Electronic Supplementary Material (ESI) for Biomaterials Science.
This journal is © The Royal Society of Chemistry 2023

Manipulation and Elimination of Circulating Tumor Cells using Multi-responsive Nanosheet for Malignant Tumor Therapy

Tao Liu[1, 3#], Bolei Cai[2#], Pingyun Yuan[1], Le Wang[2], Ran Tian[1], Taiqiang Dai[2], Lin Weng[1], Xin Chen[1]*

1. School of Chemical Engineering and Technology, Shaanxi Key Laboratory of Energy Chemical Process Intensification, Institute of Polymer Science in Chemical Engineering, Xi'an Jiao Tong University, Xi'an 710049, P. R. China

2. State Key Laboratory of Military Stomatology & National Clinical Research Center for Oral Diseases & Shaanxi Key Laboratory of Oral Diseases, Department of Oral and Maxillofacial Surgery, School of Stomatology, The Fourth Military Medical University, Xi'an 710032, China

3. National & Local Joint Engineering Research Center of Biodiagnosis and Biotherapy, The Second Affiliated Hospital of Xi'an Jiaotong University, Xi'an, China

* Corresponding Authors (E-mail: chenx2015@xjtu.edu.cn)

[#] These authors contributed equally to this work.

1. Materials

Trisodium citrate dihydrate ($Na_3C_6H_5O_7.2H_2O$), Sodium hydroxide (NaOH), Sodium nitrate ($NaNO_3$) and ferrous sulfate tetrahydrate ($FeSO_4$•$4H_2O$) were purchased from Tianjin Tianli Chemical Reagent Co., Ltd (Tianjin, China). Tetraethylorthosilicate (TEOS, 99.0%) and $NH_3 \cdot H_2O$ (AR, 25% ~ 28%) were purchased from Sinopharm Chemical Reagent Co., Ltd (Xi'an, China). Absolute ethanol (AR, 99.7%) and methanol were purchased from Tianjin HengXing Chemical Reagent Co., Ltd (Tianjin, China). 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC•HCl), n-hydroxysuccinimide (NHS), Aminopropyltriethoxysilane (APTES) and doxorubicin hydrochloride (DOX•HCl) were purchased from Energy Chemical (Shanghai, China). Graphene oxide (GO) was purchased from Goodfellow trading co., Ltd (Shanghai, China). Dual epoxy functionalized polyethylene glycol (epoxy-PEG-epoxy, MW 5k) and dual aldehyde functionalized polyethylene glycol (CHO-PEG-CHO, MW 5k) were purchased from Peng Sheng Biological Co., Ltd (Shanghai, China). Ethylenediamine was purchased from Xi'an Security Chemical Co., Ltd (Xi'an, China). Folic Acid (FA) was purchased from Shanghai Titan Technology Co., Ltd (Shanghai, China). Antimicrobial peptide (AMP, MW 3k-4k) was from Nanjing Tech University. MilliQ water was prepared using a MilliQ system (Bedford, MA, America).

2. Synthesis of silica-coated magnetic nanoparticles (Fe_3O_4@SiO_2)

A typical synthesis of hydrophilic magnetite NPs according to the literature[1] is as follows: 1 mmol of $Na_3C_6H_5O_7.2H_2O$, 4 mmol of NaOH, and 0.2 mol of $NaNO_3$ were mixed in 19 mL of deionized water, then heated to 100 °C and formed a pellucid solution. After adding 1 mL of 2 M $FeSO_4 \cdot 4H_2O$ (2mmol) solution, the mixed solution was kept at 100 °C for 1 h and cooled down to room temperature naturally. The Fe_3O_4 NPs were separated and purified from solvent by a magnet for several times. The resulted black precipitations were washed with ethanol and water three times each, and then dried under vacuum at 60 °C for 12 h.

The as-synthesized Fe_3O_4 nanoparticles were dispersed in 100 mL of mixed solvent containing 80 ml of ethanol and 20 ml of water. After adding 1 mL of ammonia, 200 μL of TEOS was added with rapid stirring. The reaction was maintained at room temperature for 8 h. The resulted Fe_3O_4@SiO_2 nanoparticles were washed with ethanol and water three times each, then dried under vacuum at 60 °C for 12 h.

3. Synthesis of Fe_3O_4@SiO_2-GO

Firstly, the as-synthesized Fe_3O_4@SiO_2 nanoparticles were dispersed in 100 mL of mixed solvent containing 100 mL of ethanol. After adding 1 mL of ammonia, 400 μL of APTES was added with rapid stirring. The reaction was maintained at room temperature for 8 h. The resulted amino-decorated Fe_3O_4@SiO_2 nanoparticles (Fe_3O_4@SiO_2-NH_2) were washed with ethanol and water three times each, then dried under vacuum at 60 °C for 12 h.

Then, 30 mg of the above Fe_3O_4@SiO_2-NH_2 nanoparticles were dispersed in 20 mL water. After adding 10 mg of GO, the reaction was maintained at room temperature for 12 h. The resulted Fe_3O_4@SiO_2-GO nanosheets were separated and purified from solvent by a magnet for several times, and then washed with ethanol and water three times each, then dried under vacuum at 60 °C for 12 h.

4. Synthesis of Fe_3O_4@SiO_2-GO-PEG-FA

The Fe_3O_4@SiO_2-GO-PEG-epoxy was firstly synthesized as follows: the above Fe_3O_4@SiO_2-GO was dispersed in 20 mL water, then 100 mg of the epoxy-PEG-epoxy was added to the dispersion and reacted at 30 °C for 12 h. The resulted Fe_3O_4@SiO_2-GO-PEG-epoxy was separated and purified from solvent by a magnet for several times, and then washed with water three times each.

