Supporting Information

Dendrimer nanoclusters loaded with gold nanoparticles for enhanced tumor CT imaging and chemotherapy *via* an amplified EPR effect

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Experimental Section

Materials. Generation 3 (G3) and generation 5 (G5) poly(amidoamine) (PAMAM) dendrimers were purchased from Dendritech (Midland, MI). 4,4'-Dithiodibutryic acid (DA), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC), doxorubicin hydrochloride (DOX·HCl), N-hydroxy succinimide (NHS), dimethyl sulfoxide (DMSO), acetic anhydride, triethylamine, sodium borohydride ($NaBH_4$), bovine serum albumin (BSA), and 1,3-propane sultone (1,3-PS) were from Sigma-Aldrich (St. Louis, MO). Glutathione (GSH) was obtained from J&K Scientific (Shanghai, China). Gold chloride trihydrate ($HAuCL_4 \cdot 4H_2O$) and all other chemicals were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Dialysis membranes with a molecular weight cut-off (MWCO) of 500 or 1000 Da were supplied from Fisher Scientific (Pittsburgh, PA). 4T1 cells were supplied from Institute of Biochemistry and Cell Biology, the Chinese Academy of Sciences (Shanghai, China). Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were from Gibco (Carlsbad, CA). Penicillin and streptomycin were from Gino Biomedical Technology Co., Ltd. (Hangzhou, China). Cell Counting Kit-8 (CCK-8) was from 7Sea Biotech Co., Ltd. (Shanghai, China). 4',6- Diamidino-2-phenylindole (DAPI) was acquired from BestBio Biotechnology Co., Ltd. (Shanghai, China). Water used in all experiments was purified using a PURIST UV Ultrapure Water System (RephiLe Bioscience, Ltd., Shanghai, China) with a resistivity higher than 18.2 MΩ·cm.

Characterization Techniques. Proton-nuclear magnetic resonance (^{1}H NMR) spectra were recorded using a Bruker AV600 NMR spectrometer (Fällanden, Switzerland). Samples were dissolved in D_2O before measurements. UV-vis spectra were collected using a Lambda 25 UV-vis spectrophotometer (PerkinElmer, Waltham, MA). Samples were dissolved in water before measurements. Dynamic light scattering (DLS) and zeta potential measurements were carried out

using a Malvern Nano-ZS Nano series Zetasizer model ZEN3600 (Worcestershire, UK) equipped with a standard 633-nm laser. To assess the colloidal stability, the samples were dispersed in water or phosphate buffered saline (PBS) at room temperature for different time periods and analyzed by DLS. Atomic force microscopy (AFM) was performed using a 3D molecular force probe instrument (Asylum Research, Santa Barbara, CA) to observe the height and morphology of the samples. Samples were dropped onto silicon wafers and air-dried before measurements. Transmission electron microscopy (TEM) imaging was performed using a JEOL 2100F electron microscope (Tokyo, Japan) at an operating voltage of 200 kV. One drop of the Au@DNCs-PS complex in water (100 μg mL^{-1}) was deposited onto a carbon-coated copper grid and air-dried before measurements. The particle size distribution was measured using Image J 1.40 software (http://rsb.info.nih.gov/ij/download.html). The Au concentration within Au@DNCs-PS or Au@G5NHAc-PS and the cellular uptake of the complexes with respect to Au contents were analyzed *via* inductively coupled plasma-optical emission spectroscopy (ICP-OES, Leeman Prodigy, Hudson, NH).

Preparation of Au@DNCs-PS. The DNCs were first synthesized following the protocol described in our earlier report.[1] Briefly, DA (3.1 mg) in dimethyl sulfoxide (DMSO, 5 mL) was activated using 1-3-dimethylaminopropyl-3-ethylcarbodiimide hydrochloride (EDC, 49.92 mg) and N-hydroxysuccimide (NHS, 29.98 mg) with continuous stirring for 4 h. Subsequently, G3 PAMAM dendrimers (30 mg, 5 mL DMSO) were dropwise added to the activated DA solution under vigorous stirring at room temperature for 24 h. The resulting reaction mixture was subjected to dialysis against water and lyophilized to obtain the DNCs.

To synthesize DNCs-PS, DNCs (10 mg, in 5 mL water) were added with an aqueous solution of 1,3-PS (3.53 mg in 5 mL water) under continuous stirring at room temperature. After 3 days of

reaction, the mixture was dialyzed against phosphate buffered saline (PBS, 2L) and water (2L) each for 3 times through a 1000 Da MWCO membrane for 3 days, and freeze dried to generate DNCs-PS product.

