Supporting Information

Protein Sulfenic Acid-Mediated Anchoring of Gold Nanoparticles for Enhanced CT Imaging and Radiotherapy of Tumors *In Vivo*

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Chemicals and instruments: All the chemical reagents were purchased from Sigma-Aldrich (MO, USA) and Sinopharm Chemical Reagents (Shanghai, China). They were used as received without further purification. Methyl thiazolyl tetrazolium (MTT) cell proliferation cytotoxicity assay kit from Sigma-Aldrich, Live/dead cell staining kit (calcein-AM-propidium iodide) from Invitrogen (CA, USA), apoptosis detection kit (Annexin V-FITC/PI Apoptosis Detection Kit, Beyotime, Shanghai, China) and Cell Cycle and Apoptosis Analysis Kit (Beyotime, Shanghai, China) were used as received. SYBR Green I (Beijing Solarbio Science&Technology Co., Ltd, Beijing, China) and Comet Assay Single Cell Gel Electrophoresis Assay (R&D Systems, Minneapolis, USA) were used as received. Hoechst 33342 was purchased from Yeasen (Shanghai, China). UV-Vis absorption spectra were taken on UV spectrometer (UV-3600, Shimadzu, Kyoto, Japan). Dynamic light scattering (DLS)

measurements were carried out using a particle size analyzer (Nano ZS90, Malvern Panalytical, Malvern, UK) at room temperature. Transmission electron microscopy (TEM) images were taken under an electron microscope (Tecnai G2 Spirit, FEI, OR, USA). Fourier transform infrared spectroscopy (FT-IR) was taken on a fourier transform infrared spectrometer (Thermo Scientific Nicolet iS50 FT-IR Spectrometer, USA). The Computed Tomography (CT) image was taken by U-SPECT/CT (MILabs, Utrecht, The Netherlands).

Preparation of dAuNP-FA: Au nanoparticles were prepared according to previous reported method in the literature [49]. Into 100 mL aqueous solution of the as-prepared AuNPs, 20 mg of methoxy-PEG_{5000}-SH and 20 mg of NH_2- PEG_{5000}-SH were introduced. The mixture was then kept under stirring overnight to enable the particle surface PEGylation. The resulting PEGylated Au nanoparticles, denoted as Au@PEG-NH_2, were purified by repetitive ultrafiltration centrifugation (5000 rpm per 10 min) to remove the unreacted PEG, and then resuspended in 1 mL Milli-Q water for use.

3,5-dioxocyclohexanecarboxylic acid (DHCA, 3.1 mg) was firstly activated with N-hydroxysuccinimide (NHS) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) for 2 h to give NHS-DHCA which was subsequently added into 1 mL of above prepared Au@PEG-NH_2 solution. After 4 h stirring at room temperature, the reaction mixture was subjected to centrifuge (14000 rpm per 15 min) once and ultra-filtered (5000 rpm per 10 min) for three times to afford the desired DHCA modified Au nanoparticles (dAuNP).

Folic acid (FA, 5.3 mg) was activated with NHS and EDC for 2 h to give NHS-FA which was then added into 0.5 mL of dAuNP solution, and the reaction mixture was kept under stirring overnight. The final reaction system was centrifuged three times to afford the desired probe dAuNP-FA. The dAuNP-FA were finally resuspended in Milli-Q water and stored at 4°C for further experiment use.

Crosslinking of dAuNP toward protein sulfenic acids: The reactivity of dAuNP to protein sulfenic acids was studied as follows: BSA powder (8 mg) was dissolved in water followed by addition of tris(2-carboxyethyl)phosphine (TCEP) solution (20 μL,

50 mM) to reduce the disulfide bond to thiols. The reduced BSA was subsequentlyt washed with water to remove the excessive TCEP by ultra-centrifugation, and the final volume was made to 1 mL. The reaction system of total volume of 100 μL was made up as follows: dAuNP/BSA-SOH was prepared by mixing dAuNP (50 μL, 40 μg/mL), pre-reduced BSA (25 μL), H_2O_2 (5 μL, 1 mM), and PBS buffer to the final volume of 100 μL. The dAuNP/BSA (without H_2O_2) and dAuNP (without BSA and H_2O_2) solutions were set as negative controls. The reaction solutions were incubated at 37°C for 1 h followed by characterization.

Studying PSA-mediated immobilization of dAuNP in cells: Cells were grown onto glass coverslips in a 12-well plates at a density of 1.5×10^5 cells/well for 24 h. The cells were washed two times using PBS (10 mM, pH 7.4) and incubated with medium containing different concentration of H_2O_2 (0, 10, 100 M) for 10 min at 37°C. Then the cells were washed 3 times using PBS followed by addition of the medium containing dAuNP with the concentration of 50 g/mL. After 24 h, the cells were fixed with 4% paraformaldehyde and stained with Hoechst 33342. The Au content was determined by dark-field microscopy. Finally, the cells of each groups were collected, and treated with aqua regia. The Au content was determined by ICP–MS.

