Supporting Information

For

Cisplatin Prodrug-Conjugated Gold Nanocluster for Fluorescence Imaging and Targeted Therapy of the Breast Cancer

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Materials and Methods

1. Materials

BSA fraction V (*Mw* 66.0 kDa), 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDCI), 1-hydroxybenzotriazole (HOBT), Tris(2-carboxyethyl) phosphine (TCEP) and $HAuCl_4$ were all ordered from Sigma-Aldrich (Shanghai, China). Heterobifunctional poly(ethylene glycol) (MAL-PEG5k-COOH) and methoxy poly(ethylene glycol) carboxyl (mPEG5k-COOH) were both purchased from JenKem Technology Co. Ltd (Beijing, China). Dialysis tubing with a molecular weight cut off (MWCO) of 3.5 or 7.0 kDa was ordered from Spectrum Inc. Co. Ltd (Shanghai, China). Cisplatin or cis-diamminedichloroplatinum(II) (CDDP) was ordered from Shandong Boyuan Pharmaceutical Co. Ltd (Jinan, China). All other chemicals were purchased from Sinopharm, Inc. Co. Ltd (Shanghai, China) and used as-received. Mini-Q water was used throughout the experiments. All glassware was washed with aqua regia, and then rinsed with ethanol and Milli-Q water before use.

2. Synthesis of the BSA-protected GNC nanoparticles

BSA-protected GNC nanoparticles were synthesized by following a protocol reported elsewhere.(1) In brief, 5 mL of aqueous $HAuCl_4$ solution (10 mM) was quickly added to 5 mL of BSA solution (50 mg/mL) under vigorous stirring. Two minutes later, 0.5 mL of NaOH solution (1.0 M) was quickly introduced, and the reaction was allowed to proceed under vigorous stirring at 37 °C for 12 h. The reactant was dialyzed against Milli-Q water and then lyophilized to obtain final product.

3. Synthesis of cisplatin prodrug conjugated GNC nanoparticles

Mono-carboxylated cisplatin prodrug MDDP was synthesized according to a literature report (**Fig. S1**).(2) Briefly, cisplatin was firstly oxidized with hydrogen peroxide to obtain intermediate product cis,cis,trans-$[Pt(NH_3)_2Cl_2(OH)_2]$. Afterwards, cis,cis,trans-$[Pt(NH_3)_2Cl_2(OH)_2]$ (0.2 g, 0.6 mmol) was reacted with succinic anhydride (0.06 g, 0.6 mmol) in DMSO for 12h at 45 °C. The reactant was then precipitated, thoroughly washed with acetone and cold ether, and dried under vacuum to obtain the final product as light yellow powder (0.21 g, 0.48 mmol, yield 79.9%). The successful synthesis of the target product was confirmed by ^{1}H-NMR and

electrospray ionization mass spectrometric (ESI-MS) examination (**Fig. S2&3**).

GSH-mediated reduction of MDDP to CDDP was confirmed by HPLC-MS examination (**Fig. S4**). Briefly, MDDP was dissolved in PBS at a platinum concentration of 2.3 mM. The MDDP solution was added with 5.0 mM GSH and incubated at 37℃ under constant shaking. The samples were collected 1 min, 1 h or 12h post GSH addition and diluted by 50 folds before used for HPLC-MS examination (HPLC1260-QQQ6460A system, C18 column 4.6 × 250 mm, Waters, USA). A mixture solution of methanol and water (65:35, v/v) was used as the mobile phase (flow rate 0.7mL/min).

MDDP-conjugated GNC nanoparticles were synthesized by coupling the amino group of BSA lysine residue with the carboxyl group of MDDP. Typically, MDDP (3.65 mg, 8.43 μmol) was dissolved in 200 μL of DMSO with the addition of 2-fold excess of EDCI (3.3 mg, 16.9 μmol) and HOBT (2.3 mg, 16.9 μmol). The mixture was stirred at room temperature (RT) for 2h. Afterwards, the resulting solution was introduced in 20 mL of Hepes buffer solution (20 mM, pH 7.4) of GNC (5.0 mg/mL). The molar ratio between the amine group of BSA and the carboxyl group of MDDP was 10:1 since 59 lysine residues are found in each BSA molecular. The mixture was stirred for 24h at RT. Excess salt and free MDDP were removed by ultracentrifugation. The final product was obtained by lyophilizing. Three MDDP molecules were conjugated on each GNC nanoparticles in average as determined by using inductively coupled plasma mass spectrometry (ICP-MS) examination.