Then, the DNCs-PS were entrapped with Au NPs to form the Au@DNCs-PS nanocomplex. Briefly, DNCs-PS (30 mg) were dispersed in 10 mL of water, dropwise added with $HAuCl_4 \cdot 4H_2O$ (30 mg/mL, 1.35 mL) with an Au salt/dendrimer molar ratio of 25: 1 under vigorous stirring for 30 min. Then, an icy $NaBH_4$ solution (18.37 mg, in 5 mL water) was rapidly added to the above mixture under stirring for 4 h. The mixture was then dialyzed against water (2L) using a membrane with an MWCO of 1000 Da for 3 days (9 times), and lyophilized to obtain the Au@DNCs-PS product. For comparison, Au@G5NHAc-PS were also synthesized based on our previous protocols.[2, 3] In brief, the amine-terminated G5 PAMAM dendrimers (10 mg, in 5 mL water) were modified with 1,3-PS (1.2 mg, in 5 mL water) under stirring at room temperature. After 3 days reaction, the resulting mixture was subjected to dialysis and lyophilization to generate G5-NH_2-PS_{20} product. Then, the G5NHAc-PS dendrimers (50 mg, dissolved in 10 mL water) were dropwise added with $HAuCl_4 \cdot 4H_2O$ (30 mg/mL, 593 μL in water) with an Au salt/dendrimer molar ratio of 25: 1 under vigorous stirring for 30 min, and icy $NaBH_4$ (8.2 mg, in 5 mL water) was rapidly added to the mixture solution under stirring for 4 h. Then, triethylamine (125.07 μL) was added to Au@G5NH_2-PS mixture solution with continuous stirring for 30 min, followed by mixing with acetic anhydride (85.06 μL) under stirring for 24 h. Finally, the mixture solution was subjected to dialysis and lyophilization process to get the Au@G5NHAc-PS product.

Preparation of DOX/Au@DNCs-PS. DOX was encapsulated into Au@DNCs-PS based on the previous protocols.[4] In brief, DOX·HCl (4 mg) was dispersed in 300 μL methanol, neutralized with 5 μL of triethylamine to generate free DOX, which was then mixed with Au@DNCs-PS (4

mg in 1.5 mL water) at the different dendrimer/DOX molar ratios. The resulting mixture was stirred overnight to allow for the evaporation of methanol. Then, the mixture was subjected to centrifugation (1000 rpm, 10 min) to collect both supernatant and the precipitate containing the unbound free DOX. The precipitate was then dissolved in methanol and quantified using UV-vis spectroscopy. The drug loading content and efficiency were calculated according to equations (1) and (2), respectively. Similarly, DOX/Au@G5NHAc-PS complex was prepared based on the above method.

$$Loading\ content\ (\%) = \frac{weight\ of\ loaded\ DOX}{total\ weight\ of\ DOX/Au@DNCsPS} \times 100\% \quad (1)$$

$$Loading\ efficiency\ (\%) = \frac{weight\ of\ loaded\ DOX}{initial\ weight\ of\ DOX} \times 100\% \quad (2)$$

Stimuli-Responsive Release of DOX from the NCs. In order to assess the redox-responsive DOX release *in vitro*, DOX/Au@DNCs-PS (2 mg) were dispersed in 1 mL of phosphate buffer (pH = 7.4 or 6.5) in the presence or absence of GSH (10 mM). Each solution was placed in a dialysis bag with a molecular weight cut-off (MWCO) of 1000 Da and then submerged in 9 mL of the corresponding buffer medium, and the whole system was kept at 37 °C. At each predetermined time interval, 1 mL of medium was taken out from different samples, and the DOX concentration was determined by UV-vis spectroscopy at 480 nm. To maintain a constant volume of the outer phase, 1 mL of the corresponding buffer solution was replenished. To ensure a valid comparison between groups, the amount of DOX remained constant and experiment was performed in triplicate. We also checked the drug release profile of the DOX-loaded Au@G5NHAc-PS complex at acidic or physiological pH.

X-Ray Attenuation Property. The X-ray attenuation property and computed tomography (CT) phantom study of Au@DNCs-PS and Au@G5NHAc-PS were carried out at different Au

concentrations (2.5, 5, 10, 20 or 40 mM) using a dual-source SOMATOM Definition Flash CT system (iCT 256, Philips Medical Systems, Amsterdam, The Netherlands) at 120 kV and a slice thickness of 1.00 mm. For each sample, the X-ray attenuation intensity was determined in Hounsfield units (HU) by a standard display program.

Protein Resistance Assay. Protein resistance assay was used to investigate the antifouling property of Au@DNCs-PS using UV-vis spectroscopy.[5] Briefly, the BSA (1 mg/mL in PBS solution) and Au@DNCs or Au@DNCs-PS at different concentrations (0, 0.125, 0.5 or 2 mg/mL) were incubated at 37 °C. After 4 h incubation, each mixture was centrifuged at 8000 rpm for 5 min to obtain the non-adsorbed BSA in the supernatant. Then, the reduced absorbance at 278 nm for the BSA/Au@DNCs or BSA/Au@DNCs-PS mixture was calculated to quantify the antifouling properties of the corresponding particles.

