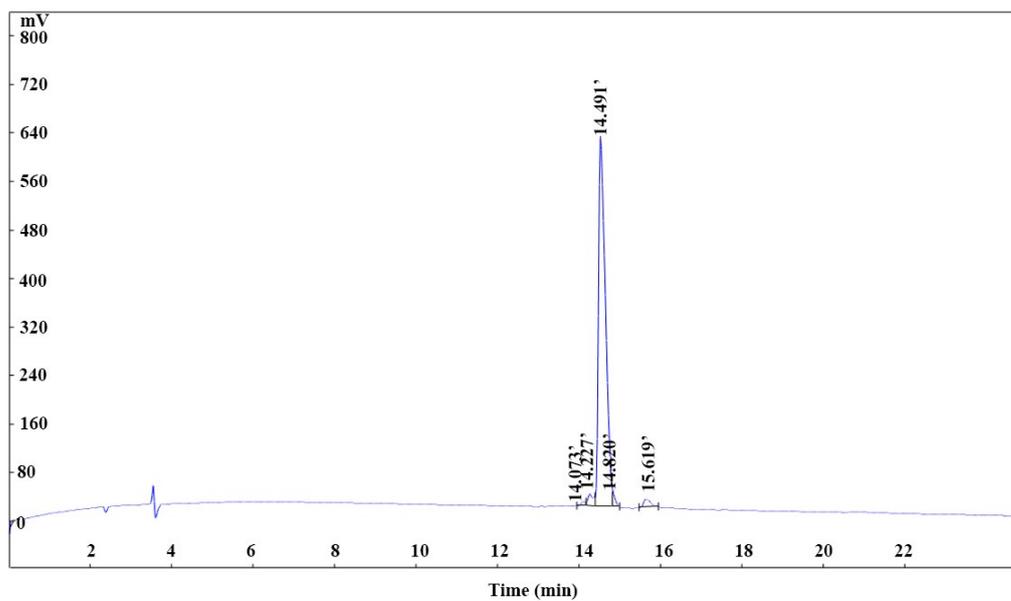


Fig. S5 HPLC analysis of CPP.

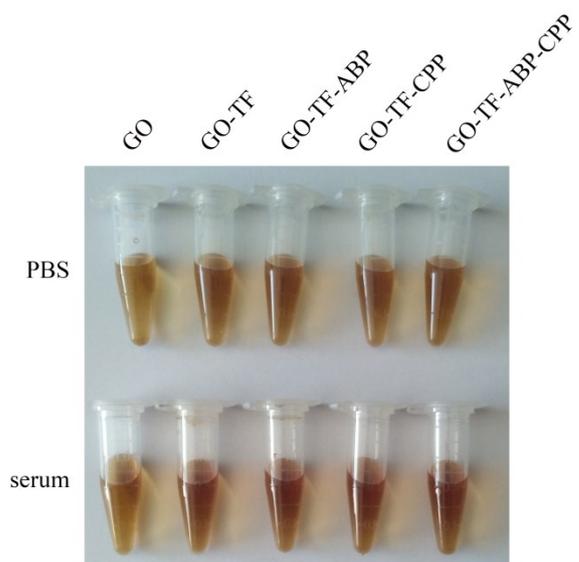


Fig. S6 Stability of the synthesized GO nanosheets.