4T1 cells were grown onto glass coverslips in a 12-well plates at a density of 1×10^5 for 24 h. The cells were washed two times using PBS (10 mM, pH 7.4) and incubated with medium containing different concentration of H_2O_2 (0, 10, and 100 μM) for 10 min at 37°C. The cells were washed 3 times using PBS buffer followed by addition of medium containing dAuNPs with the concentration of 50 g/mL. The cells were divided into four groups, i.e., control (X-ray), X-ray+dAuNP, X-ray+10 M H_2O_2 +dAuNP, and X-ray+100 M H_2O_2 + dAuNP. All above groups of cells were irradiated by 4 Gy X-ray. After 2 h incubation, the cells were fixed with 4% paraformaldehyde and permeabilized with 1% Triton X-100 for 10 minutes. The fixed cells were then incubated with 200 L rabbit monoclonal anti-phosphohistone γ-H2AX antibody (ab81299, Abcam Inc.) overnight at 4°C, followed by incubation with the secondary antibody Cy3-labeled goat anti-rabbit IgG (H+L) for 1 h at 37°C. Finally, the cells

were stained with Hoechst and then examined using an Olympus confocal microscope (Olympus, Tokyo, Japan) for analyzing the red phospho-H2AX signals.

***In vitro* DNA damage study:** 4T1 cells were grown onto glass coverslips in a 12-well plates at a density of 1×10^5 for 24 h. Cells receiving 4 Gy X-ray irradiation in absence of gold nanoparticles were denoted as X-ray group, cells receiving 4 Gy X-ray irradiation after incubation with dAuNP-FA (50 μg/mL) were denoted as dAuNP-FA+X-ray group, cells receiving 4 Gy X-ray irradiation after incubation with AuNP-FA (50 μg/mL) were denoted as AuNP-FA+X-ray group and cells subjected none of treatment were denoted as control group.

Colony Formation: 4T1 cells were seeded in 6-well plates and cultured for 24 h .Three groups (n = 3 per group) were divided: control, AuNP-FA+X-ray, and dAuNP-FA+X-ray. 4T1 cells were cultured with the same concentration (50 μg/mL) of dAuNP-FA and AuNP-FA for 24 h, and subsequently irradiated with 0, 2, 4, 6, and 8 Gy X-rays, respectively. After 10 days incubation, the cells were fixed by formalin and stained with Giemsa dye for 1.5 h. Colonies counted and the survival fractions were calculated.

The number of colonies was counted and a survival curve was drawn using a one-click multi-target model. The sensitization enhancement ratio (SER) is calculated using the following formula: y = 1-(1-exp (-k*D))^N, where y is the survival rate, k is the passivation constant of the cell survival curve, and N is extrapolated number, D is the dose received. Sensitivity enhancement ratio (SER) is a very important parameter for evaluating radio enhancement efficiency and can be calculated by the following equation: SER = D_0 (with radiosensitizer)/D_0 (without radiosensitizer). D_0 can be calculated by the following equation: $D_0=1/k$.

CT imaging performance of dAuNP-FA *in vivo*: All animal studies were performed in strict accordance with the Guidelines for Care and Use of Laboratory Animals of Soochow University (Suzhou, China) and was approved by the Animal Ethics Committee of the Soochow University Laboratory Animal Center (Suzhou, China). The CT imaging capability of dAuNP-FA was first compared with commercial iopromide *in vitro*. Different mass concentrations (0, 0.25, 0.5, 1, 2, and 4

mg/mL) of contrast agents were used. To study the protein sulfenic acid-reacted immobilization of the dAuNP-FA probes on the enhancement of CT signal *in vivo*, two groups of tumor-bearing mice were intravenously injected with 200 μL aqueous solution of dAuNP-FA or AuNP-FA (5 mg/mL), respectively. For *in vivo* CT imaging, 4T1 tumor-bearing mice were prepared and placed in an animal bed under anesthetic for CT imaging. The CT imaging was taken at 0, 4, 8, 12, 24 and 36 h post-injection. The CT images were collected before and after injection of contrast agent. All the CT images were recorded on an imaging system (MILabs, Utrecht, The Netherlands).

Radiotherapeutic application of dAuNP-FA *in vivo*: 4T1 tumor-bearing mice were randomly divided into five groups (n = 5 in each group) and treated with different combinations, including PBS (control), X-ray radiation (RT), intravenous AuNP followed by radiation therapy (AuNP+RT), AuNP-FA followed by radiation therapy (AuNP-FA+RT), dAuNP-FA followed by radiation therapy (dAuNP-FA+RT).