Cell culture and *in Vitro* Cytotoxicity Assay

4T1 cells were regularly cultured and passaged in DMEM with fetal bovine serum (10%) and penicillin-streptomycin (1%) at 37 °C in a humidified cell incubator with 5% CO_2. To determine the cytotoxicity of drug-free Au@DNCs-PS or Au@G5NHAc-PS, cell counting kit-8 (CCK-8) assay was performed. Briefly, 1×10^4 4T1 cells were seeded into each well of a 96-well plate and culture overnight. Then, the medium of each well was replaced with fresh medium containing drug-free Au@DNCs-PS or Au@G5NHAc-PS at different concentrations. After 24 h of incubation, the medium of each well was discarded and the cells were washed with PBS for three times. Then, the cells in each well were added with 100 μL medium containing 10 μL CCK-8 and incubated for another 2 h. A Thermo Scientific Multiskan MK3 ELISA reader (Thermo Scientific, Waltham, MA) was used to record the absorbance at 450 nm for each well. Similarly, the therapeutic efficacy of drug loaded materials were also evaluated with fresh medium containing

free DOX·HCl (for short in cell culture assays and animal experiments, DOX), DOX/Au@DNCs-PS, or DOX/Au@G5NHAc-PS at different DOX concentrations and the cells were incubated for 24 h. For each sample, 5 parallel wells were tested and the data were presented as mean ± SD.

***In Vitro* Cellular Uptake**

Fluorescence microscopy imaging (an Axio Vert.A1 Carl Zeiss fluorescence microscope) was used to qualitatively evaluate the cellular uptake of DOX-loaded materials. In brief, 4T1 cells were seeded into 12-well plates (2×10^5 cells per well) with 1 mL medium for each well overnight. The next day, the medium of each well was replaced with fresh DMEM containing free DOX, DOX/Au@DNCs-PS, or DOX/Au@G5NHAc-PS at a DOX concentration of 12.5 μg mL^{-1} and the cells were incubated at 37 °C for 4 h. Then, the culture medium was removed and the cells were washed with PBS for three times, fixed with 2.5% glutaraldehyde for 15 min at room temperature and stained with DAPI for 15 min before fluorescence microscopic imaging.

The cellular uptake of DOX-containing materials within 4T1 cells was analyzed quantitatively using a flow cytometer (Becton Dickinson Facscan analyzer, Franklin Lakes, NJ). Typically, 4T1 cells were seeded into 12-well plates (2×10^5 cells per well within 1 mL medium) overnight. The next day, cells in each well were washed and replaced with fresh DMEM containing DOX/Au@G5NHAc-PS or DOX/Au@DNCs-PS complexes at different DOX concentrations. After 4 h, the medium in each well was removed and the cells were washed with PBS for 3 times, trypsinized, centrifuged, and resuspended in 500 μL PBS before flow cytometry analysis. For each sample, 10000 cells were counted, and each measurement was done in triplicate. Similarly, the cellular uptake of DOX-free complexes was analyzed using ICP-OES. The experimental protocols were the same as described above, and cells were treated with Au@DNCs-PS or Au@G5NHAc-PS at different Au concentrations (0, 0.1, 0.25, 0.5, and 1.0 mM, respectively) for 4 h, then the cell

number of each well was counted and then the cells were digested by *aqua regia* for 4 h. The samples were diluted with water before ICP-OES analysis of Au contents.

Penetration and Antitumor Therapy of 3D Tumor Spheroids *in Vitro*

The 4T1 tumor spheroids were constructed using standard literature protocol with some modifications.[6-9] Briefly, 500 μL of agarose solution (2%, w/v, sterile saline) was dropped into the mold in the 3D petri dish. Air bubbles were removed *via* pipet suction. After that, the solidified gels were separated from the mold, placed to each well of a 12-well plate, and equilibrated for more than 15 min with DMEM. Then, a cell suspension (190 μL) containing 5×10^5 4T1 cells was slowly added to each well of the 12-well plate, and after standing for 10 min, 2.5 mL of fresh cell culture medium was slowly added to each well to let the cells to aggregate and grow. When the 4T1 tumor spheroids reached an appropriate volume, the penetration capacity and the therapeutic efficacy of free DOX or DOX-loaded materials were evaluated according to protocols describe in the literature.[6, 8, 9]

To evaluate the tumor penetration depth, the 4T1 tumor spheroids were incubated with free DOX, DOX/Au@DNCs-PS or DOX/Au@G5NHAc-PS at a DOX concentration of 12.5 μg mL^{-1}. After 4 h of incubation, the 3D-tumor spheroids were washed carefully with PBS and observed *via* confocal laser scanning microscopy (CLSM) with Z-stack scanning. The *in vitro* antitumor efficacy was evaluated by incubating the 4T1 3D-tumor spheroids with PBS, free DOX, DOX/Au@DNCs-PS or DOX/Au@G5NHAc-PS for 0, 1, 3, 5 and 7 days with the same DOX concentration (12.5 μg mL^{-1}), and the volume changes in different groups were observed *via* a phase contrast microscopy.