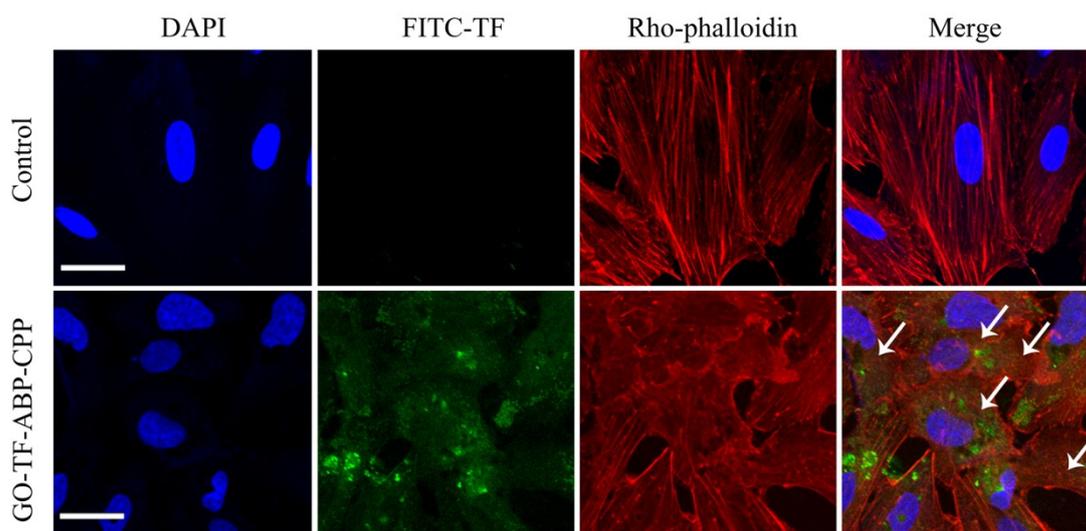


Fig. S7 Effect of GO-TF-ABP-CPP on actin organization. The white arrows indicate the cells with disrupted actin cytoskeleton. Bar = 20 μ m.

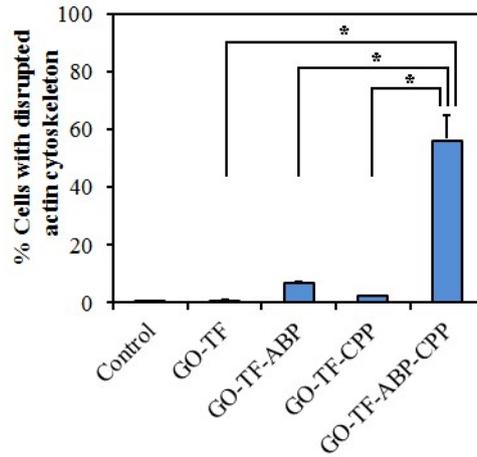


Fig. S8 Effect of the GO nanosheets on the actin cytoskeleton in A549 cells. * indicates significant difference between the treatments ($p < 0.05$).

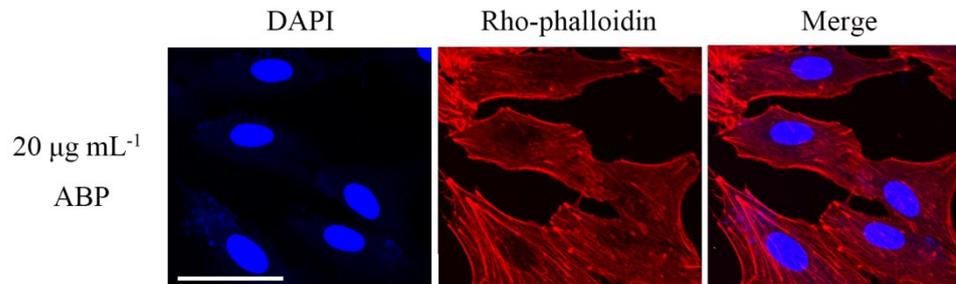


Fig. S9 ABP alone has no impact on actin organization. The A549 cells were treated with 20 $\mu\text{g mL}^{-1}$ ABP for 24 h, stained by DAPI and Rhodamine B-phalloidin, and observed using a fluorescence microscopy. Bar = 50 μm . Note that the treated cells display normal actin filaments.

