Supporting Information

Glutathione-triggered “Off−On” Release of Anticancer Drugs from Dendrimer-encapsulated Gold Nanoparticles

Xinyu Wang[1], Xiaopan Cai[2], Jingjing Hu[3], Naimin Shao[1], Fei Wang[1], Qiang Zhang[1], Jianru Xiao[2], Yiyun Cheng[1]

[1]*Shanghai Key Laboratory of Regulatory Biology, School of Life Sciences, East China Normal University, Shanghai, 200062, P.R. China*

[2]*Department of Orthopedic Oncology, Changzheng Hospital, the Second Military Medical University, Shanghai, 200003, P.R. China*

[3]*Department of Bioscience and Biotechnology, Dalian University of Technology, Dalian, 116024, P.R. China.*

EXPERIMENTAL SECTION

Materials. G3-G5 ethylenediamine (EDA)-cored and hydroxyl-terminated PAMAM dendrimers (G3-OH, G4-OH, and G5-OH) with 32, 64 and 128 surface functionalities were purchased from Dendritech Inc. (Midland, MI). CPP, 6-MP, MSA, and 2-iminothiolane hydrochloride were purchased from Sigma-Aldrich Inc. (St. Louis, MO). DOX, CPT, chloroauric acid ($HAuCl_4$), sodium borohydride ($NaBH_4$) were purchased from Aladdin Inc. (Shanghai, China). Acetonitrile and methanol (HPLC grade) were purchased from Fisher Scientific Ltd. The dendrimers were distilled to remove the solvent (methanol) and obtained as white gels. All other products were used as received without further purification.

Preparation of DEGNPs. DEGNPs were prepared using G3-OH, G4-OH, and G5-OH as templates. The complexation of aqueous $AuCl_4^-$ to the interior tertiary amine groups of PAMAM dendrimer was followed by reduction of the complexed Au^{3+} ions with $NaBH_4$ in water. Specifically, 25, 50, and 100 equivalent molars of $HAuCl_4$ were mixed with G3-OH, G4-OH, and G5-OH dendrimer, respectively. The solutions were vigorously stirred for 2 min, followed by the dropwise addition of 5-fold molar excess amount of $NaBH_4$ in 0.3 M NaOH to the above solution. A conversion of the pale yellow solution to the characteristic red color of gold nanoparticles was observed during the reduction of Au^{3+} ions. After that, the solutions of G3-OH/Au NPs, G4-OH/Au NPs, and G5-OH/Au NPs were transferred into dialysis bags with a molecular weight cut off of 3500 Da and the solution was extensively dialyzed against deionized water. The products were lyophilized and stored at 4 ^oC before drug loading experiments.

Characterization of the Synthesized DEGNPs and Drug-loaded DEGNPs. The size and morphology of G3-OH/Au NPs, G4-OH/Au NPs and G5-OH/Au NPs were examined using a JEM 2010 transmission electron microscope (JEOL). The UV-Vis spectra of the synthesized DEGNPs were measured by a UV-2600 spectrophotometer (Techcomp, Shanghai). The samples of G5-OH/Au NPs loaded with 6-MP and

G5-OH loaded with 6-MP were characterized by ^{1}H NMR on a Varian 699.804 MHz NMR spectrometer at 298.2 ± 0.1 K.

Preparation of DOX-SH and CPT Loaded DEGNPs. DOX-SH was synthesized by mixing DOX and 2-iminothiolane under argon protection. The solution was stirred for 48 h at dark to ensure the high conversion efficiency of DOX to DOX-SH (ESI-MS calculated $[M+1]^+$ for DOX-SH: 645.6, observed $[M+1]^+$: 646.2, **Figure S7**).The samples of DOX-SH and G5-OH/Au NPs were mixed together and stirred for 12 h to prepare DOX-SH loaded nanoparticles. For CPT, 10-fold molar excess amount of MSA were added into the G5-OH/Au NPs solution and the mixture was allowed to react for 12 h, followed by the addition of CPT to the resulting G5-OH/Au-MSA NPs solution for another 12 h. The samples were then lyophilized and dissolved in PBS buffer for further use. The prepared DOX-SH and CPT-loaded DEGNPs were directly used in further cytotoxicity, flow cytometry, and CLSM studies.