Animal Experiments

All animal experiments were approved by the Committee of Experimental Animal Care and

Use of Donghua University (Approval No. DHUEC-STCSM-2023-10) and also in accordance with the policy of the National Ministry of Health of China. Female BALB/c nude mice (4-6 weeks old, 15-20 g) were purchased from Shanghai Slac Laboratory Animal Center (Shanghai, China). The tumor model was established by subcutaneous injection of 4T1 cells (2×10^6, 100 μL in PBS) into the right flank region of each mouse. When the tumor volume reached 100 mm^3 at one week post-injection, the tumor-bearing mice were used for subsequent CT imaging and chemotherapy experiments.

***In Vivo* CT Imaging of Tumors**

For *in vivo* CT imaging, each tumor-bearing mouse was anesthetized by pentobarbital sodium solution (1%, w/w), and peritumorally injected with the Au@G5NHAc-PS or Au@DNCs-PS ([Au] = 40 mM, in 100 μL of PBS), and CT images of tumors were collected at different time points (0, 15, 30, 60, 90, 150 and 240 min, respectively) post-injection by a CT system (iCT 256, Philips Medical Systems, Amsterdam, The Netherlands) at 120 kV and 97 mA. The CT value in Hounsfield unit (HU) was determined by a standard display program.

***In Vivo* Antitumor Therapy**

The tumor-bearing mice were randomly divided into six groups (n = 7 for each), which were then injected with 100 μL PBS as control, or 100 μL PBS containing Au@G5NHAc-PS, Au@DNCs-PS, free DOX, DOX/Au@G5NHAc-PS or DOX/Au@DNCs-PS. For the DOX containing groups, 10 mg/kg of the DOX dose was used for each mouse, while for the Au@G5NHAc-PS or Au@DNCs-PS, the amount is equivalent to the DOX-containing groups. The mice were administered intratumorally with the above materials every other day (five injections) for 15 days. During the treatment process, the tumor volume and mouse body weight

were recorded every 2 days. The tumor volume was calculated according the formula of $V = W^2 \times L/2$, where V, W and L represents the volume, width and length of tumor, respectively.

In Vivo Histological and Biosafety Evaluation

On the last day of treatment, the tumor-bearing mice from different groups were sacrificed to evaluate the antitumor efficacy and the biosafety profile according to standard protocols.[10, 11] Briefly, the tumor tissues were collected, and fixed with 4% paraformaldehyde overnight, and processed for hematoxylin and eosin (H&E), TdT-mediated dUTP nick-end labeling (TUNEL) and ki-67 staining to observe the histological changes. Meanwhile, main organs (lung, kidney, heart, liver and spleen) were also extracted, processed, and H&E stained according to standard protocols.

Statistical Analysis

All quantitative data were presents as mean ± standard deviation (SD) with at least three parallel trials or measurements. One-way of variance statistical method was adopted to analyze the experimental results using IBM SPSS Statistics 25 software (IBM, Armonk, NY). A p value of 0.05 was set as the significance level, and the data were marked with * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$, respectively.

Table S1. The hydrodynamic sizes and zeta potentials of Au@DNCs-PS and Au@G5NHAc-PS complexes (Mean ± SD, n = 3)

Samples	**Hydrodynamics size (nm)**	**Zeta potential (mV)**
Au@DNCs-PS	182.3 ± 5.9	21.3 ± 0.9
Au@G5NHAc-PS	191.0 ± 11.6	31.5 ± 0.8
DOX/Au@DNCs-PS	249.4 ± 7.0	10.2 ± 2.1
DOX/Au@G5NHAc-PS	210.8 ± 6.4	5.9 ± 1.1

Table S2. The DOX loading efficiency (DLE, %) and loading content (DLC, %) of DOX/Au@DNCs-PS at the different dendrimer/DOX molar ratios.

Samples (dendrimer:DOX)	**DLC (%)**	**DLE (%)**
1:1	2.55	71.08
1:2.5	5.85	78.61
1:5	6.78	79.87
1:10	17.62	82.40

Table S3. The IC_{50}s of DOX, DOX/Au@DNCs-PS or DOX/Au@G5NHAc-PS that were used to treat 4T1 cells for 24 h (n =3).