The injection dose of gold nanoparticles was 300 μg in 0.2 mL volume. After 8 h, a portion of the tumor was taken for fixation, and the tissue slice was prepared for TEM measurement. The rest of the mice were irradiated by 8 Gy X-rays. Then, tumor size was monitored and measured to evaluate the therapeutic effect of X-ray. Next, to further assess the radiosensitization efficacy, tumor tissues were extracted 2 days after X-ray radiation and eventually subjected to H&E staining. We dewaxed the tissues using xylene and ethanol and rinsed them with tap water. Stain sections with Hematoxylin solution for 3-5 min, rinse with tap water. Then treat the sections with Hematoxylin Differentiation solution, rinse with tap water. Treat the section with Hematoxylin Scott Tap Bluing, rinse with tap water. Treat the section with ethanol and stain sections with Eosin dye for 5 min. The sections were dehydrated with ethanol and xylene. Finally seal with neutral gum.Observe with microscope inspection, image acquisition and analysis.

Hemolysis Assay: The blood samples from healthy adult BABL/C mice were used to evaluate the blood compatibility of AuNP and dAuNP-FA. The red blood cells (RBCs) were collected by removing the serum from the blood after centrifugation and

suction. RBCs were then purified by washing with PBS for five times, and diluted to 10 times of their initial volume with PBS solution. A 0.5 mL of diluted RBCs suspension was subsequently mixed with 0.5 mL 0.9% NaCl as a negative control; 0.5 mL Mili-Q water as a positive control; or 0.5 mL of dAuNP-FA and AuNP suspension (0.9% NaCl) at concentrations ranging from 10 to 640 μg/mL. The mixtures were shaken slightly and then kept for 2 h at room temperature. The samples were centrifuged (10000 rpm, 5 min), photographed, and measured the absorbance of the supernatants at 570 nm.

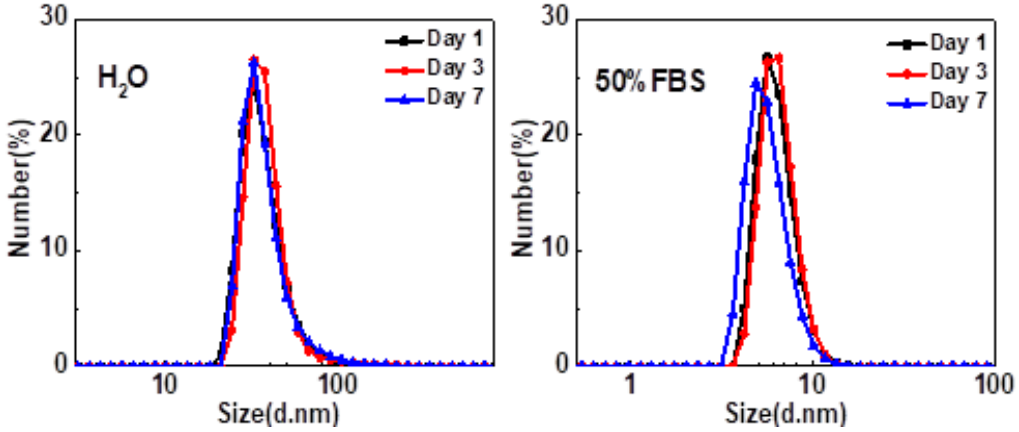

Figure S1. The hydrodynamic sizes of dAuNP-FA dispersed in aqueous solution (a) and 50% fetal bovine serum (FBS) solution (b).

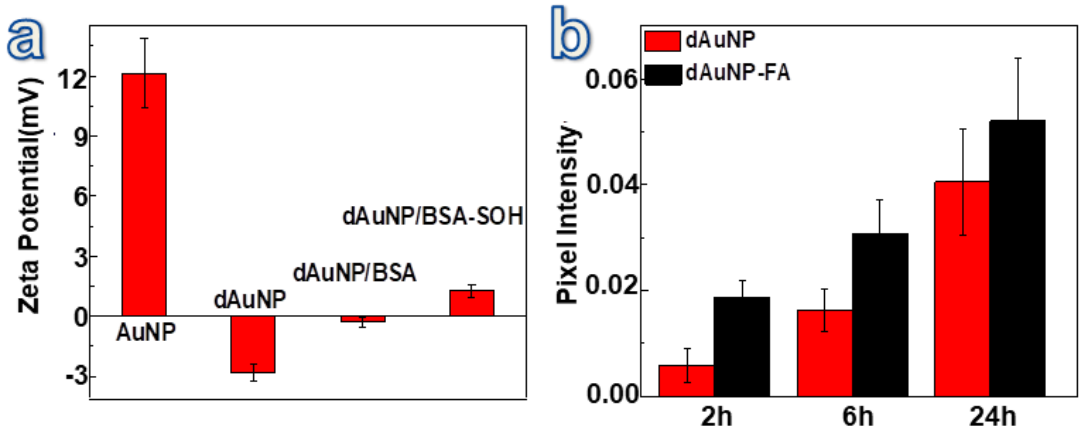

Figure S2. Zeta potential (a) in the presence of BSA or BSA-SOH. Quantification of fluorescence intensities of 4T1 cells incubated with AuNP-FA and dAuNP-FA (b).