4. Synthesis of folic acid functionalized PEG spacer

FA-PEG5k-COOH was synthesized by conjugating thiolated FA onto the PEG spacer (**Fig. S5**). We firstly synthesized 2-aminoethyl disulfide pyridine folic acid according a procedure described previously.(3) The ^{1}H-NMR spectra of 2-aminoethyl disulfide pyridine folic acid was shown in **Fig. S6**. To synthesize FA-conjugated PEG5k-COOH, 2-aminoethyl disulfide pyridine folic acid (21.6 mg, 3.6 μmol) and Tris(2-carboxyethyl) phosphine (TCEP, 44 mg, 7.2 μmol) were dissolved in 5 mL of DMAC and 5 mL of phosphor buffer (PB) solution, respectively. Afterwards, the DMAC solution was slowly added into the PB solution of TCEP. The mixture was

stirred at room temperature for 2 h. The reactant was introduced into 10 mL PB solution of MAL-PEG5k-COOH (150 mg, 0.03 mmol). After 24 h reaction at room temperature, the reactant was purified by dialyzing against DI water and lyophilized to obtain 150.3 mg of the final product (26.8 μmol, yield 89.5%).The successful synthesis of FA-PEG-COOH was confirmed by ^{1}H-NMR spectrometric and UV-Vis spectrophotometric measurement as shown in **Fig. S7&8, respectively**.

5. Synthesis of folic acid modified GNC-Pt (FA-GNC-Pt) nanoparticles

The carboxyl group of FA-PEG5k-COOH (47.2 mg, 8.43 μmol) was activated with EDCI and HOBT at RT for 2h. The mixture was then added to a Hepes solution (20 mM, pH 7.4) of GNC-Pt (5 mg/mL, 20 mL) under constant stirring. After 24 h reaction at RT, the reactant was purified by ultracentrifugation (MWCO 30 kDa). And the final product FA-GNC-Pt was obtained by lyophilizing. MDDP-conjugated GNC nanoparticles with PEG modification, namely GNC-Pt were synthesized by following the similar procedure and used as the negative control. The grafting ratio of FA-PEG_{5k}-CPOOH was measured by using UV-Vis spectroscopic examination (**Fig. S8**).

6. Nanoparticle characterization

The fluorescence emission of GNC nanoparticles was examined using fluorescence spectrophotometer (Ex 415 nm, Em 550-800 nm, Hitachi F-4600, Hitachi Co. Ltd., Japan). Size distribution and the surface charge of various formulations were examined using dynamic light scattering (DLS) measurement (Nanosizer, Malvern Instruments, UK). The morphology of the GNC or FA-GNC-Pt nanoparticles was investigated using transmission electron microscopy (TEM) examination.

7. Cisplatin release from the GNC-Pt nanoparticles

To determine CDDP release profile from the GNC-Pt nanoparticles, GNC-Pt was dissolved in 0.5 mL of 5 mM GSH aqueous solution at a final concentration of 20 mg/mL. The mixture was put into the dialysis tubing (MWCO 7.0 kDa) and then dialyzed against 50 mL of GSH solution (5 mM). The dialysate was collected at 37 ℃ for desired time duration (30 min, 1 h, 2 h, 4 h, 8 h, 16 h, and 24 h) and then examined for platinum content by using ICP-MS measurement. CDDP released from

the GNC-Pt nanoparticles without GSH addition was used as control (**Fig. S9**).

8. Cell line and animals

4T1 murine breast cancer cell line was obtained from cell bank of Chinese Academy of Sciences, Shanghai, China. 4T1 cells with luciferase expression (4T1-Luc) were produced by lectiviral vector (encoded with a pGL3 luciferase promoter) mediated transfection. Both 4T1 and 4T1-Luc cells were cultured in complete RPMI 1640 cell culture medium containing 10% fetal bovine serum (FBS). All experiments were performed on cells in the logarithmic phase of growth.

Female Balb/c nude mice (4-5 weeks, 18±2 g) were purchased from Shanghai Experimental Animal Center (Shanghai). All animal procedures were carried out under the guidelines approved by the Institutional Animal Care and Use Committee of the Shanghai Institute of Material Medica, Chinese Academy of Sciences.