Samples	**IC_{50} for 4T1 cells (μg/mL)**
DOX	13.56 ± 0.50
DOX/Au@DNCs-PS	26.19 ± 0.30
DOX/Au@G5NHAc-PS	27.27 ± 0.29

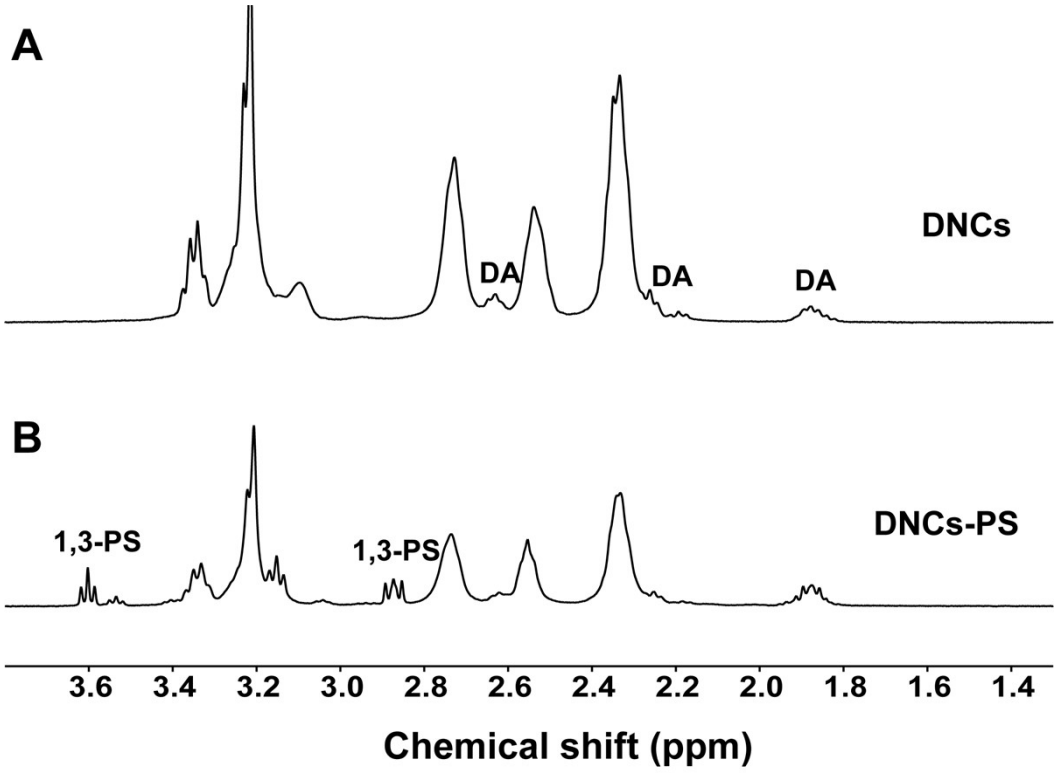

Figure S1. ^{1}H NMR spectra of (A) DNCs and (B) DNCs-PS in D_2O.

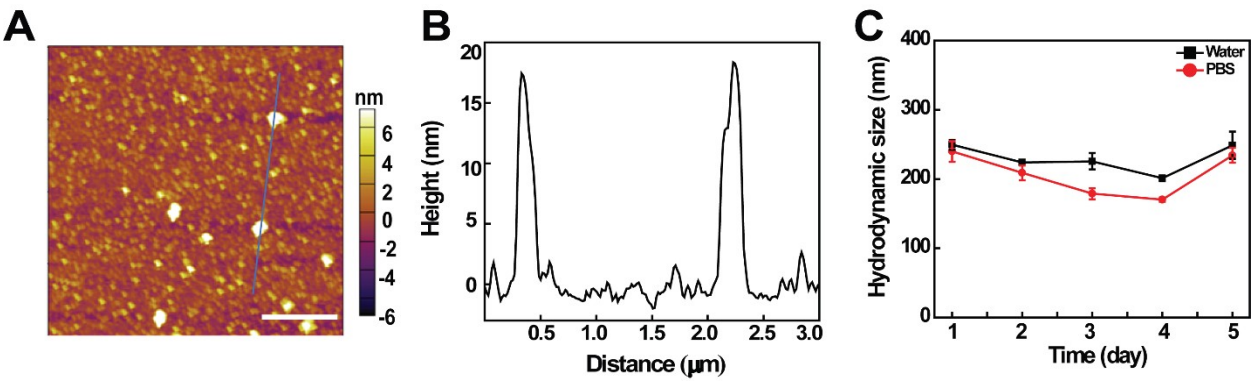

Figure S2. AFM image (A) and corresponding height profile (B) of DOX/Au@DNCs-PS deposited onto silicon wafers. Scale bar is 1 μm. (C) Hydrodynamic size of the DOX/Au@DNCs-PS dispersed in water and PBS at room temperature at different storage time periods.