After that, the obtained Fe_3O_4@SiO_2-GO-PEG-epoxy was re-dispersed into 20 mL of water, and continued to react with 100 μL of ethylenediamine at 30 °C overnight to convert epoxy groups into amino groups for the further connection with

FA, which named Fe_3O_4@SiO_2-GO-PEG-NH_2. Subsequently, 5 mg of FA, 20 mg of EDC•HCl and 10 mg of NHS were mixed in 10 mL water for activation by incubation at room temperature for 2 h. And then, the above Fe_3O_4@SiO_2-GO-PEG-NH_2 was added into the mixture to react at 30 °C for 12 h, which was purified by a magnet for several times and then washed with water three times each.

5. Synthesis of Fe_3O_4@SiO_2-GO-PEG-FA/CHO

In order to fabricate pH cleavable Schiff base bonds, the resulted Fe_3O_4@SiO_2-GO-PEG-FA was first reacted with ethylenediamine to convert the carboxyl groups of GO to amino groups and then connected with CHO-PEG-CHO, which denoted as Fe_3O_4@SiO_2-GO-PEG-FA/CHO. Specifically, the obtained Fe_3O_4@SiO_2-GO-PEG-FA, EDC•HCl (20 mg) and NHS (10 mg) were mixed in 10 mL water for activation by incubation at room temperature for 2 h. After that, 100 μL of ethylenediamine was added into the mixture to react for 12 h. Subsequently, the resulted product was re-dispersed into 20 mL of methanol. After adding 100 mg of CHO-PEG-CHO and 2 μL of acetic acid, the mixed solution was kept at 30 °C for 24 h. Finally, the resulted Fe_3O_4@SiO_2-GO-PEG-FA/CHO was purified by a magnet for several times and then washed with ethanol and water three times each.

6. Synthesis of Fe_3O_4@SiO_2-GO-PEG-FA/AMP-DOX (FGPFAD)

The prepared Fe_3O_4@SiO_2-GO-PEG-FA/CHO was then reacted with DOX-labeled AMP (AMP-DOX) to fabricate a pH-responsive drug delivery system, which would release in the acidic environment of the tumor because of the generation of Schiff base bonds.

Firstly, DOX-labeled AMP (AMP-DOX) was synthesized as follows: 200 mg of AMP, 64 mg of EDC•HCl and 20 mg of NHS were mixed in 10 mL water with stirring. Then, 4 mg of DOX•HCl was added to the mixture and continued to react overnight in the dark. After the reaction, the product was dialyzed against distilled water for 3 d in a dialysis bag with a molecular weight cutoff (MWCO) of 1000 Da and then dried with a freeze dryer.

After that, the prepared Fe_3O_4@SiO_2-GO-PEG-FA/CHO was dispersed into 20 mL of water, which was reacted for 24 h in the dark after the addition of 100 mg of the resulted AMP-DOX. Lastly, the resulted Fe_3O_4@SiO_2-GO-PEG-FA/AMP-DOX (FGPFAD) was purified by a magnet for several times and then washed with water three times each, then dried under vacuum at 60 °C for 12 h in the dark.

7. Tumor cells capture experiments

2058 cells (A-2058 human melanoma malignum cells) and HUVEC cells (Human Umbilical Vein Endothelial Cells) used in this study were obtained from the National Institutes of Health (NIH). These cells were cultured in Dulbecco's modification of Eagle's medium (DMEM, Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS, Gibico, USA) and penicillin-streptomycin (100 U/mL and 100 μg/mL, Gibico, USA), and incubated at 37°C in 5% CO_2.

The 2058 cells were seeded at 2×10^4 per well onto 24-well plates containing glass coverslips for 24 hours before treatment. FGPFAD and Fe_3O_4@SiO_2-GO-PEG-AMP-DOX (FGPAD, nanosheets without FA ligand) were incubated with 2058 cells (pH 7.4, simulate blood environment). After incubation, the cells were rinsed with PBS for 5 times, fixed with 4% paraformaldehyde for 20 min, permeabilized in 0.1% Triton X-100 for 5 min, and then stained with 4′, 6-diamidino-2-phenylindole (DAPI, Life Technologies, USA) and phalloidin (sigma-aldrich, USA). Afterwards, the cells were rinsed, mounted and the fluorescence was observed under a fluorescence microscope (Olympus BX51, Olympus, Japan).

8. Magnetically-induced aggregation of tumor cells *in vitro*

The 2058 cells were seeded at 2×10^4 per well onto 24-well plates for 24 hours before treatment. Fe_3O_4@SiO_2-GO-PEG-FA/AMP (nanosheets without fluorescence) was incubated with 2058 cells (pH 7.4). The external magnetic field was applied at one side for 24 hours and the aggregation of cells before and after the addition of a magnetic field was stained with DAPI and observed.

9. Cell uptake experiments

To observe the selective cell uptake of FGPFAD nanosheets, 2058 cells and HUVEC cells were seeded at 2×10^4 per well into 24-well plates. The FGPFAD were incubated with 2058 cells (pH 6.8) and HUVEC cells (pH 7.4) for 10 mins, 2 hours and 24 hours. After rinsing with PBS for 5 times, cells were fixed with 4% paraformaldehyde for 20 min, and then stained with DAPI and phalloidin. The stained cells were observed under a fluorescence microscope (Olympus BX51, Olympus, Japan).