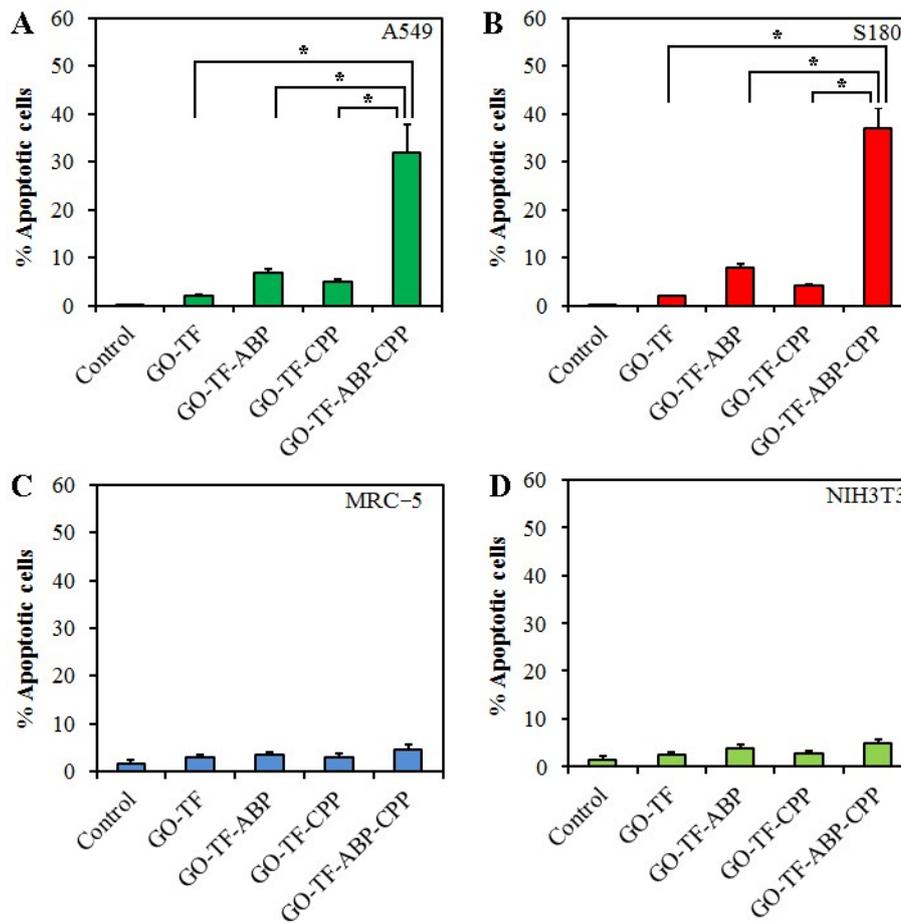


Fig. S10 Effect of the grafted GO nanosheets on apoptosis in tumor cells (A, B) and normal cells (C, D). * indicates significant difference between the treatments ($p < 0.05$).

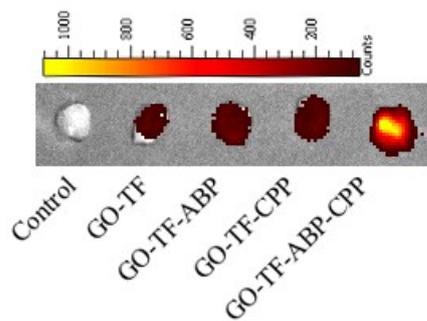


Fig. S11 Distribution of the GO nanosheets in the S180 tumors. The tumors were sampled after 24 h post injection, and then used for imaging.

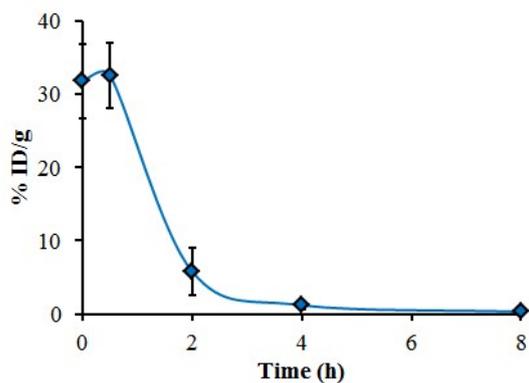


Fig. S12 Blood circulation of intravenously injected GO-TF-ABP-CPP in S180 tumor-bearing mice. The blood was sampled from the mice at different time points post injection and used for determination of fluorescence intensity.