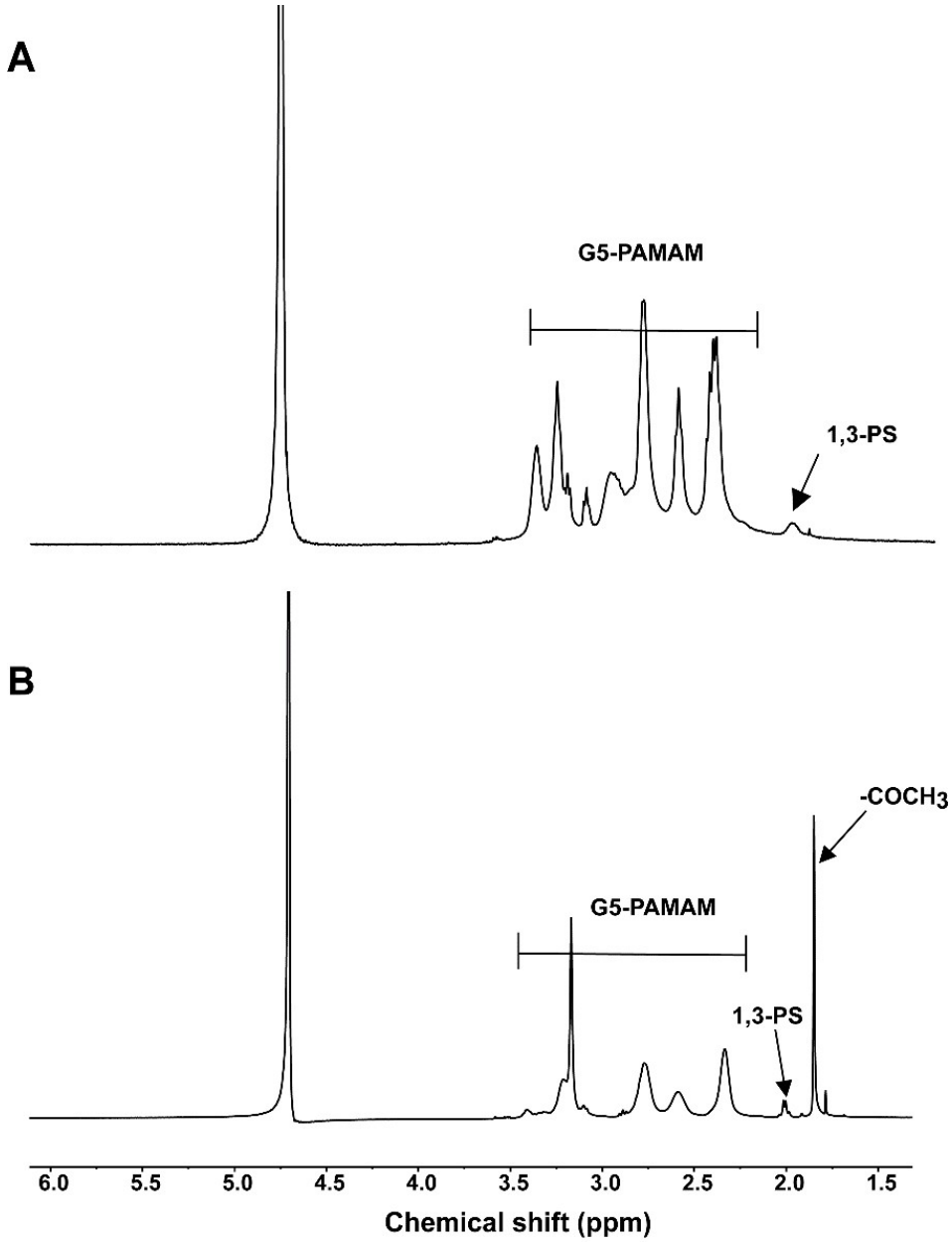

Figure S3. ^{1}H NMR spectra of (A) Au@G5-PS and (B) Au@G5NHAc-PS complexes in D_2O.

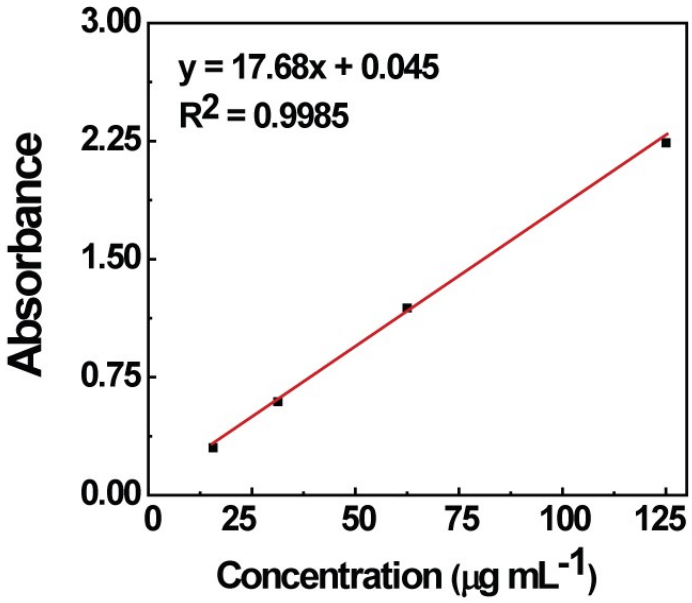

Figure S4. The calibration curve of DOX absorbance at 480 nm *versus* concentration in methanol solution.