For quantitative analysis of cell uptake of FGPFAD, 2058 cells (pH 6.8) and HUVEC cells (pH 7.4) were seeded in a 6 well plate at a density of 1×10^6 per well for 24 hours. Then the FGPFAD was incubated with cells at the Pt concentration of 1 μM at 37 °C for 24 h. After incubation, the cells were washed by PBS 3 times then detached by trypsin (0.25 %). The cell suspensions were spun down and wash by PBS twice. Finally, the cells were counted by flow cytometer (FC500; Beckman Counter, CA, USA).

10. *In vitro* cytotoxicity analysis

2058 cells and HUVEC cells were seeded at 3×10^3 per well in 96-well plate for 24h before treatment. Then the cells were exposed to different concentrations of Fe_3O_4@SiO_2-GO (group 1), Fe_3O_4@SiO_2-GO-PEG-FA/AMP (group 2), FGPFAD nanosheets before (group 3) and after 808 nm NIR irradiation (group 4) as well as DOX+AMP (group 5) for 48 h. Cell viability was measured by using the Cell Counting Kit 8 (CCK-8, Dojindo Co., Ltd. Japan) according to the manufacture's protocol. The absorbance of the wells was read at 450 nm by using Varioskan Flash multimode reader (Thermo Fisher Scientific, USA).

11. *In vivo* tumor metastasis inhibition experiments

All experimental protocols were approved by the Ethics Committee of the Fourth Military Medical University Health Science Center, Xi'an, China. To set up the tumor

xenograft model, BALB/c male nude mice (5 weeks old) were prepared and a total of 5×10^6 2058 cells were injected subcutaneously into the back, respectively. When the tumor reached approximately 60 mm^3 in volume, 5×10^5 2058 cells with D-Luciferin (Promega, Madison, WI, USA) were injected intravenously *via* tail vein in nude mice to obtain metastatic lung tumors. After the luciferase-labeled 2058 cells were injected, the mice were injected *via* the tail vein with i: PBS; ii: Fe_3O_4@SiO_2-GO-PEG/AMP-DOX; iii: Fe_3O_4@SiO_2-GO-PEG-FA/AMP; iv: FGPFAD, respectively (dosage: 10 mg/kg mice). After the vein injection, an external magnetic field was employed at the *in-situ* tumor for 24 hours. Then, the luminescence and fluorescent signal were recorded by using the Xenogen-IVIS Imaging System. In the supine position, the metastatic area in lungs was observed *in vivo* using the Xenogen-IVIS Imaging System on the 24th day after the vein injection.

12. *In vivo* Antitumor experiments

When the tumor reached approximately 60 mm^3 in volume, 5×10^5 2058 cells with D-Luciferin (Promega, Madison, WI, USA) were injected intravenously *via* tail vein in nude mice to obtain metastatic lung tumors.

After the 2058 cells were injected, thirty-six tumor-bearing mice were randomly divided into six groups (n = 6) and the mice were injected *via* the tail vein with I: PBS; II: DOX+AMP; III: FGPFAD; IV: Fe_3O_4@SiO_2-GO-PEG-FA+NIR; V: FGPFAD+NIR. Specifically, a volume of 100 μL of PBS, the mixture of DOX and AMP (DOX+AMP, 5 $\mu g\cdot mL^{-1}$), aqueous solution of FGPFAD, and Fe_3O_4@SiO_2-GO-PEG-FA (10 mg/kg mice) were used. After the vein injection, an external magnetic field was employed at the *in-situ* tumor for 24 hours，the tumorous areas were exposed to 808 nm NIR irradiation (1.1 W/cm^2) for 5 mins to investigate the photothermal therapeutic effect *in vivo*. The tumor size ($V = W^2 \times L/2$ mm^3) was measured, and the body weight were recorded every 3 days for 21 days. At day 21st, the tumors were collected and fixed in 10% formalin overnight, embedded in paraffin,

and sectioned at a thickness of 5 μm. The sections were stained with a DeadEnd Fluorometric or Colorimetric TUNEL system (Promega Corporation, Madison, Wis) and hematoxylin and eosin (H&E).

Another five groups were further investigated for lung metastasis and survival (I: PBS; II: DOX+AMP; III: FGPFAD; IV: Fe_3O_4@SiO_2-GO-PEG-FA+NIR; V: FGPFAD+NIR) until the mice were either naturally died or were sacrificed when the tumor volume grew to 2000 mm^3 for the survival analysis, according to the animal ethical requirement. The lung tissues were collected and fixed in 10% formalin overnight, embedded in paraffin, and sectioned at a thickness of 5 μm. The sections were stained with a hematoxylin and eosin (H&E) to observe the metastatic area in lungs. The histological observation of the organs was used to verify the *in vivo* toxicity of FGPFAD nanosheets.

13. Statistical analysis

All results are representative of data generated in three independent experiments. All numerical values were expressed as the mean ± SD. For multiple comparisons, statistical analysis was performed using one-way ANOVA followed by a Bonferroni post-test. For individual comparisons, statistical analysis was performed using two-tailed t-test. Data analysis was performed using SPSS 22.0 software and considered statistically significant at $P < 0.05$.

14. Characterization

The infrared (IR) spectra were measured by Nicolet iS50 FT-IR using KBr pellets. The thermogravimetric curves are measured by synchronous thermal analyzer (TG) using NETZSCH STA449F5. The zeta potential of nanosheets is examined by Malvern nanoparticle size with zeta potentiometer (Zetasizer Nano ZSE). Transmission electron microscopy (TEM) images were recorded on a TECNAI G2 spirit BioTwin Transmission electron microscope operated at 200 kV. For the TEM observation, samples were obtained by dropping 10 μL of solution onto carbon-coated copper grids. All the TEM images were visualized without staining. The XPS analysis

of nanosheets is examined by Thermo Fisher ESCALAB Xi+. The temperature measurement was carried out on near infrared laser light source (808NL-2W) and thermal imager (Testo AG ATS024T-W050V). The cellular uptake of FGPFAD was monitored by fluorescence microscopy using an Olympus BX51 microscope equipped with a fluorescent lamp: ex = 495 nm, em = 600 nm for DOX.