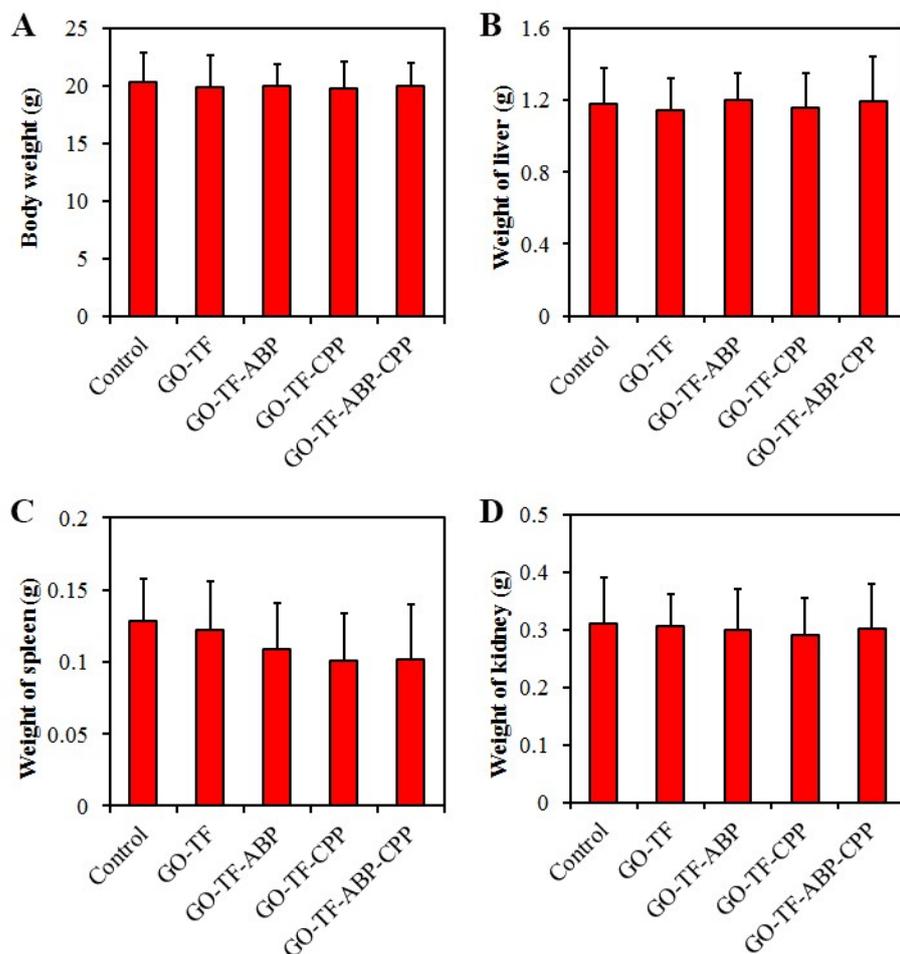


Fig. S13 Effect of the GO nanosheets on the weight of the body and the organs in the S180 tumor-bearing mice. Note that there is no significant difference in the weight of the body and the organs between the groups.

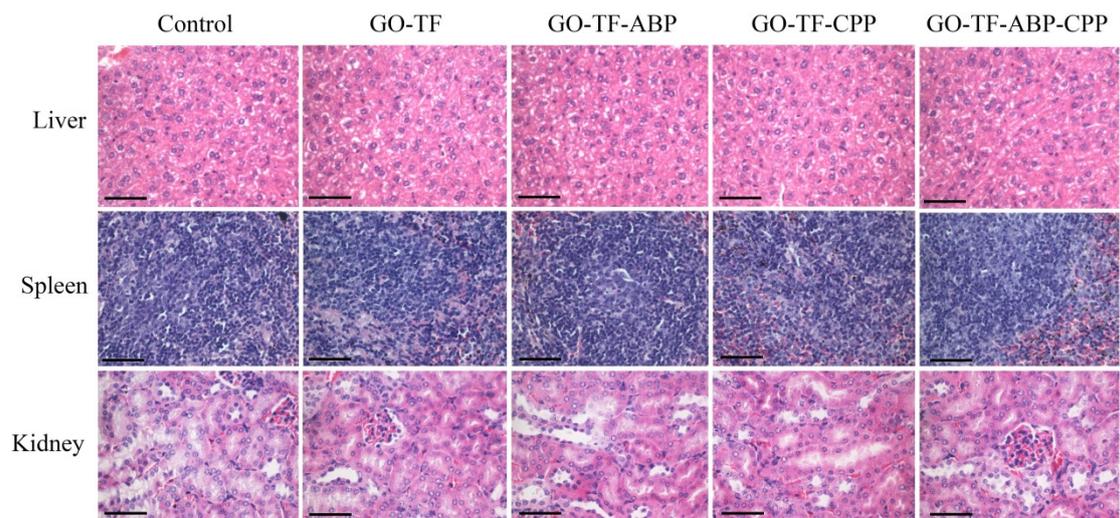


Fig. S14 H&E staining of the RES organs in the GO nanosheet-treated and S180 tumor-bearing mice. Note that the tissues of RES organs remain normal under the treatment of GO nanosheets. Scale bar, 50 μ m.