***In Vitro* Release Studies.** The *in vitro* release behaviors of CPP from G3-OH, G4-OH and G5-OH dendrimers and G3-OH/Au NPs, G4-OH/Au NPs and G5-OH/Au NPs were investigated by an equilibrium dialysis method. Generally, the CPP loaded dendrimers or DEGNPs were prepared at a CPP/dendrimer molar ratio of 5. The complex solution was vigorously stirred for 2 h for CPP to be sufficiently bound with dendrimer and nanoparticle. The samples were then transferred into dialysis bags with a molecular weight cut off of 3500 Da. The dialysis bags were immediately immersed into a container filled with 30 mL 0.01 M phosphate buffer (PBS, pH=7.4), and outer phase of the dialysis bag was stirred at 250 r.p.m. during the *in vitro* release studies. 100 μL of the samples were collected at scheduled time intervals from the outer phase and the CPP concentrations were determined by a high-performance liquid chromatography (HPLC) method. The *in vitro* release of 6-MP from dendrimer and DEGNPs is performed by a similar method. The molar ratio of 6-MP and dendrimer is kept constant at 10. The *in vitro* release of DOX-SH from DEGNPs is performed as follows. Excess amount of DOX-SH was mixed with DEGNPs in a 50% DMSO aqueous solution. The mixture was stirring for 2 h, followed by centrifuging at 15000

r.p.m. for 20 min to remove the unbound DOX-SH. The precipitate was washed with distilled water twice and dissolved in PBS buffer. The GSH triggered release sample was added with GSH at a final concentration of 10 mM. 100 μL of the samples were collected at scheduled time intervals and the samples were centrifuged at 15000 r.p.m. DOX-SH concentration in the supernatant was analyzed by HPLC.

HPLC Analysis. The concentration of CPP and 6-MP in the samples collected from the *in vitro* release studies were measured by an HPLC method as described elsewhere. Generally, the HPLC experiments were conducted on an HPLC instrument (Agilent 1200, U.S.A) equipped with a C18 column (4.6 mm diameter, 150 mm length, 5 mm particle size, ZORBAX Eclipse XDB, Agilent, U.S.A.). For CPP, the mobile phase was a mixture of acetonitrile and 0.02% (v/v) phosphoric acid (pH = 2.7) at a volume ratio of 70:30 and a flow rate of 1.0 mL/min. 10 μL of the samples were injected and the drug was detected at 227 nm. The retention time of CPP and CPP disulfide is 3.9 ± 0.2 and 5.1 ± 0.2 min. The standard curves of CPP and CPP disulfide were Area = 2.424*C* - 0.5830 (R^2 = 0.9999) and Area = 2.626*C* - 0.03069 (R^2 = 0.9999), respectively with an application concentration range from 1 to 80 μg/mL.

For 6-MP, the mobile phase was a mixture of deionized water and methanol at a volume ratio of 90:10 and a flow rate of 1.0 mL/min. 10 μL of the samples were injected and the drug was detected at 327 nm. The retention time of 6-MP is 6.3 ± 0.3 min. The standard curve is Area = 65.99*C* - 0.7863 and the application concentration ranges from 0.1 to 25.6 μg/mL.

For DOX-SH, the mobile phase was a mixture of acetonitrile and 0.2% (v/v) trifluoroacetic acid aqueous solution at a volume ratio of 35:65 and a flow rate of 1.0 mL/min. 10 μL of the samples were injected and the drug was detected at 490 nm. The standard curve for the drug was Area = 10.06C – 2.030 (R^2 = 0.9995).

Cell Culture. HeLa, U2OS, MCF-7, and NIH3T3 cells were obtained from the Institute of Life and Biomedical, School of Life Sciences, East China Normal University. The cells were grown at 37 ℃ in 5% CO_2 in DMEM medium (Gibco, Inc.)

supplemented with 10% heat inactivated FBS, penicillin (100 units/mL) and streptomycin (100 mg/mL).