References

1. Hui, C.; Shen, C.; Yang, T.; Bao, L.; Tian, J.; Ding, H.; Li, C.; Gao, H. J., Large-Scale Fe_3O_4 Nanoparticles Soluble in Water Synthesized by a Facile Method. *The Journal of Physical Chemistry C* **2008,** *112* (30), 11336-11339.

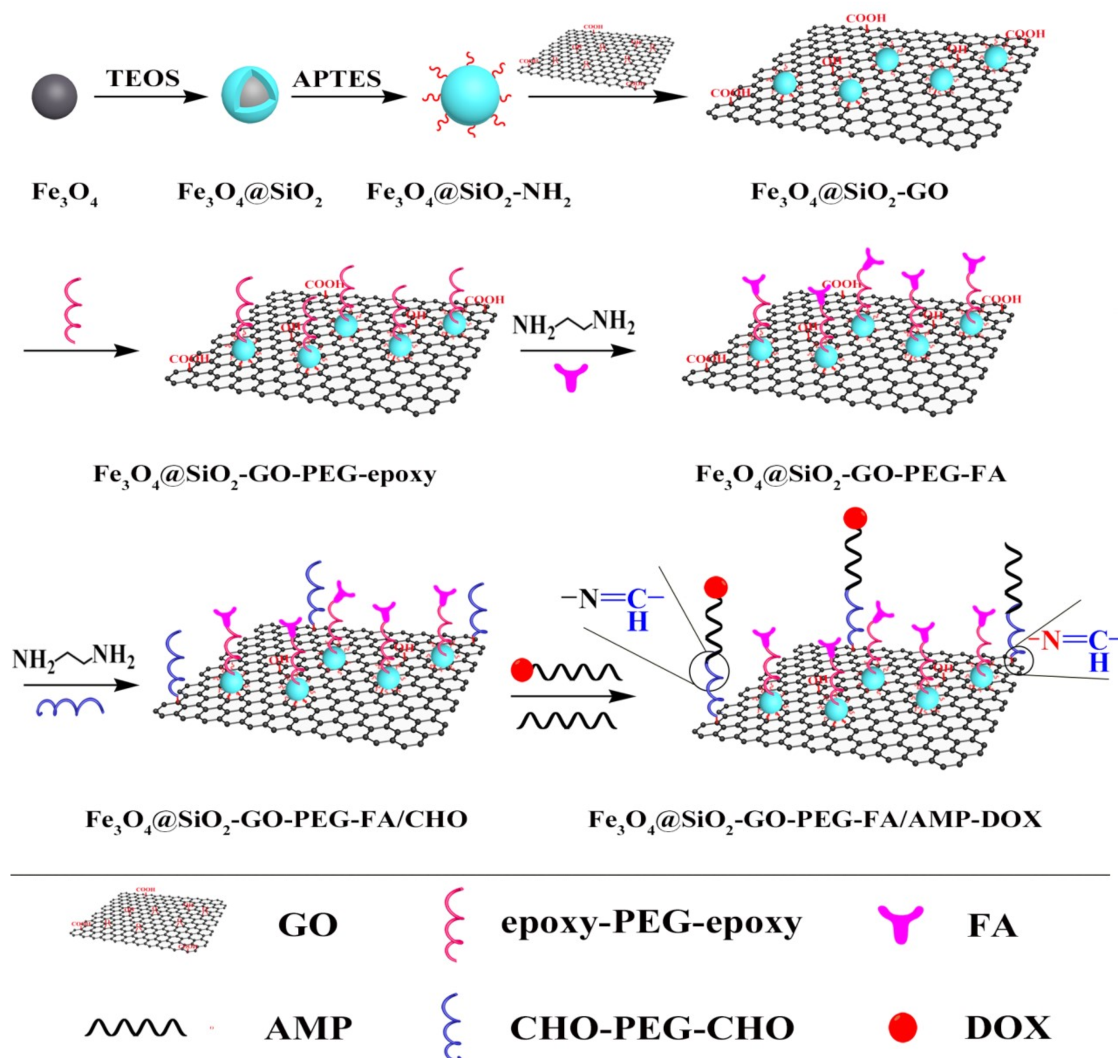

Figure S1. Schematic illustration of the Fe_3O_4@SiO_2-GO-PEG-FA/AMP-DOX (FGPFAD for short) synthesis.

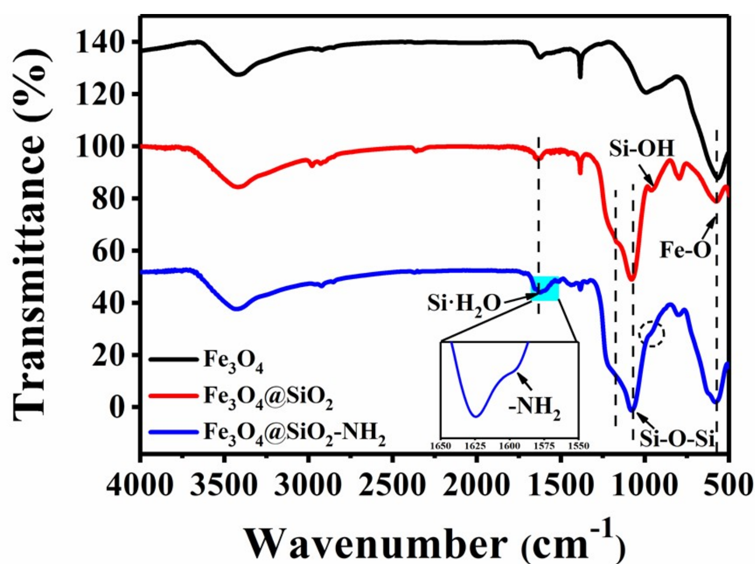

Figure S2. Fourier transform infrared spectroscopy of Fe_3O_4, Fe_3O_4@SiO_2 and Fe_3O_4@SiO_2-NH_2.