9. Cellular uptake and intracellular distribution of GNC nanoparticles

To investigate the uptake of drug carrier, 4T1 cells were seeded into 24-well plates (1×10^5 cells/well) and allowed to attach for 24 h. The cells were then incubated with GNC or FA-GNC at 37°C for different time durations (2, 6, 10 h). After that, cells were collected, washed, and then resuspended in PBS (pH 7.4). Approximately 10,000 events were acquired per sample. Thereafter, the intracellular fluorescence was monitored on a FACS Calibur system. All experiments were performed in triplicate.

To investigate the intracellular distribution of FA-GNC-Pt or PEG-GNC-Pt, 4T1 cells were seeded on 10 mm^2 glass coverslips placed in 24-well plates at the density of 5×10^4 cells/well. The cells were incubated with the FA-GNC-Pt or PEG-GNC-Pt nanoparticles for 2h, 6h or 10h, respectively. The cells were stained with Hoechst 33342 and fixed with 4% paraformaldehyde. The intracellular distribution of GNC-Pt nanoparticles was then examined using confocal laser scanning microscopic (CLSM) measurement (FluoView FV1000, Olympus, Japan).

To demonstrate folate receptor (FR)-specific recognition of the FA-GNC-Pt nanoparticles, 4T1 cells were pre-treated with 5 mM of free folic acid 1h before nanoparticle addition. Afterwards, the cells were treated with FA-GNC-Pt or PEG-GNC-Pt nanoparticles for 4h and examined by CLSM (**Fig. S10**).

10. Cytotoxicity assay *in vitro*

The cytotoxicity of GNC-Pt nanoparticles with or without FA conjugation was evaluated using MTT assay. 4T1 cells were seeded in 96-well plates at a density of 1×10^4 cells per well, then grew for 24 h before the cells were incubated with 100 μL of cell culture medium containing CDDP, MDDP, GNC-Pt or FA-GNC-Pt with various concentrations for 4 or 36h. The cell viability was then examined by using MTT assay 36h post nanoparticle addition.

To examine GNC-Pt nanoparticle induced apoptosis of the cancer cells, 4T1 cells were pre-incubated in 24-well plates at a density of 5×10^4 cells/well for 24 h. The cells were then incubated with cisplatin, MDDP, PEG-GNC-Pt or FA-GNC-Pt nanoparticles at an identical Pt concentration of 20 μM for 4h or 36h. The nanoparticles were removed by washed with PBS, and the cells were stained with Annexin V-FITC and Propidium iodide (PI) for apoptosis analysis using flow cytometric method. Background and auto-fluorescence was determined using mock-treated cells. Data analysis was carried out using the FCS Express 3 software (De Novo Software, CA, USA).

11. *In vivo* distribution and anti-tumor efficiency of GNC-Pt nanoparticles

To investigate the *in vivo* distribution of FA-GNC-Pt and GNC-Pt nanoparticles, each Balb/c nude mice was subcutaneously injected with 1×10^6 4T1 breast cells on the right mammary gland. The tumors bearing mice were randomly grouped (n = 3) when the tumor volume reached 100 mm^3. Mice at each group were intravenously (i.v.) injected with 100 μL suspension of GNC-Pt or FA-GNC-Pt nanoparticles at an identical Pt dose of 1.0 mg/kg. The biodistribution of FA-GNC-Pt or GNC-Pt nanoparticles were then examined using fluorescence imaging *in vivo*. The major organs (i.e. heart, liver, spleen, lung, kidney and tumor) were collected and the organ concentration of GNC nanoparticles was determined by normalizing the fluorescence intensity with that of organ mass.

The anti-tumor studies were performed in a 4T1 tumor model. Each Balb/c nude mice was subcutaneously injected with 1×10^6 4T1-Luc breast cells on the right mammary gland. Five days later post tumor implantation, the mice bearing 4T1

tumors around 50 mm^3 were randomly grouped into six groups (n = 6). The mice were i.v. injected with CDDP, MDDP, GNC-Pt or FA-GNC-Pt nanoparticles at an identical Pt dose of 1.0 mg/kg. The injections were repeated for three times. Two mouse groups were i.v. injected with 100 μL of saline solution and GNC suspension and used as the negative controls. The body weight and tumor volume of each mouse group were monitored and recorded every three days over a period of 21 days. The tumor volume was calculated by formula $V = L \times W \times W/2$ (L, the longest dimension; W, the shortest dimension) (**Fig. S11**). To examine the lung metastasis of the 4T1 tumor, all the lungs were collected at the end of anti-tumor study and imaged using a digital camera. For bioluminance imaging (BLI) examination, the freshly collected lungs were immersed in D-luciferin (5.0 mg/mL) for 5 mins and then imaged with the IVIS whole animal fluorescence imaging system (Xenogen, Alameda, CA). Quantitative luminescence intensity analysis was performed with the Live Image software (Xenogen, Alameda, CA). For biosafety assay, the liver kidneys were examined by using H&E staining.