The 3-(4,5-Dimethylthiazol-2-Thiazolyl)-2,5-Diphenyltrazolium Bromide (MTT) Assay. The cytotoxicities of the synthesized DEGNPs, anti-cancer drugs, and anti-cancer drug loaded DEGNPs were evaluated by an MTT assay. Generally 10^4 HeLa, MCF-7, U2OS, and NIH3T3 cells were seeded into a 96-well plate and cultured for 12 h before the cytotoxicity studies. Subsequently, the medium was replaced with fresh DMEM containing DEGNPs, drugs, and drug-loaded DEGNPs for another 24 h or 48 h, followed by replacing the medium with MTT containing DMEM for 2 h. After that, 150 μL DMSO was added into the wells in order to dissolve the insoluble purple formazan. The absorbance of each sample with 4 replicates (n=4) was detected at 490 nm with a reference wavelength of 650 nm by a microplate reader, and the obtained values were used to reflect the viability of the cells treated with each sample. Cells incubated without any materials were used as controls and the viabilities of these cells were set as 100%.

Flow Cytometry Studies. HeLa cells were first added in to a 24 well-plate at a cell density of 5×10^4 per well. After overnight incubation, 20 mM GSH-OEt was pre-treated for 2 h followed by the incubation of the cells with 10 μM DOX-SH or DOX-SH loaded G5-OH/Au NPs for 3 h. The cells were then washed by PBS, digested by trypsin (100 μL), centrifuged to remove the supernatant, and re-dissolved in PBS. The samples were analyzed by flow cytometry. Data for 1.0×10^4 gated events were collected and analysis was performed by means of a BD FACS Calibur flow cytometer and CELLQuest software.

CLSM Studies. Cell uptake of DOX-SH and DOX-SH loaded G5-OH/Au NPs was further analyzed by CLSM studies. HeLa cells were seeded into 24-well plates at a density of 5×10^4 cells per well in 0.5 mL complete DMEM and cultured for 12 h, followed by removing the complete medium and incubating with the same samples in flow cytometry studies. Subsequently, the cells were washed twice with PBS, fixed with 4% paraformaldehyde for 15 min, stained with DAPI for another 15 min at room

temperature and then the cells were rinsed with PBS twice again. After that, the cells were incubated with 0.1% Triton X-100 for 5 min, stained by phalloidin-FITC (green) for 10 min and washed for twice by PBS. Finally, the slides were mounted and observed with a CLSM (Leica TCS SP5).

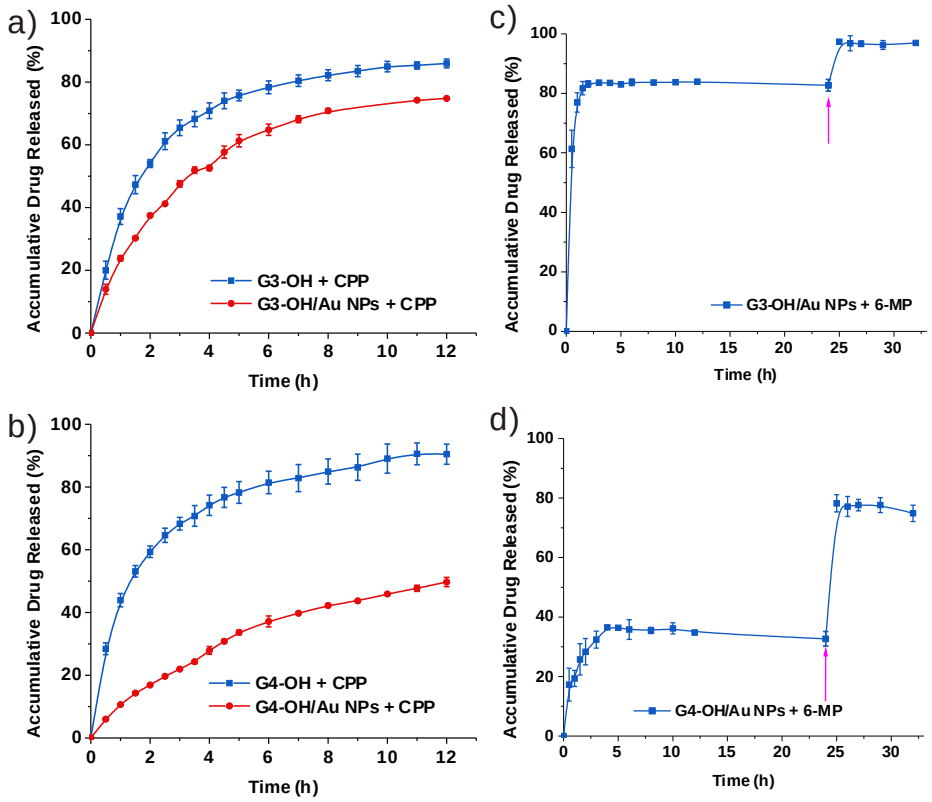


Figure S1. *In vitro* release profiles of CPP (a, b) and 6-MP (c, d) form G3-OH/Au NPs (a,c) and G4-OH/Au NPs (b, d). The purple arrow indicates the addition of GSH in to the 6-MP loaded DEGNPs.