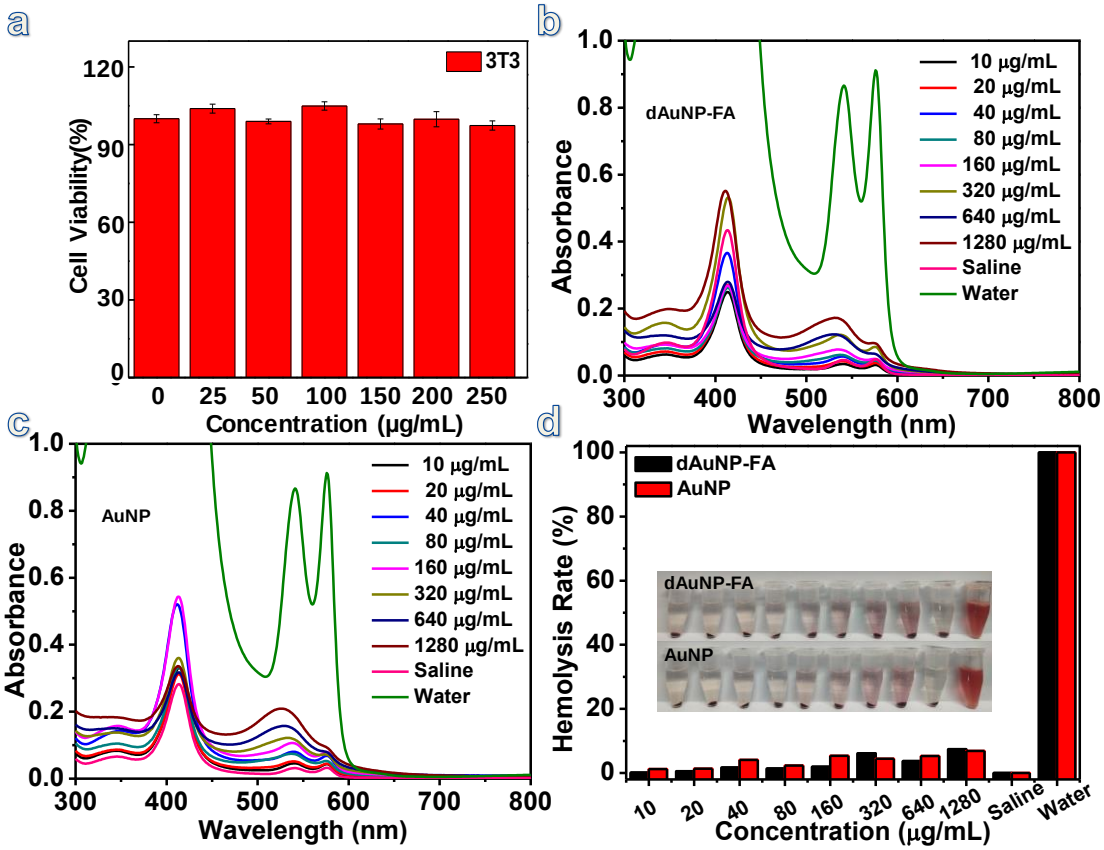

Figure S3. (a) Viability of 3T3 cells after incubation with different concentrations of dAuNP-FA for 24 h. (b-c) Hemolysis assays for dAuNP-FA and AuNP at different concentrations from 10 μg/mL to 1280 μg/mL.