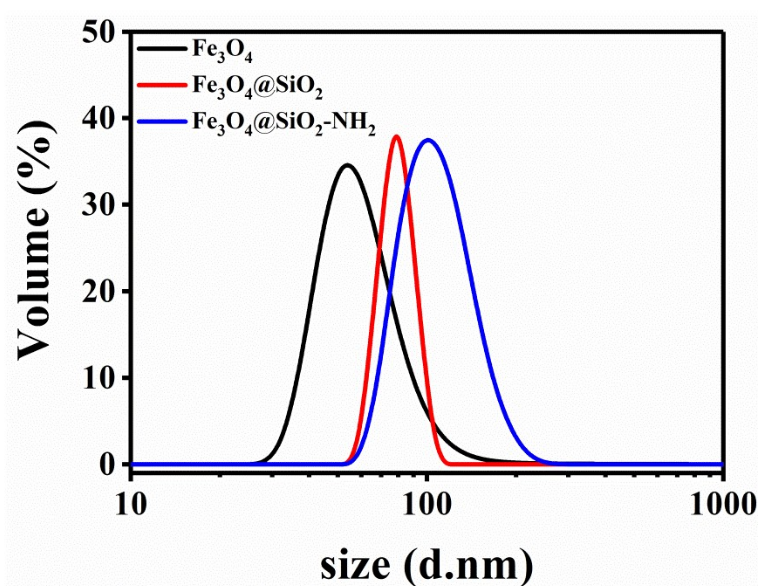

Figure S3. Dynamic light scattering of Fe_3O_4, Fe_3O_4@SiO_2 and Fe_3O_4@SiO_2-NH_2.

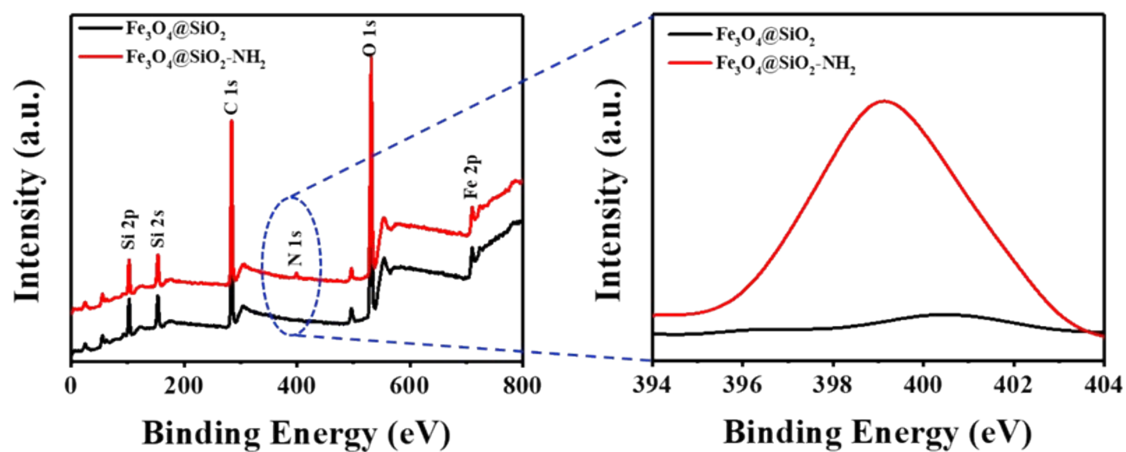

Figure S4. X-ray photoelectron spectroscopic (XPS) analysis of $Fe_3O_4@SiO_2$ and $Fe_3O_4@SiO_2$-NH_2.

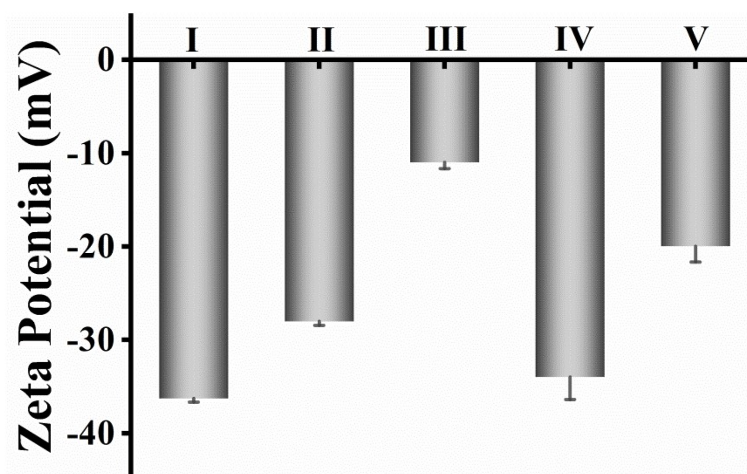

Figure S5. Surface charge of Fe_3O_4 (I), $Fe_3O_4@SiO_2$ (II), $Fe_3O_4@SiO_2$-NH_2 (III), GO (IV) and $Fe_3O_4@SiO_2$-GO (V).