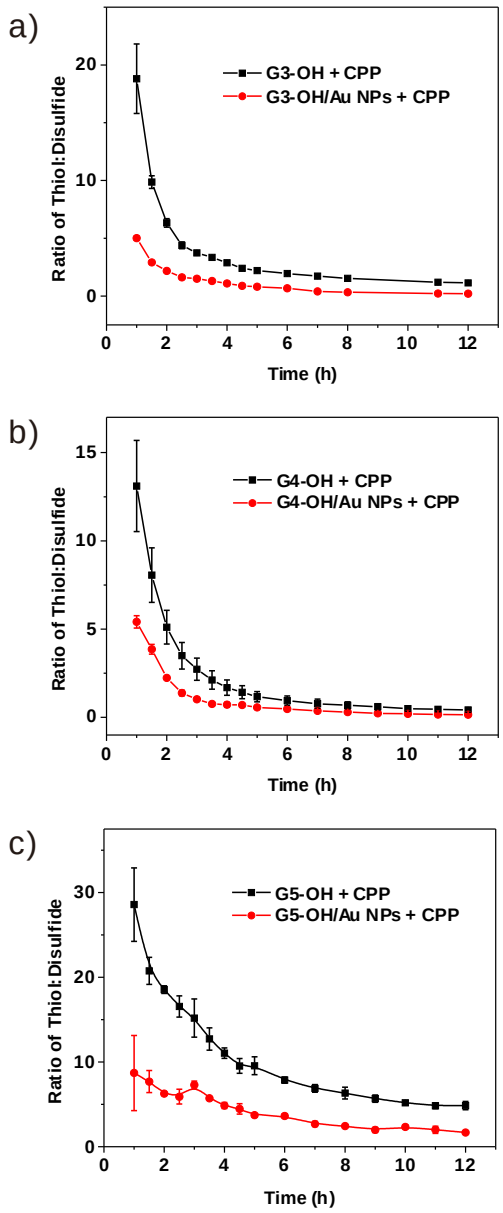
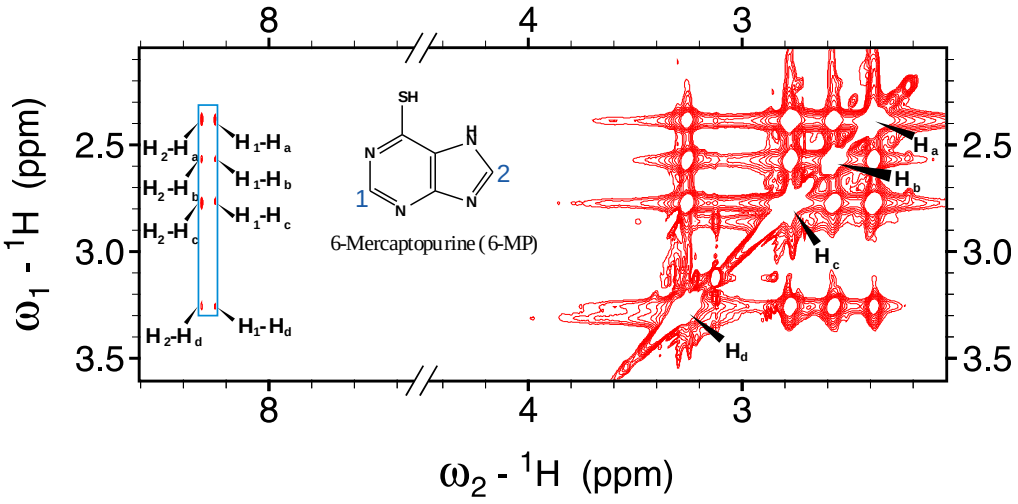
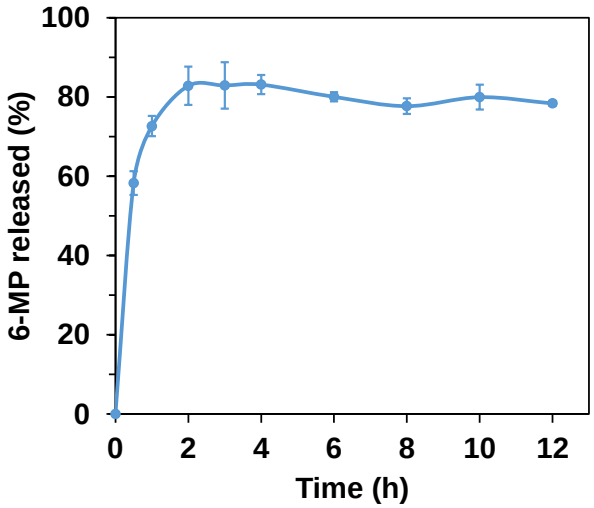