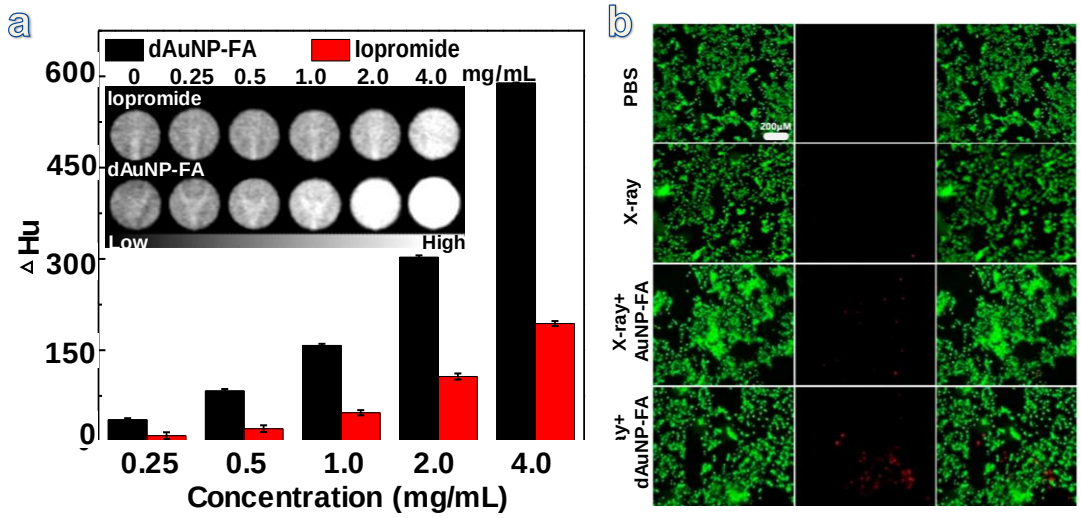

Figure S4. (a) CT images of two series of aqueous solutions containing iopromide and dAuNP-FA with different concentrations. (b) *In vitro* radiotherapy of 4T1 cancer cells. Radiosensitization effect of Au nanoparticle capped CHD and folic acid evaluated through live/dead staining. Au: 50 μg/mL, X-ray: 6 Gy, scale bar: 200 μm.

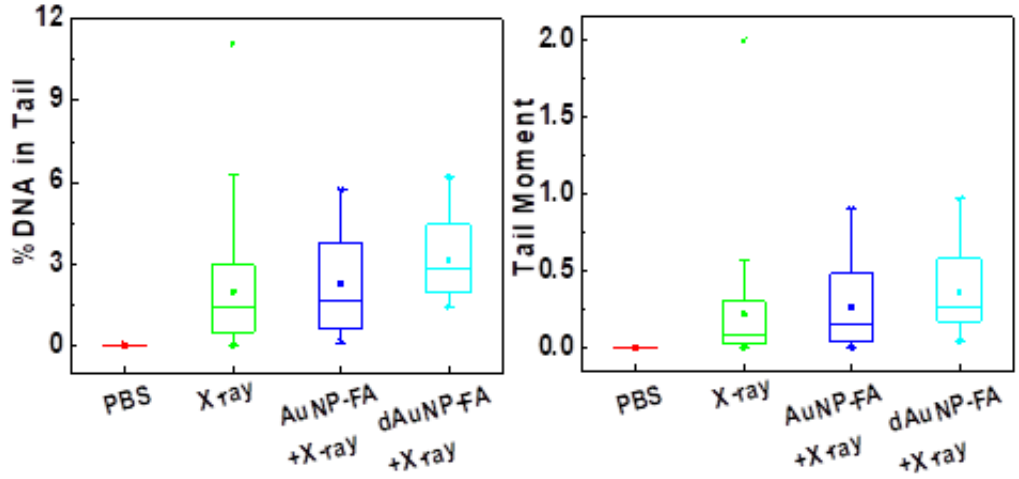

Figure S5. Analysis of single cell electrophoresis Images. n=35.

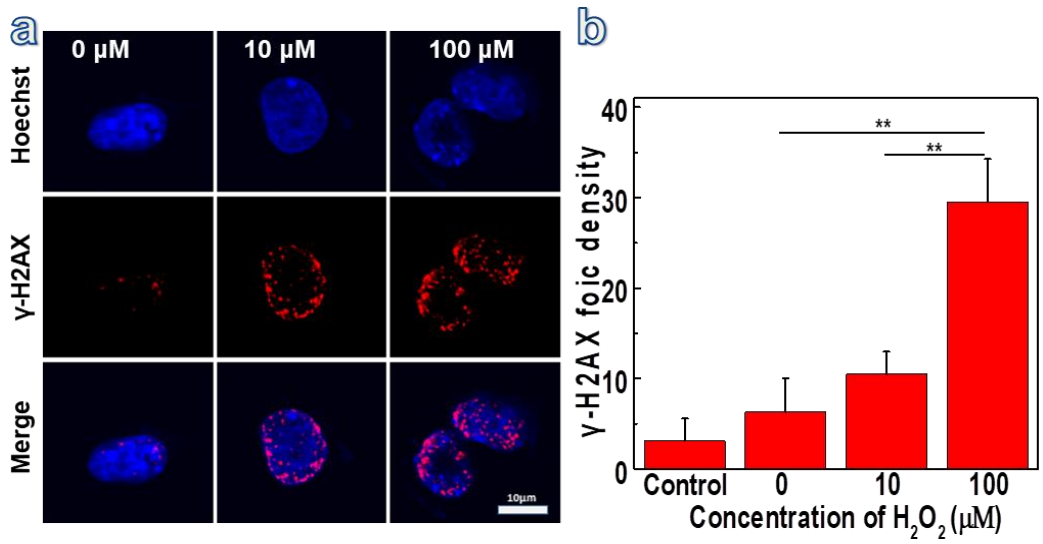

Figure S6. (a) Assessment of DNA damage through γ-H2AX immunofluorescent staining in 4T1 cells treated with different concentrations of H_2O_2. (b) Quantitative analysis of γ-H2AX foci density in 4T1 cells, scale bar: 10 μm, *$p < 0.05$, **$p < 0.01$, by t-test, $n \geq 50$ cells.