12. Statistical analysis

Data were expressed as mean ± SD, the statistical significance was determined by using the student t-test. $p < 0.05$ was considered as significant.

References

[1]. Xie J, Zheng Y, Ying J, J. Am. Chem. Soc. 2009;**131**: 888.

[2]. Xiao H, Qi R, Liu S, Hu X, Duan T, Huang Y, et al, Biomaterials. 2011;**32**:7732.

[3]. Feng B, Xu Z, Zhou F, Yu H, Sun Q, Wang D, Yin Q, Zhang Z, Li Y. Nanoscale. 2015;**7**:14854.

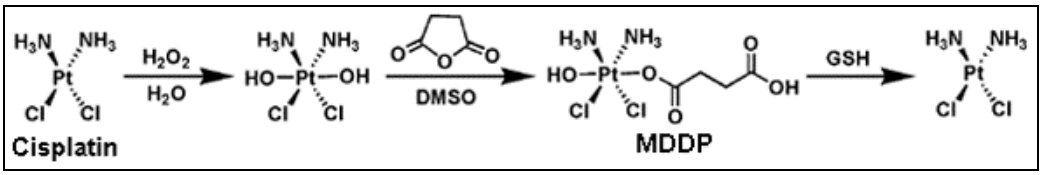

Figure S1. Schematic illustration for synthesis and glutathione (GSH)-mediated reduction of cisplatin prodrug MDDP.

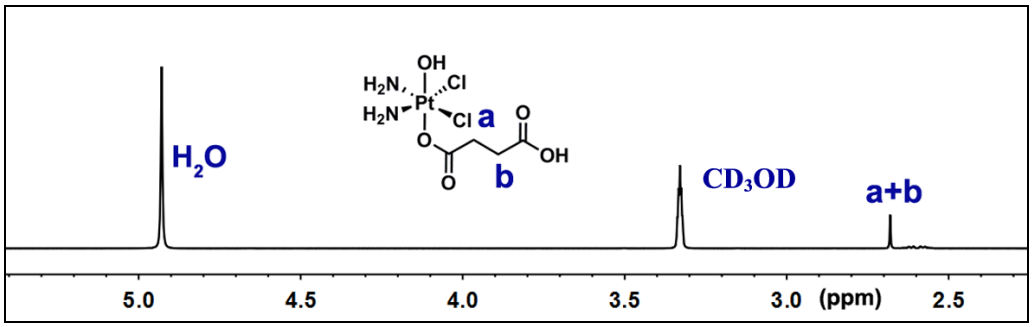

Figure S2. ^{1}H-NMR spectra of MDDP in CD_3OD. The successful synthesis of MDDP was confirmed by the presence of $H_{(a+b)}$ around 2.7 ppm.

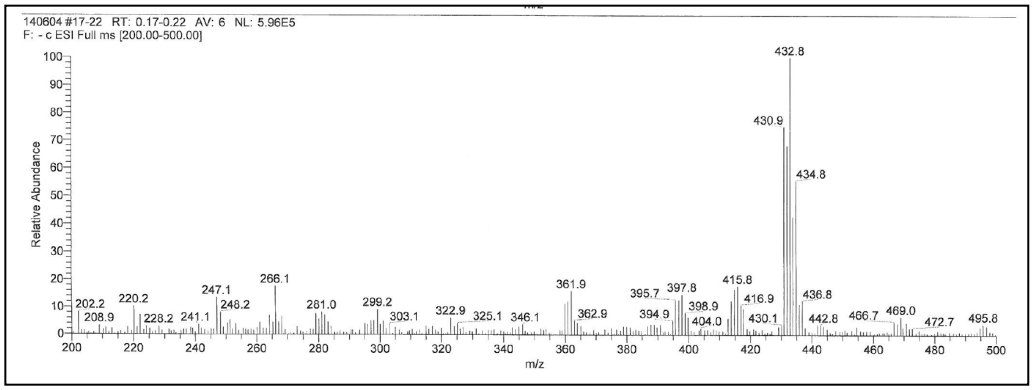

Figure S3. ESI-MS spectra of MDDP (*Mw* = 434.1 Da).