Figure S2. Ratio of CPP thiol and CPP disulfide in the samples released from G3-OH/Au NPs, G4-OH/Au NPs and G5-OH/Au NPs, respectively.

Note: The results suggest that the detected CPP molecules are mostly in the form of CPP thiol at the beginning of release and the released CPP thiol will convert to CPP disulfide during the release studies.



(a)



(b)

Figure S3. ^{1}H-^{1}H NOESY spectrum of G5-NH_2/6-MP complex using a 700 MHz NMR, at a mixing time of 300 ms (a) and *In vitro* release profiles of 6-MP from the G5-NH_2/6-MP complex (b).

Note: Strong NOE cross-peaks are observed between G5 interior pocket protons and 6-MP aromatic protons in Figure S3a, revealing the encapsulation of 6-MP molecules within G5-NH_2. Though 6-MP was encapsulated within G5-NH_2 interior pockets in the G5-NH_2/6-MP complex, the 6-MP quickly released from G5-NH_2 dendrimer within 2 h in PBS buffer (Figure S3b), just like that loaded within G5-OH. Therefore, only the drugs loaded within dendrimer via covalent interactions (e.g. the Au-S linkage) exhibit a sustained release behavior in physiological conditions (Figure 3b).

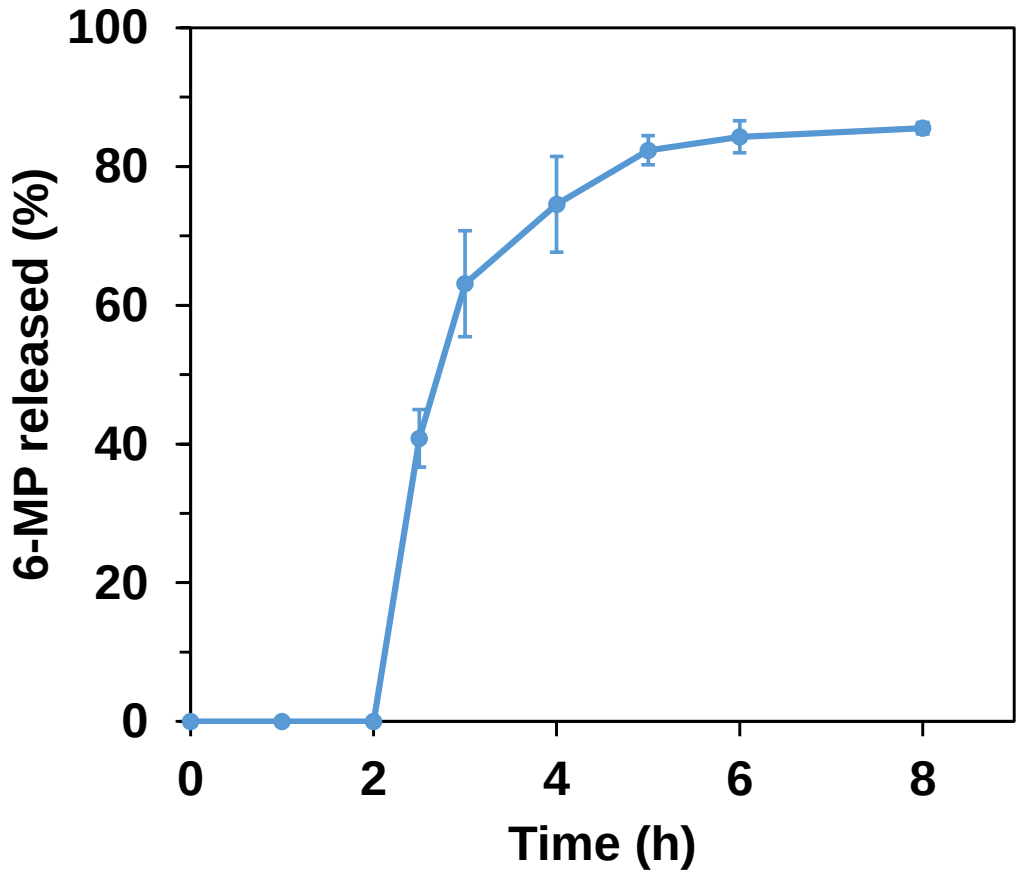


Figure S4. *In vitro* release profiles of 6-MP from the DEGNPs/6-MP complex after the remove of unbound 6-MP in Figure 3b.

Note: To confirm that the released 6-MP molecules from DEGNPs within the first several hours in Figure 3b are unbound drugs, we dialyzed the formulation against PBS buffer for 2 h to remove the unbound 6-MP and then conducted the *in vitro* release studies as demonstrated in the experimental section. GSH was added to the DEGNPs/6-MP complex solution at 2 h. The result reveals an "Off-On" GSH-triggered release behavior as shown in Figure S4, suggesting that the drugs released from the formulation within 4 h in Figure 3b are mostly unbound drugs.

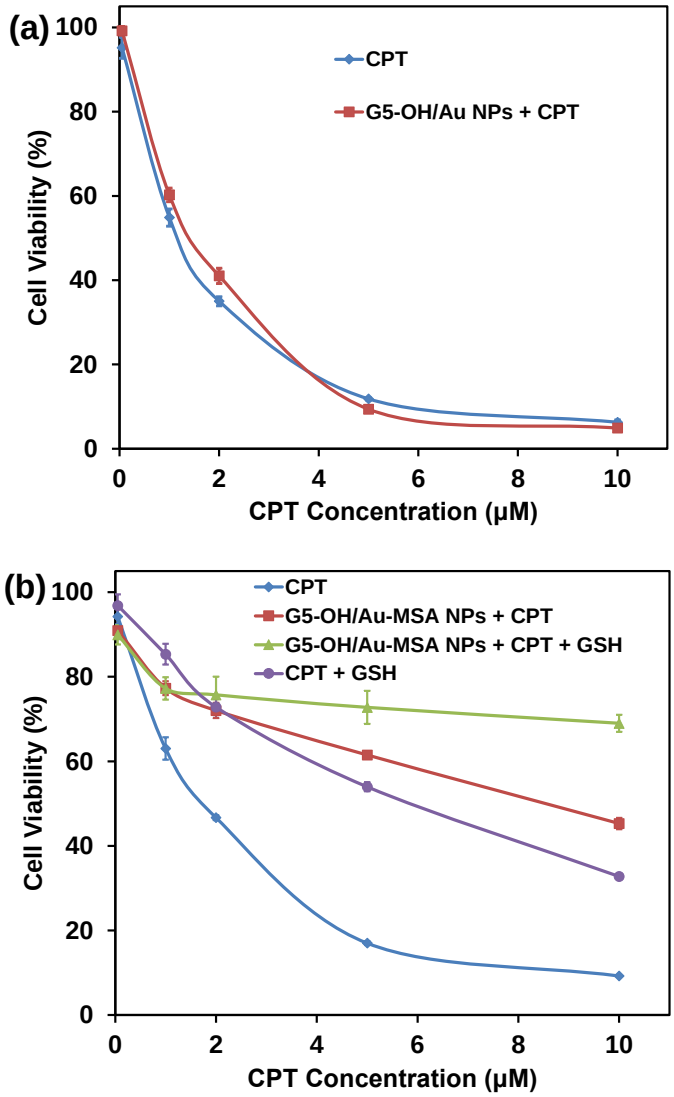


Figure S5. (a) The cytotoxicity of CPT in the presence or absence of DEGNPs without MSA. The results indicate that MSA plays an important role in the attachment of CPT onto DEGNPs. (b) Cytotoxicities of CPT loaded G5-OH/Au-MSA NPs compared with free CPT. The addition of 5 mM GSH decreased the cytotoxicity of CPT loaded within the nanoparticle.