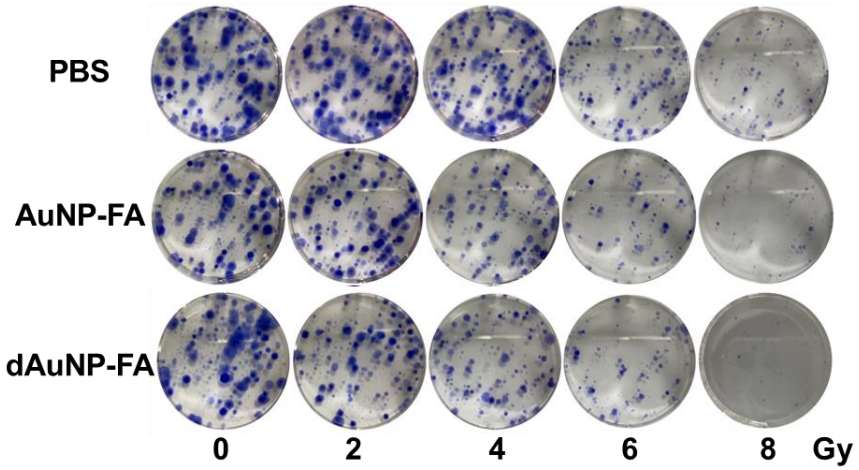

Figure S7. Photographs of colony formation assay of 4T1 cells cultured with/without AuNP-FA, dAuNP-FA undery different doses of X-ray irradiation. The Au nanoparticle concentration is 50 μg/mL.

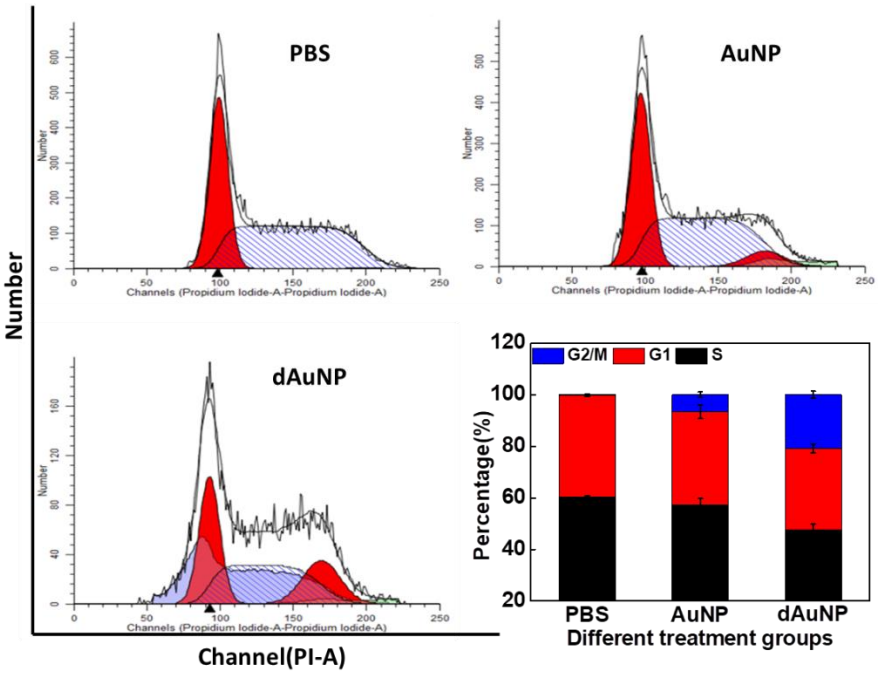

Figure S8. Status of cell cycles of 4T1 cells receiving different nanoparticales. The concentration of Au nanoparticle is 50 μg/mL.