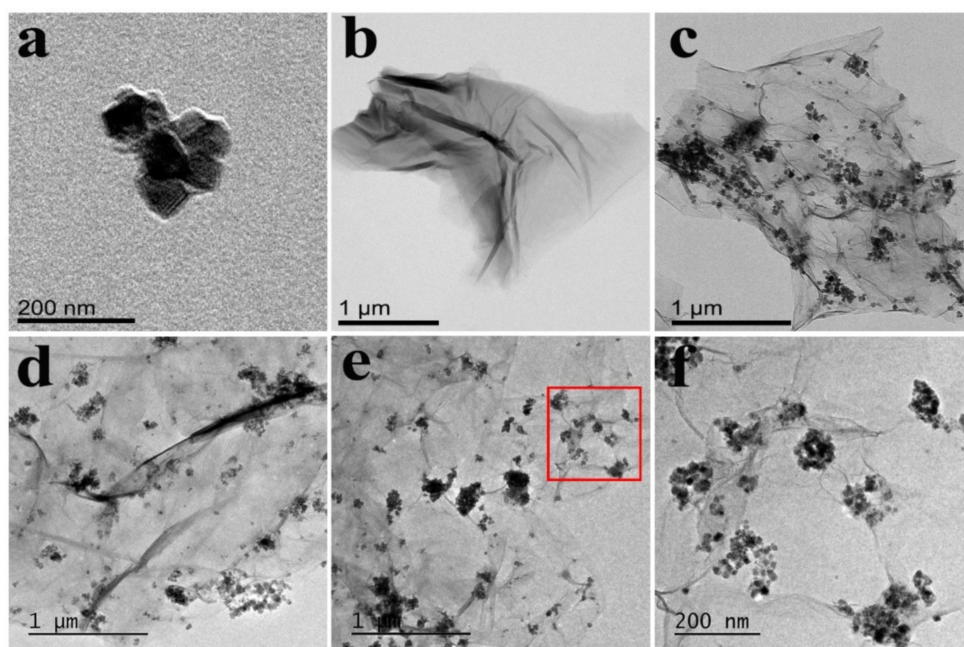

Figure S6. The transmission electron microscope (TEM) images of (a) Fe_3O_4@SiO_2-NH_2, (b) GO, (c) Fe_3O_4@SiO_2-GO, (d) Fe_3O_4@SiO_2-GO-PEG-epoxy, as well as (e-f) Fe_3O_4@SiO_2-GO-PEG-FA/CHO. Figure S6f represents the magnified image of the boxed area in figure S6e.

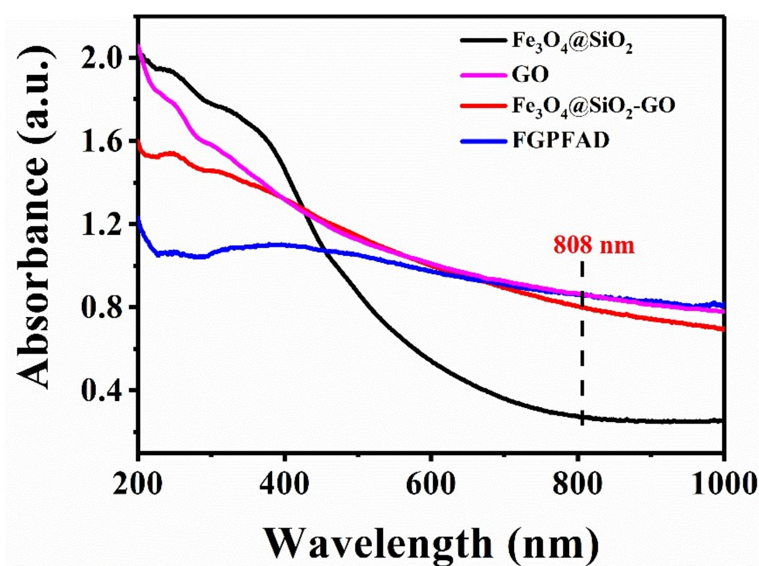

Figure S7. UV−vis spectra of Fe_3O_4@SiO_2, GO, Fe_3O_4@SiO_2-GO and FGPFAD suspension in PBS with pH 7.4.

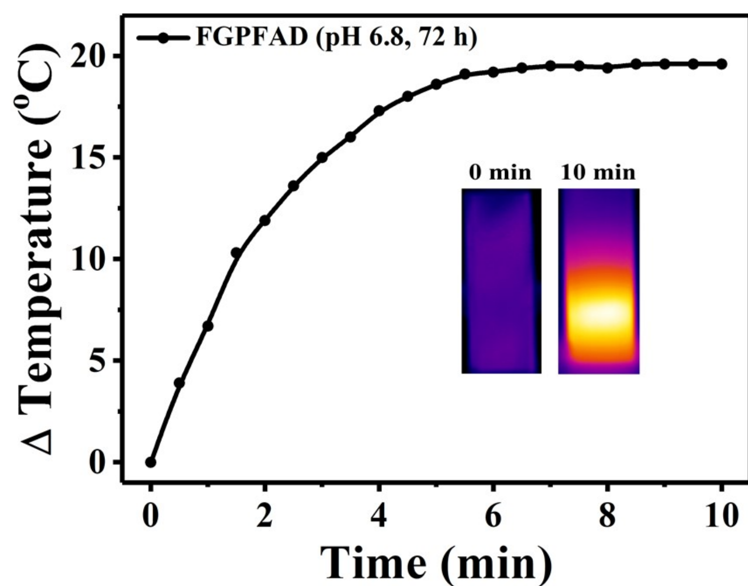

Figure S8. Photothermal heating curves of FGPFAD nanosheets after 72 h incubation in PBS at pH 6.8. The insert was the corresponding thermal images before and after 10 min NIR irradiation.