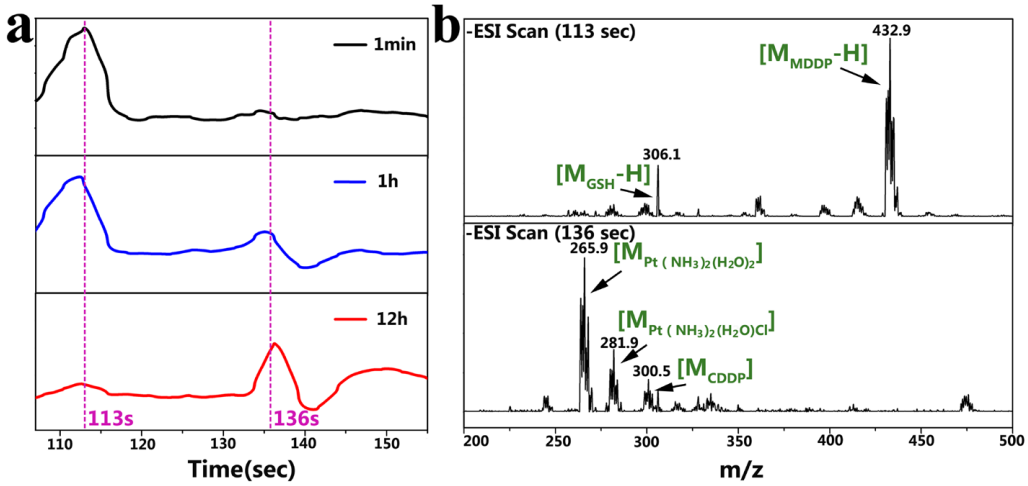

Figure S4. LC/MS examination of GSH-mediated reduction of MDDP to cisplatin. (a) Time course HPLC curves of MDDP in the presence 5.0 mM GSH examined at 1 min, 1 h or 12h post GSH addition; (b) ESI-MS spectra of the starting material MDDP and the reduction product CDDP, which were examined at 1h post GSH addition.

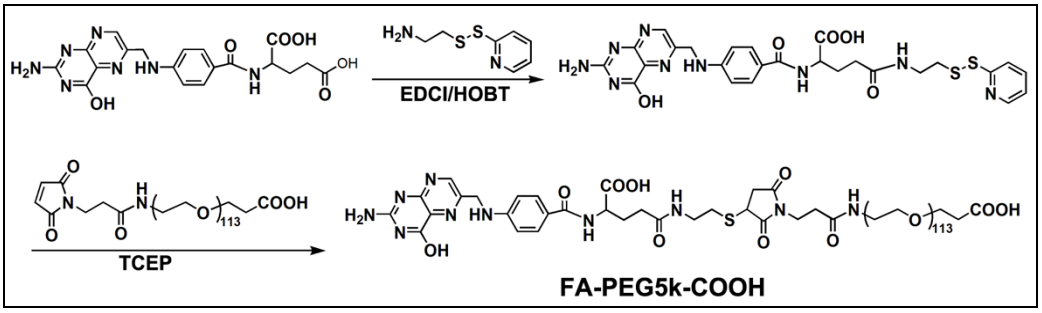

Figure S5. Schematic illustration for synthesis of FA-conjugated PEG5k-COOH.