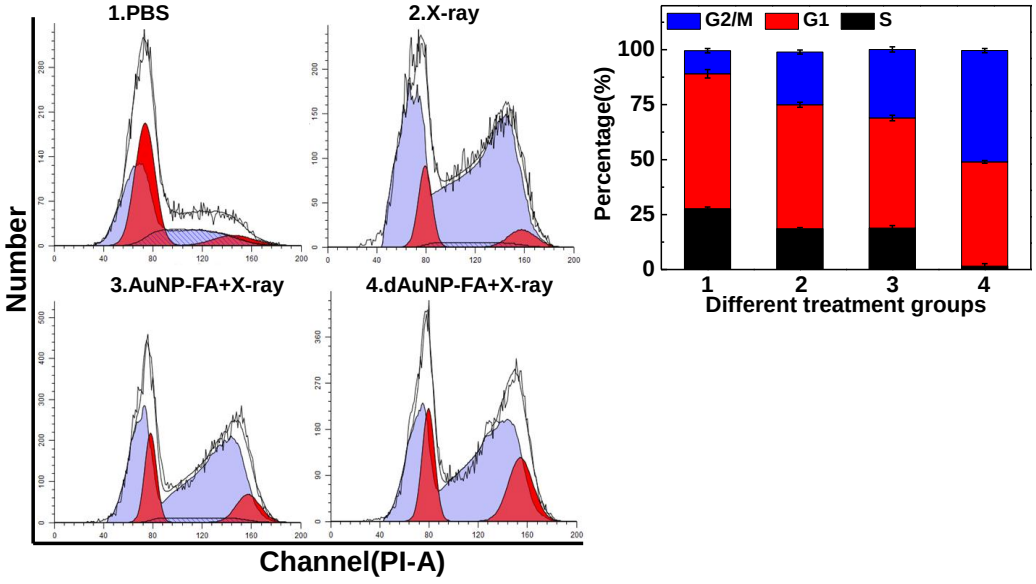

Figure S9. Status of cell cycles of 4T1 cells with AuNP-FA or dAuNP−FA irradiated by 2 Gy X-ray. The concentration of Au nanoparticle is 50 μg/mL.

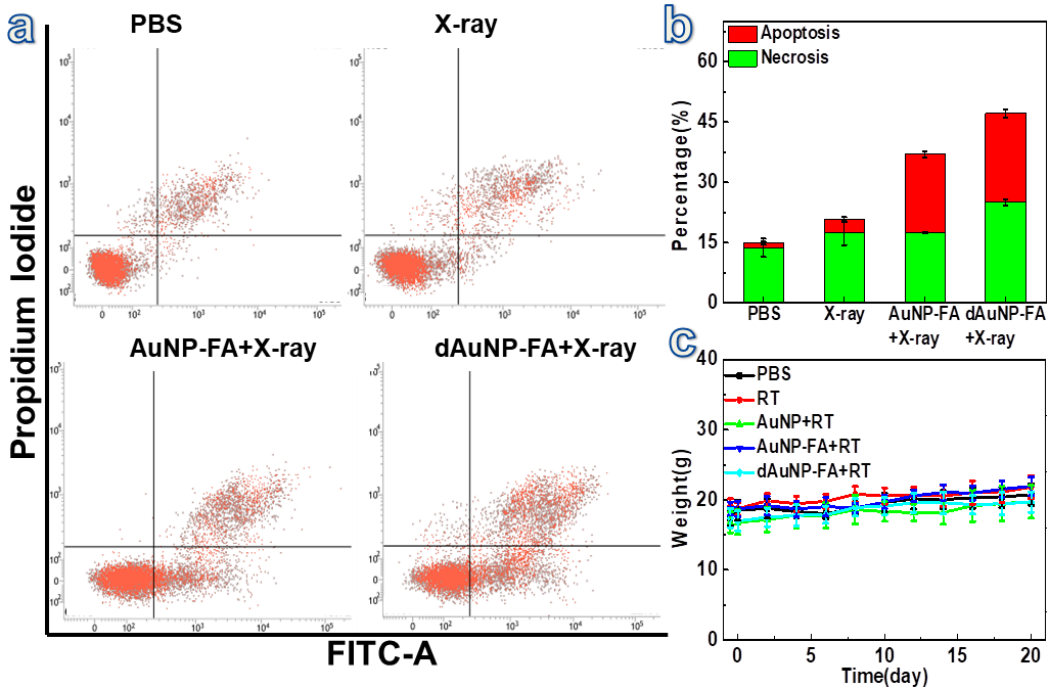

Figure S10. *In vitro* radiotherapy of 4T1 cancer cells. Apoptotic rate of 4T1 cells irradiated by 6 Gy X-rays (a&b). The concentration of Au nanoparticle is 50 μg/mL. The variations in weight of mice from the groups with different treatments (c).