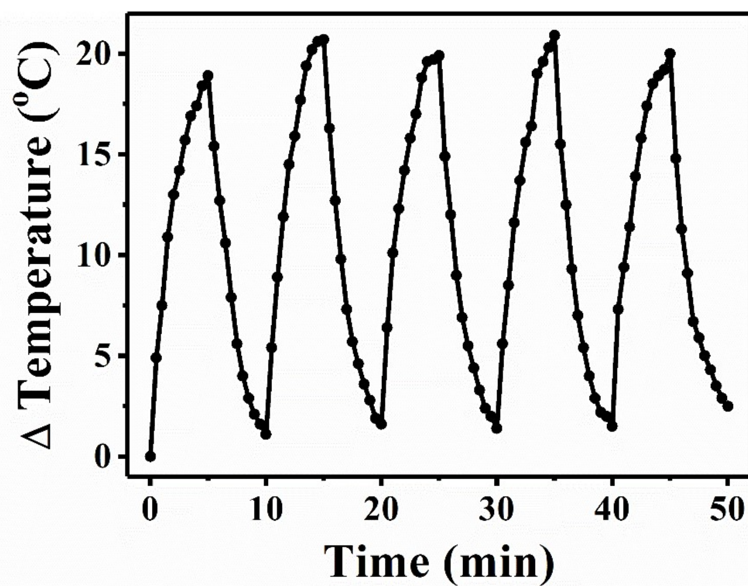

Figure S9. The photothermal stability of FGPFAD nanosheets undergoing five times on/off cycles of NIR lasers.

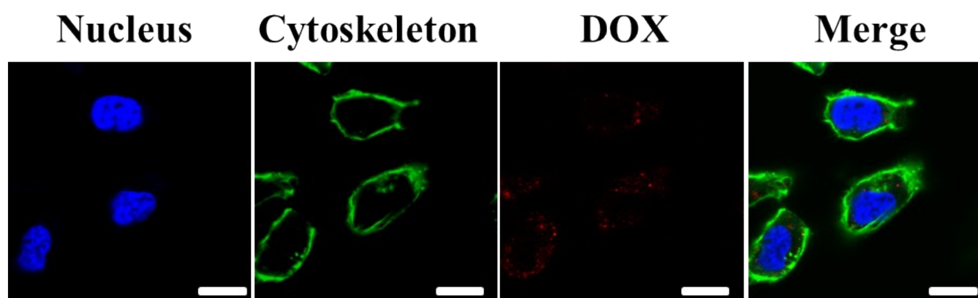

Figure S10. Confocal fluorescent microscope images of 2058 cells (model tumor cells as CTCs) after incubation with FGPAD at pH 7.4 (simulated circulating tumor cells in the blood) for 24 h (scale bars: 20 μm).

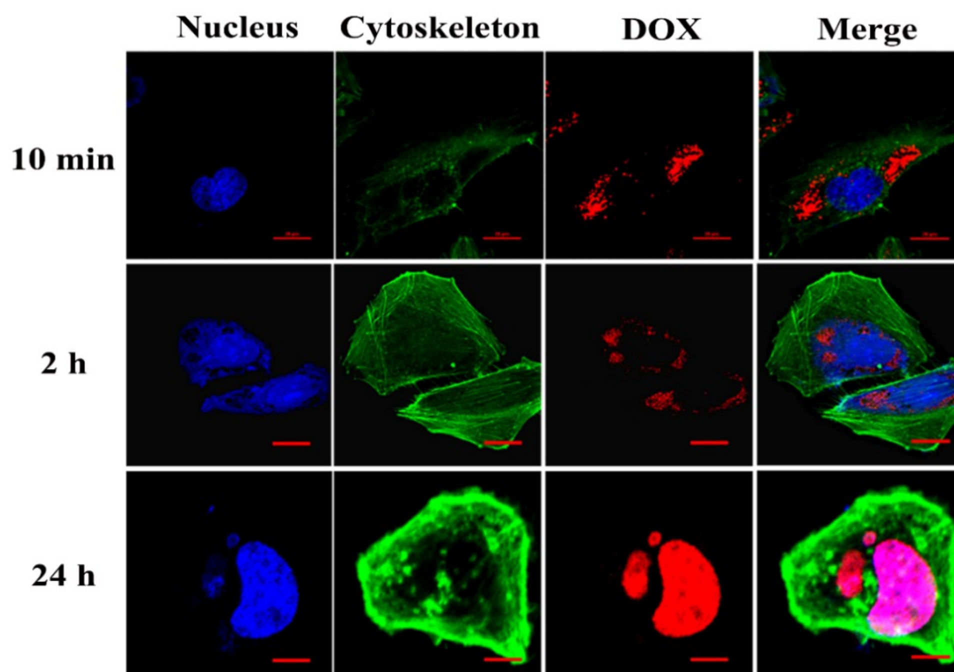

Figure S11. Confocal fluorescent microscope images of 2058 cells (tumor cells) after incubating with FGPFAD at pH 6.8 (simulated condition of primary tumor) for 10 min, 2 h and 24 h (scale bars: 20 μm).