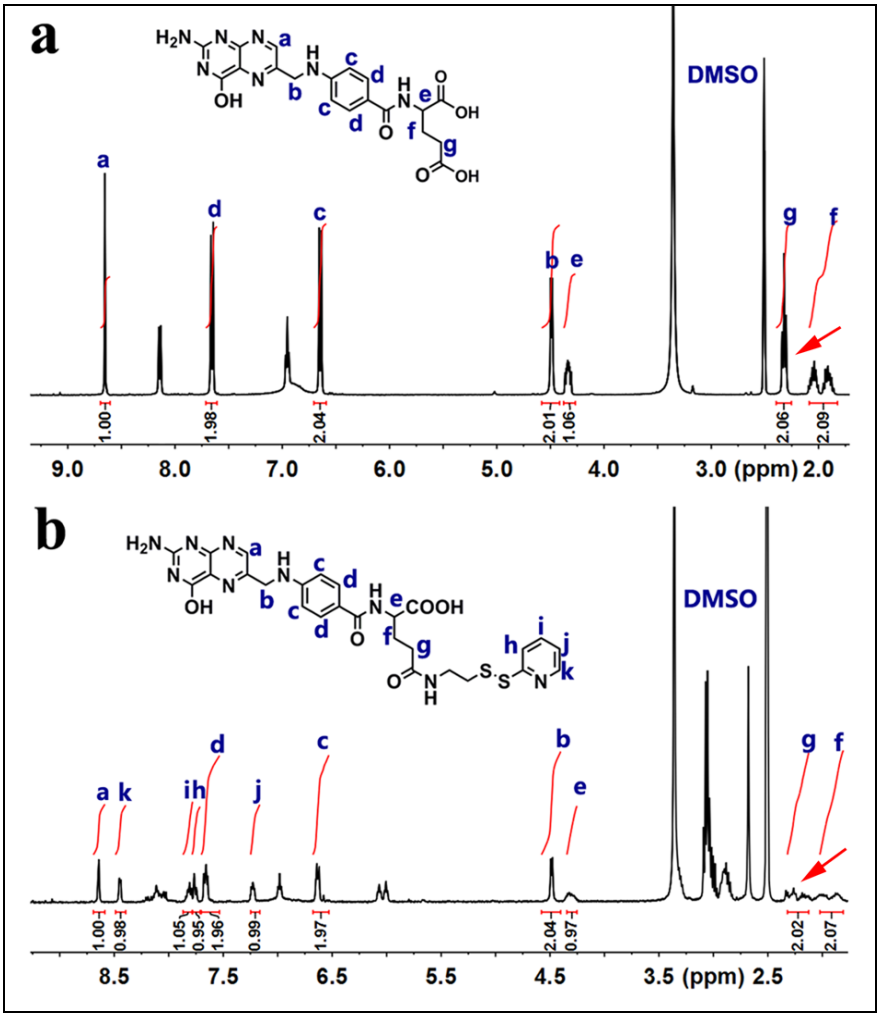

Figure S6. ^{1}H-NMR spectra of a) folic acid (folate, FA), and b) 2-aminoethyl disulfide pyridine FA in d_6-DMSO. The triplicate peak indicated by the red arrow in Figure SI5a was assigned to the H_g next to the γ-carboxylate group. H_g split into a broad peak after coupling 2-aminoethyl disulfide pyridine with FA. In contrast, no chemical shift occurred for H_e next to the α-carboxylate group. All these information confirmed the formation of the amide bond between the γ-carboxylate group of FA and the amine group of 2-aminoethyl disulfide pyridine.