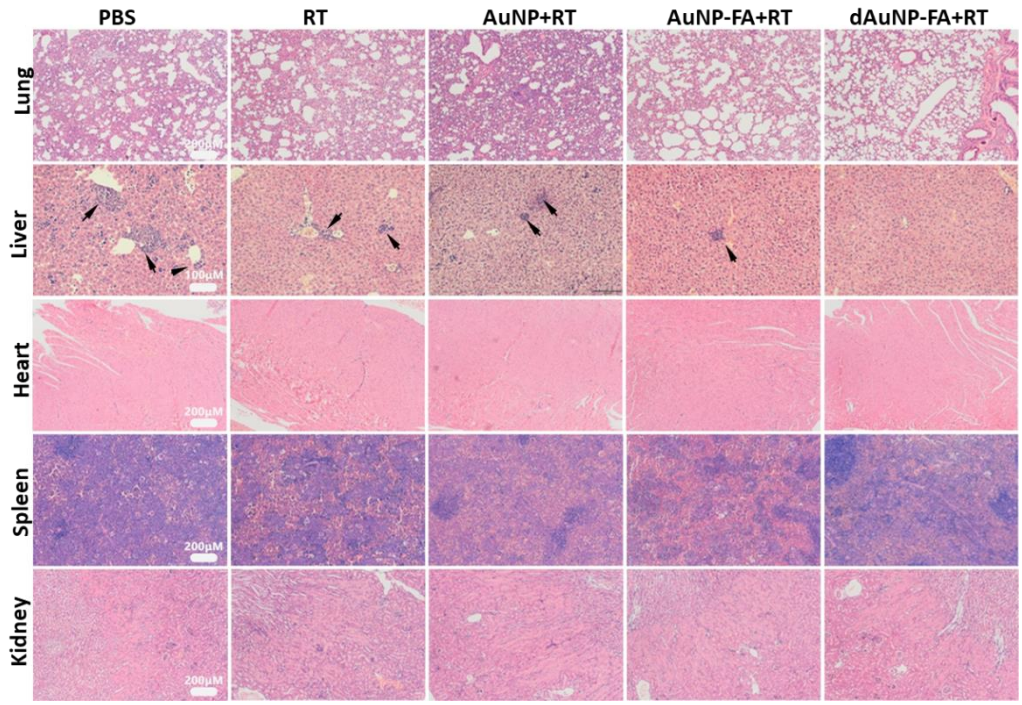

Figure S11. Slices of H&E staining containing lung, liver, heart, kidney, and spleen of tumor-bearing mice after radiation therapy for 20 days.

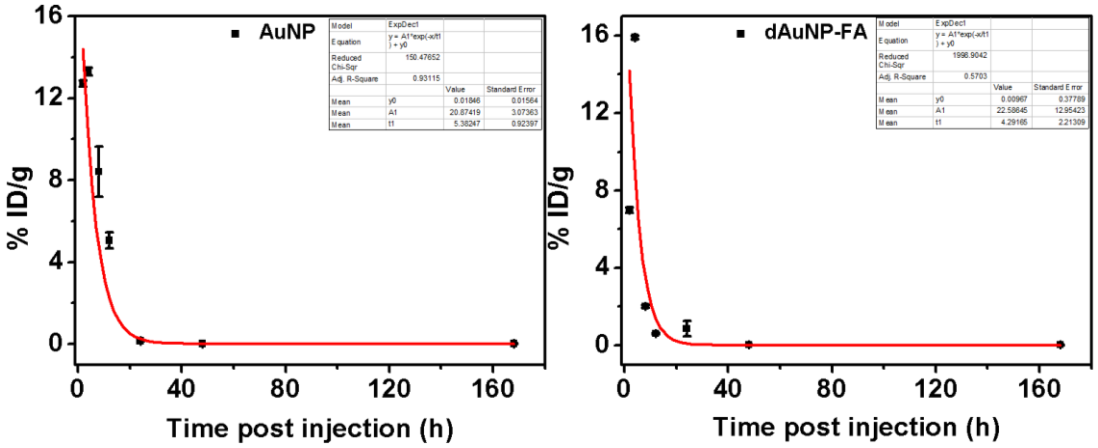

Figure S12. Blood pharmacokinetic profile of AuNP and dAuNP-FA. The $T_{1/2}$ of AuNP and dAuNP-FA is 5.38 h and 4.29 h, respectively.

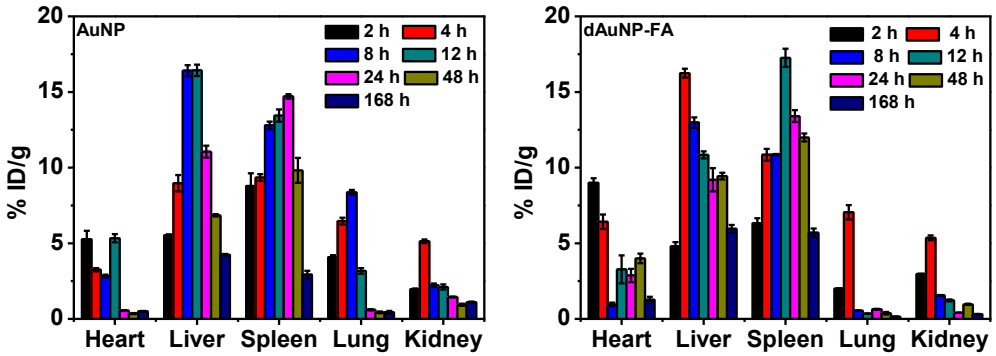

Figure S13. Time-dependent biodistribution of AuNP and dAuNP-FA in major organ tissues (n=3).