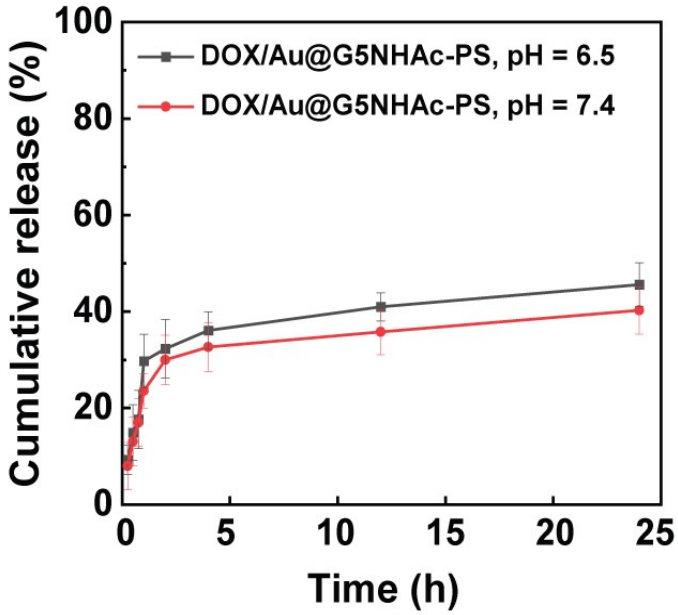

Figure S5. Cumulative DOX release profiles from the DOX/Au@G5NHAc-PS complex dispersed in phosphate buffer incubated under pH 6.5 or pH 7.4 at 37 °C.

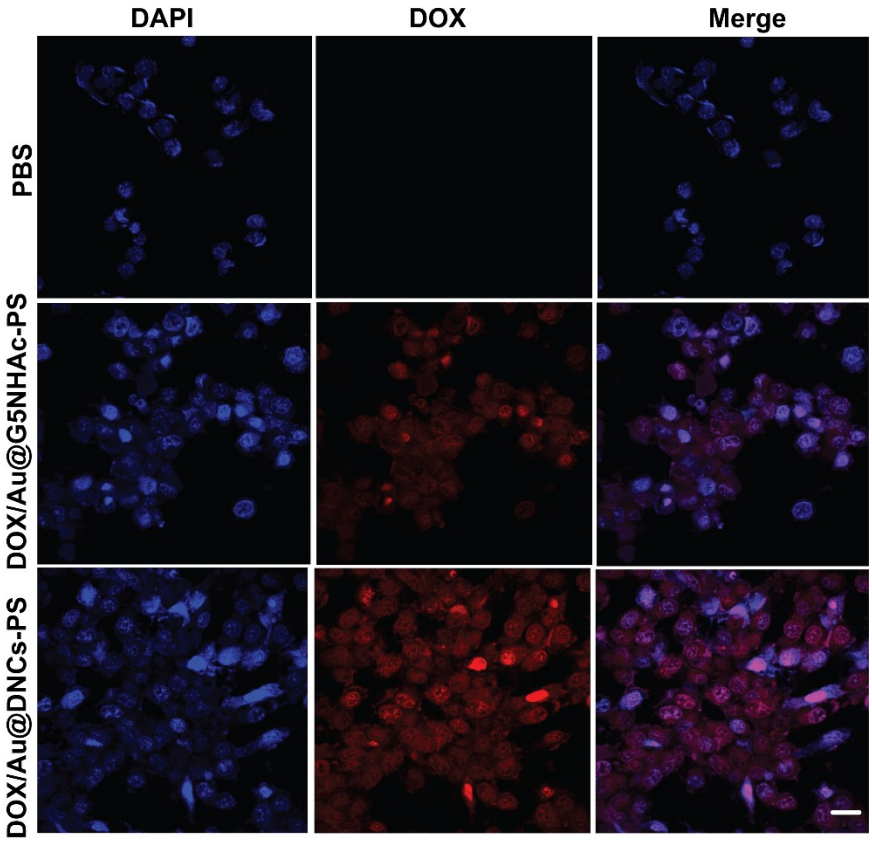

Figure S6. CLSM imaging of 4T1 cells incubated with PBS, DOX/Au@G5NHAc-PS or DOX/Au@DNCs-PS at the same DOX concentration (12.5 μg mL^{-1}) for 4 h (scale bar is 20 μm for each panel).