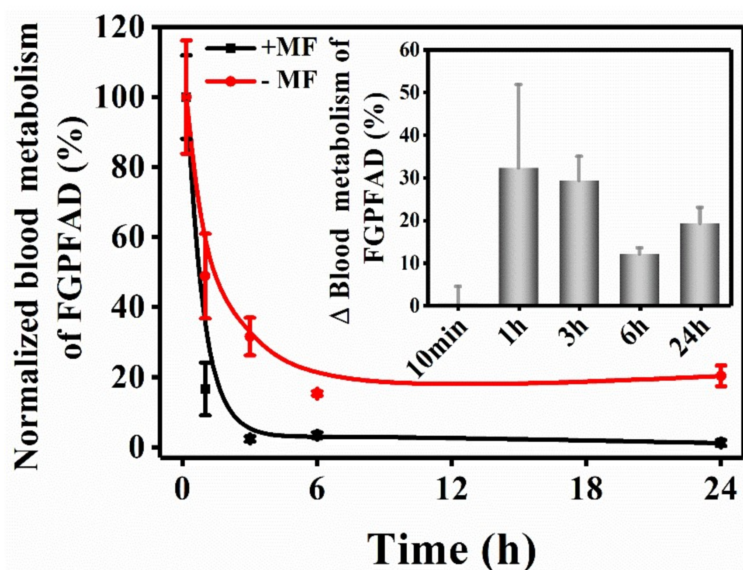

Figure S12. Blood clearance and the amount difference (insert) of FGPFAD nanosheets removed from blood circulation within 24 h after *in vivo* injection with and without magnetic field (MF).

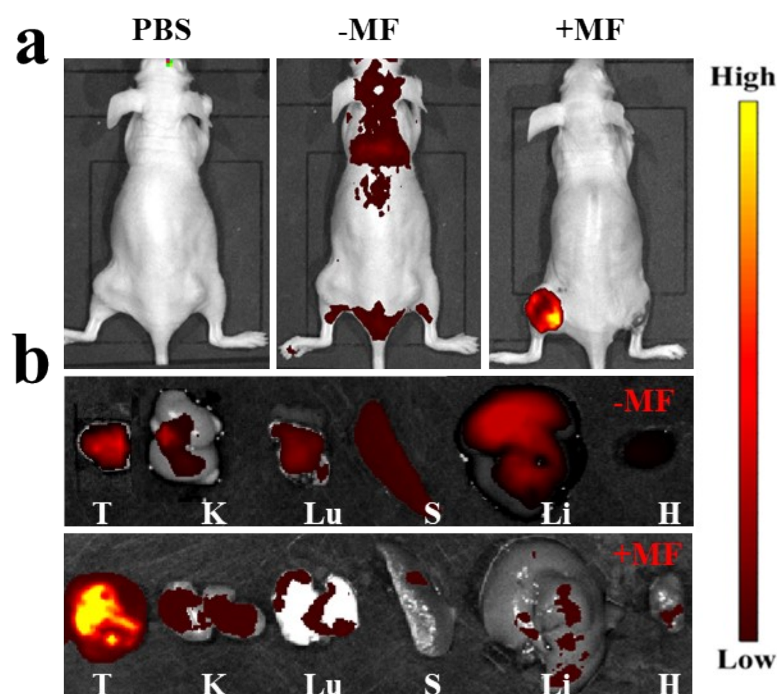

Figure S13. (a) *In vivo* fluorescence images of tumor-bearing mice and (b) corresponding *ex vivo* fluorescence images of major organs and tumors at 24 h after injection of FGPFAD nanosheets with and without magnetic field (MF). T, H, Li, S, Lu and K stand for tumor, heart, liver, spleen, lung and kidney, respectively.

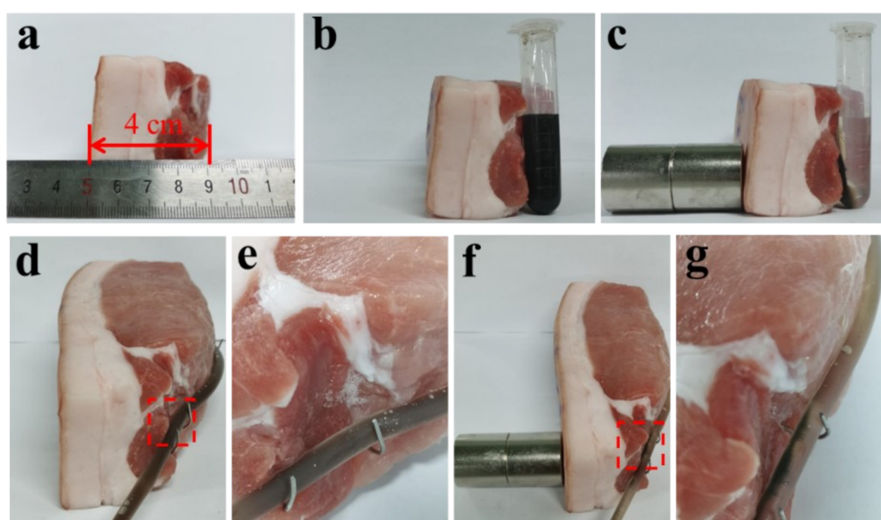

Figure S14. (a) The selected pork with a thickness of 4 cm. (b-c) Magnet-induced accumulation of FGPFAD nanosheets under static condition without (b) or with (c) an external magnetic field through the pork. (d-g) Magnet-induced collection and retention of FGPFAD nanosheets in simulated blood circulation without (d, e) or with (f, g) external magnetic field through the pork. Figure S14e and figure S14g presented the magnified images of the boxed areas from figure S14d and figure S14f, respectively.