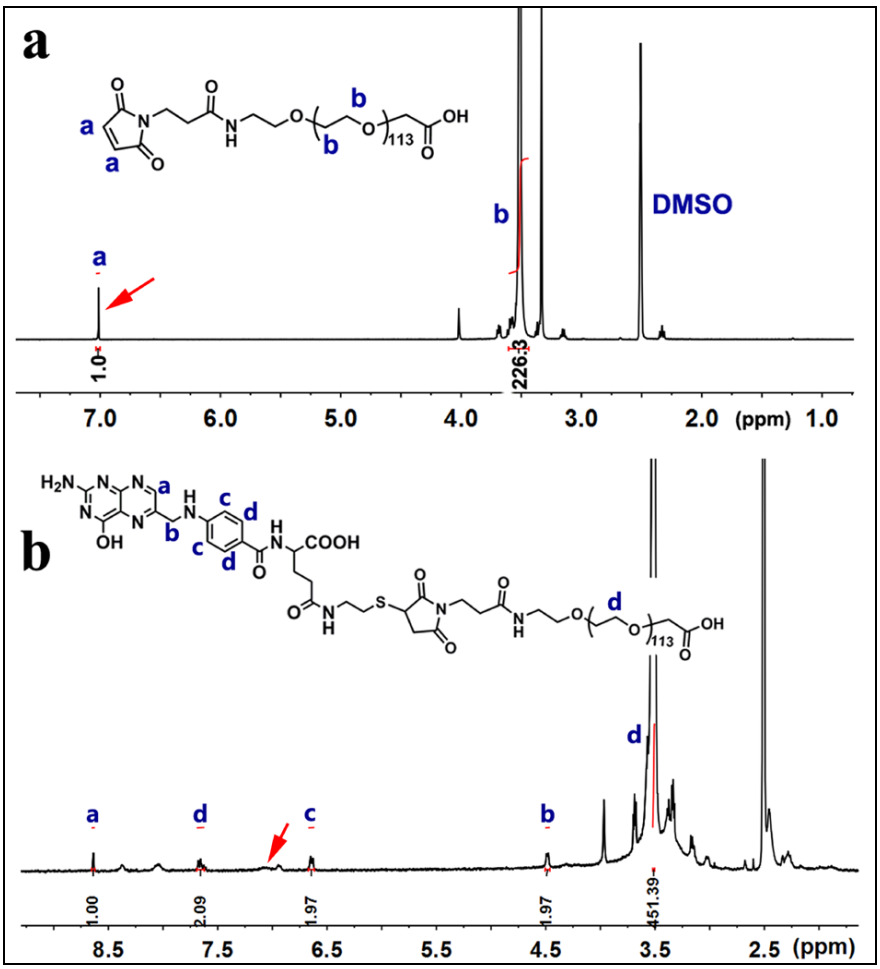

Figure S7. ^{1}H-NMR spectra of (a) MAL-PEG$_{5k}$-COOH, and (b) FA-PEG$_{5k}$-COOH in d_6-DMSO. H_a presented in Figure SI7a disappeared in Figure SI7b due to addition of the thiol group of FA to the double bond of the maleimid group.

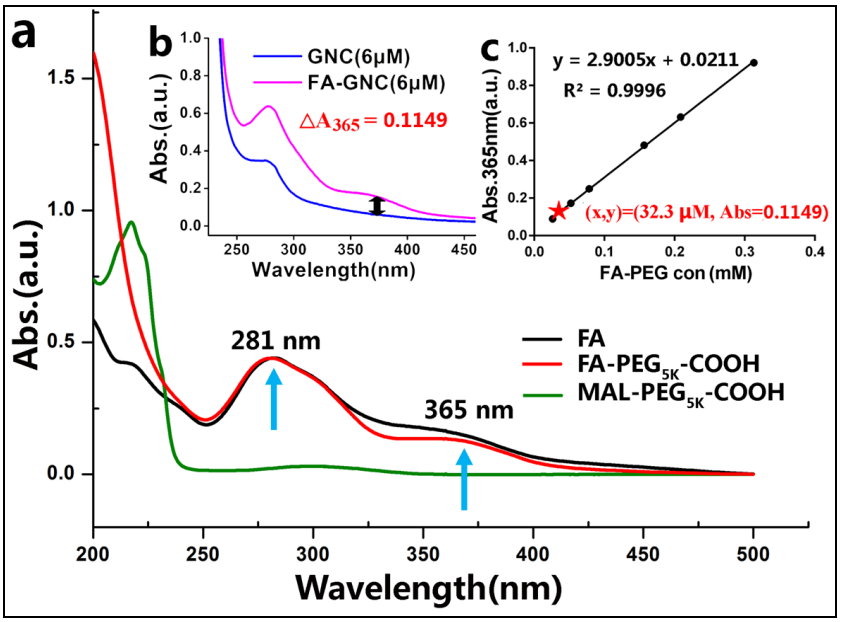

Figure S8. (a) UV-Vis spectra of MAL-PEG$_{5k}$-COOH, folic acid (folate, FA) and FA-PEG$_{5K}$-COOH in water. The insert figures were the (b) UV-Vis spectrum of GNC and FA-GNC nanoparticles, and (c) the standard curve for FA-GNC, respectively. The FA conjugation density was calculated by using UV-Vis spectroscopic examination. The standard curve was established by measuring the Absorbance of FA-PEG-COOH at 365 nm versus its molar concentration.