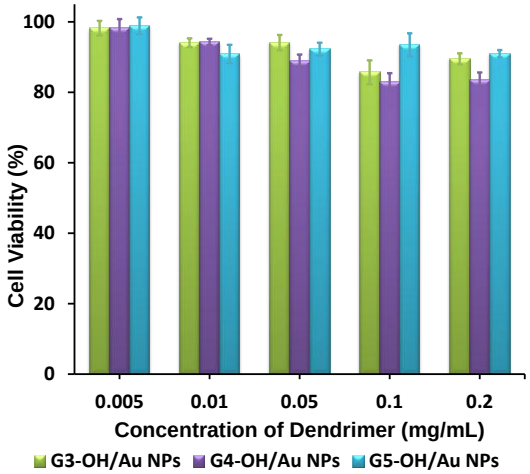


Figure S6. Viability of HeLa cells incubated with G3-OH/Au NPs, G4-OH/Au NPs and G5-OH/Au NPs for 48 h.

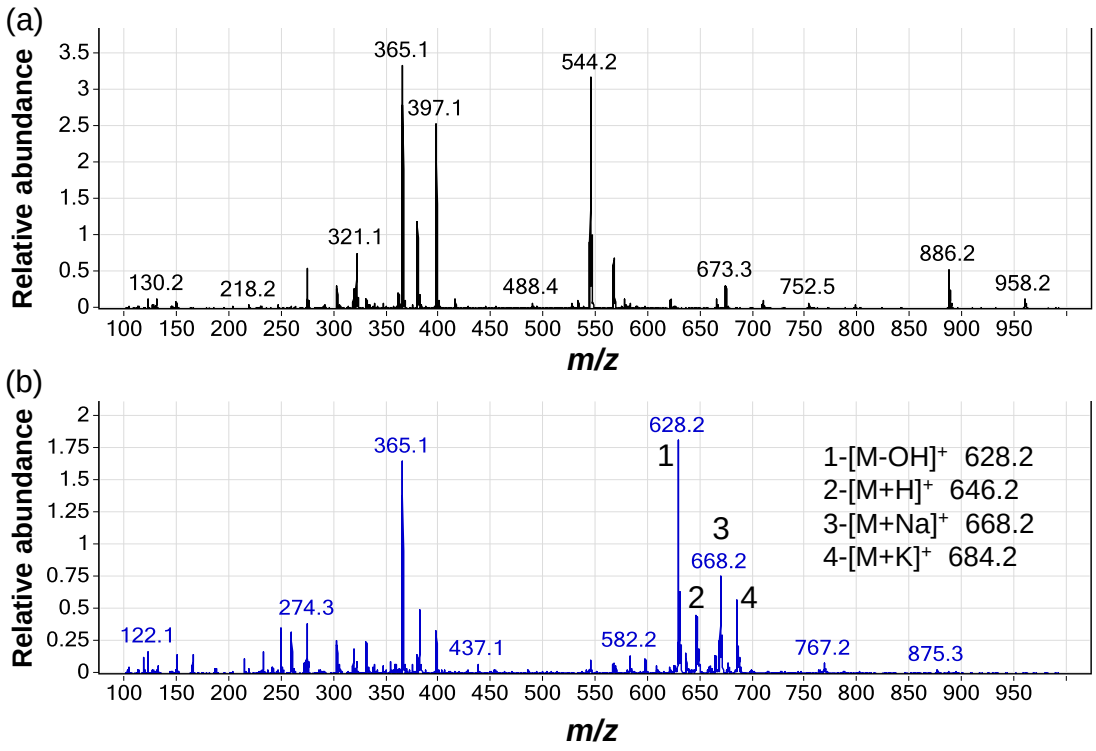


Figure S7. ESI-MS spectra of unmodified doxorubicin (a) and thiolated doxorubicin (b).

Note: Unmodified DOX has an observed $[M+1]^+$ value of 544.2, which is in accordance with its calculated molecular weight. The synthesized DOX-SH has an observed molecular weight of 645.2 (ESI-MS: $[M+1]^+$=646.2), and the theoretical molecular weight DOX-SH is 644.6 (calculated). The disappearance of the peak at 544.2 for DOX in Figure S7b suggests that most of the DOX were converted to DOX-SH after the reaction.