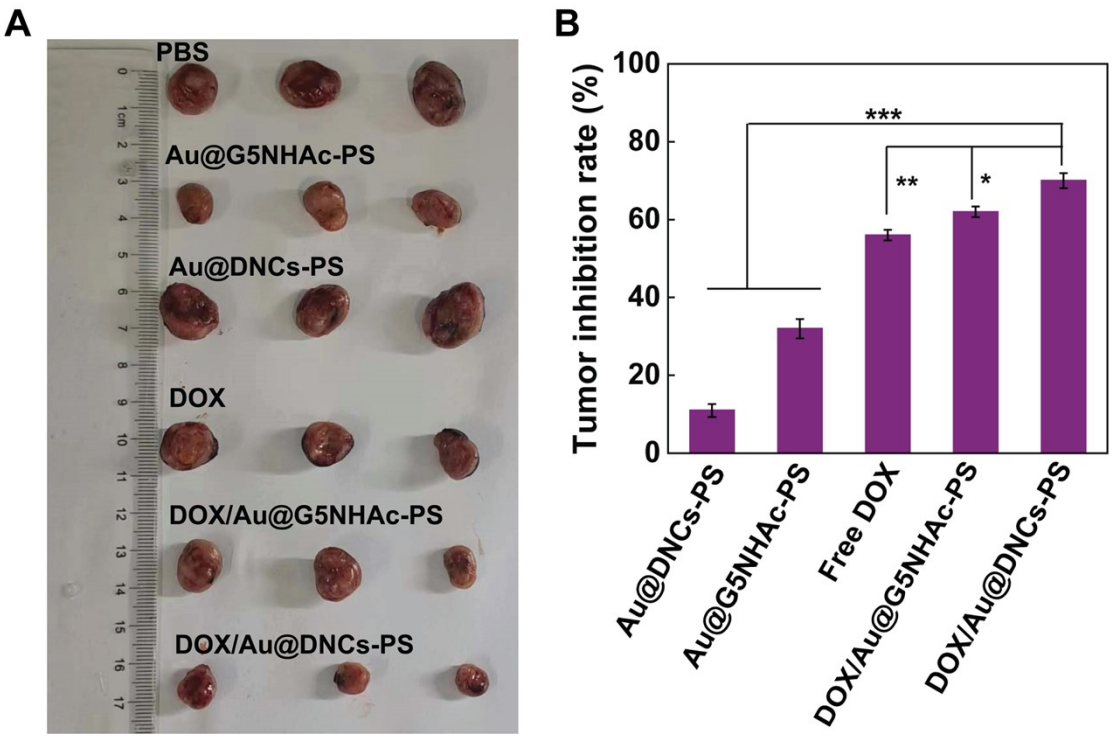

Figure S7. (A) Representative photographs of tumor tissues and (B) tumor inhibition rates of 4T1-tumor bearing mice after treated with different materials.

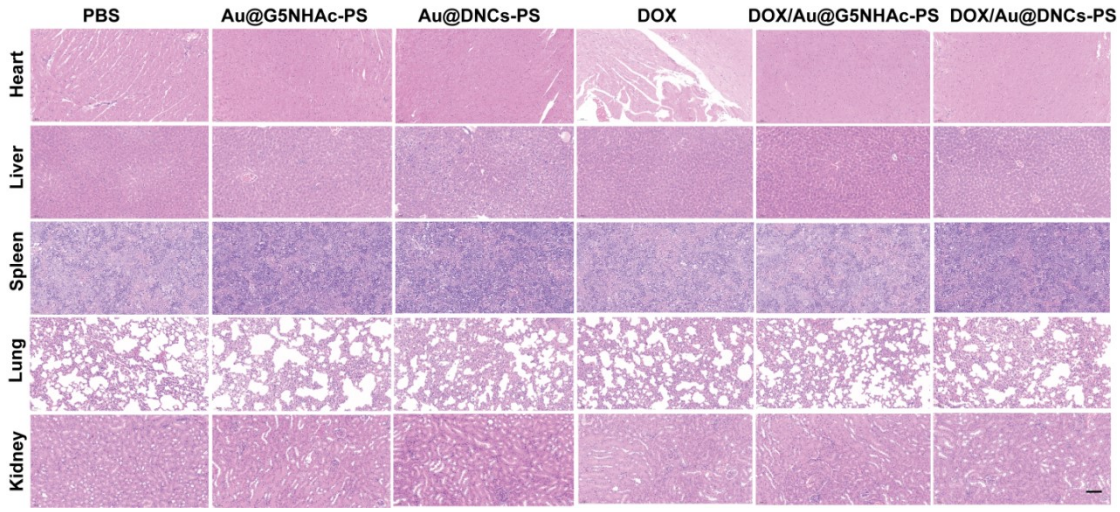

Figure S8. H&E-stained sections of major organs of 4T1 tumor-bearing mice after different treatments for 15 days. Scale bar for each panel represents 50 μm.

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