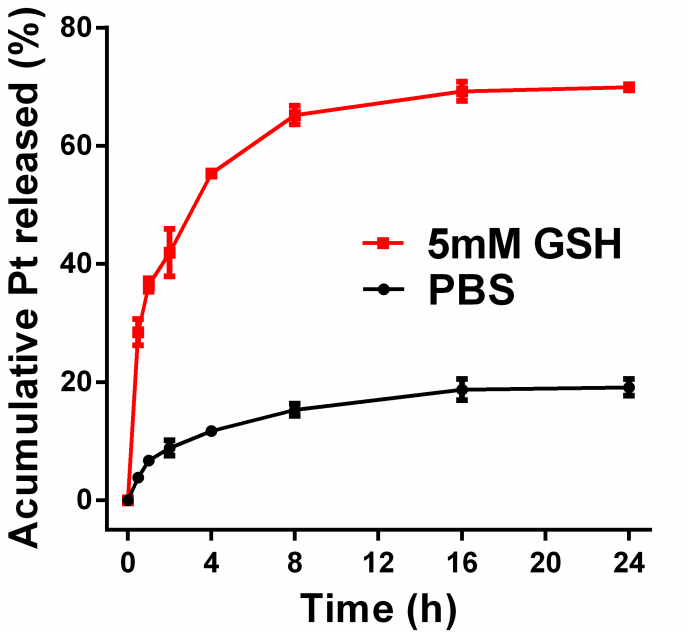

Figure S9. Accumulative platinum release from the GNC-Pt nanoparticles in PBS or 5.0 mM GSH solution.

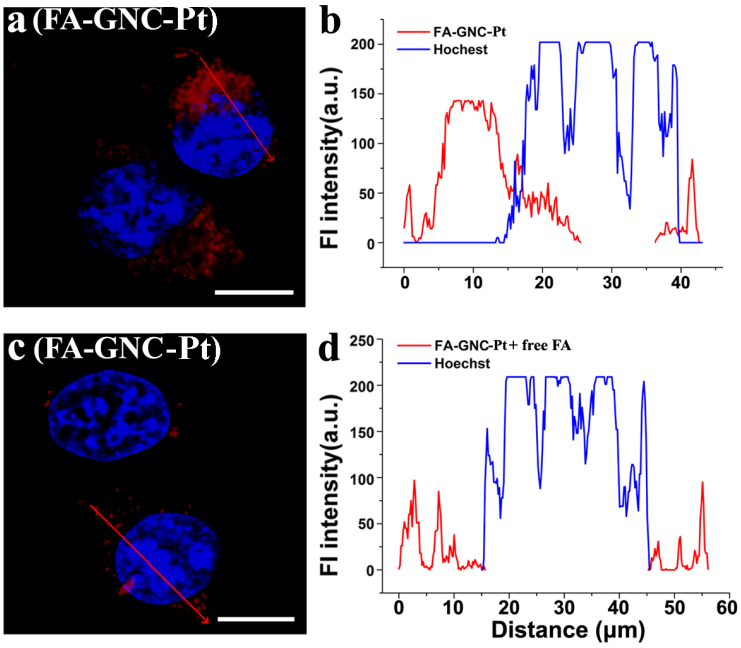

Figure S10. (a) CLSM image of FA-GNC treated 4T1 cells after 4h incubation; (b) Representative intracellular fluorescence intensity of FA-GNC treated cells; (C) CLSM image FA-GNC treated 4T1 cells after 4h incubation, the cells were pre-treated with FA for 1h before FA-GNC addition; (d) Intracellular fluorescence intensity of FA-GNC treated cells with FA pre-blocking of the folate receptor.

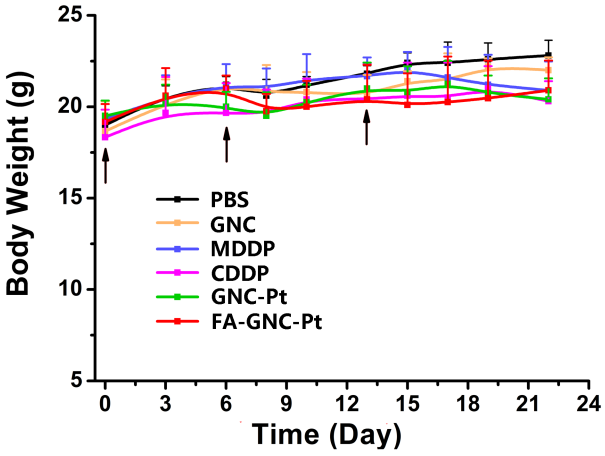

Figure S11. Body weight change recorded during the anti-tumor study (the black arrows indicated the time point for nanoparticle administration).