Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information for

Reduction-Responsive Dithiomaleimide-Based Nanomedicine with High Drug Loading and FRET-Indicated Drug Release

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Experimental section

Materials

2,3-Dibromomaleimide and other materials were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted. Triphosgene was purchased from Alfa Aesar (Ward Hill, MA, USA). TCEP·HCl (tris(2-carboxyethyl)phosphine hydrochloride) was purchased from Thermo Scientific (Waltham, MA, USA). Anhydrous dichloromethane (DCM), hexane, tetrahydrofuran (THF) and dimethylformamide (DMF) were purified by passing them through alumina columns and kept anhydrous by storing them in the presence of molecular sieves. Spectra/Por 6 dialysis tubing (MWCO 1K) was purchased from Spectrum Laboratories Inc (Rancho Dominguez, CA, USA). Phosphate-Buffered Saline (PBS) and Dulbecco's Modified Eagle Medium (DMEM) were obtained from Invitrogen (Carlsbad, CA, USA). LS174T colon cancer cell line was purchased from American Type Culture Collection (Manassas, VA, USA). Fetal Bovine Serum (FBS) was obtained from Lonza Walkersville Inc (Walkersville, MD, USA). 96 well BD Falcon culture plates were purchased from Fisher Scientific (Hampton, NH, USA).

Measurements

Nuclear magnetic resonance (NMR) analyses were conducted on a Varian U500 (500 MHz) or a VXR500 (500 MHz) spectrometer. HPLC analyses were performed on a Shimadzu CBM-20A system (Shimadzu, Kyoto, Japan) equipped with SPD20A PDA detector (190nm-800nm) and RF10Axl fluorescence detector, and an analytical C18 column (Shimadzu, 3 μm, 50*4.6 mm, Kyoto, Japan). The size and size distribution of NPs were determined by ZetaPlus dynamic light scattering (DLS) detector (15 mW laser, incident beam = 676 nm, Brookhaven Instruments, Holtsville, NY, USA). Infrared spectra were recorded on a PerkinElmer 100 serial FTIR

spectrophotometer. Fluorescence spectra of compounds were measured on a LS55 fluorescence spectrometer (Perkin Elmer, Santa Clara, CA, USA). TEM images were collected on a JEOL 2100 cryo TEM.

Synthesis of CPT-S-S-CPT. CPT (2.0 mmol, 696.7 mg) was suspended in anhydrous DCM (30 mL), followed by addition of triphosgene (0.8 mmol, 237.4 mg) and 4-(dimethylamino)pyridine (DMAP, 2.0 mmol, 244.3 mg). The mixture was stirred at room temperature for 4 h until the solution became clear, at which time point 2-hydroxyethyl disulfide (0.8 mmol, 123.4 mg) in THF/DCM (1/3, v/v, 2 mL) was added in one portion. The reaction mixture was stirred at room temperature for another 24 h. Solvent was removed under reduced pressure and the crude product was purified by silica gel column chromatography using ethyl acetate to ethyl acetate/methanol (9/1, v/v) as the eluent. A light yellow solid was obtained (62% yield). LRMS (ESI) *m/z*: calculated for $C_{46}H_{39}N_4O_{12}S_2$ $[M+H]^+$ 903.2, found 903.4.

Synthesis of *N*-propargyl-2,3-dibromomaleimide. 2,3-dibromomaleimide (1.0 mmol, 254.9 mg) and propargyl bromide (2.5 mmol, 297.4 mg) were dissolved in acetone (60 mL), followed by addition of potassium carbonate (4.0 mmol, 552.8 mg). The mixture was stirred at room temperature for 48 h. The precipitate was filtrated off, and the solvent was removed. The crude product was purified by silica gel column chromatography using hexane/ethyl acetate (1/1, v/v) as the eluent (50% yield). LRMS (ESI) *m/z*: calculated for $C_7H_4Br_2NO_2$ $[M+H]^+$ 293.9, found 293.8.

Synthesis of $(CPT)_2$-Mal-alkyne. CPT-S-S-CPT (0.2 mmol, 180.4 mg) and *N*-propargyl-2,3-dibromomaleimide (0.18 mmol, 52.7 mg) were dissolved in THF/methanol (1/3, v/v, 30 mL), followed by addition of triethylamine (0.56 mmol, 56.7 mg) and TCEP·HCl (0.2 mmol, 57.3 mg).

The mixture was stirred at room temperature for 24 h. Solvent was removed under reduced pressure, and the solid residue was purified by silica gel column chromatography using ethyl acetate to ethyl acetate/methanol (9/1, v/v) as the eluent. A yellow solid was obtained (40% yield). LRMS (ESI) *m/z*: calculated for $C_{53}H_{42}N_5O_{14}S_2$ $[M+H]^+$ 1036.2, found 1036.5.

Synthesis of $(CPT)_2$-Mal-PEG. $(CPT)_2$-Mal-alkyne (0.025 mmol, 25.9 mg) and PEG-N_3 (0.02 mmol) were dissolved in anhydrous DMF (5 mL), followed by addition of copper bromide (0.01 mmol, 1.4 mg) and *N*, *N*, *N'*, *N''*, *N''*-pentamethyldiethylenetriamine (PMDETA, 0.01 mmol, 1.8 mg). The mixture was stirred at 40°C for 24 h. Copper salt was removed by passing the reaction mixture through an aluminum column. The crude product was purified by silica gel column (short PEG segment) or by ultracentrifugation (long PEG segment).

Preparation of $(CPT)_2$-Mal-PEG_{1k} NPs. $(CPT)_2$-Mal-PEG_{1k} in DMF (10 mg/mL, 100 μL) was stirred vigorously and slowly added nanopure water (4 mL) dropwise in 30 min. After the addition of nanopure water was complete, the solution was stirred for another 4 h prior to dialysis against DI water for 48 h.

General procedures for the synthesis of 2,3-dithiomaleimides. 2,3-dibromomaleimide (1 molar equivalent) was added to a mixture of thiol (2.1 molar equivalents) and triethylamine (2.1 molar equivalents) in anhydrous methanol, and the mixture was stirred at room temperature for 2 h-6 h, at which point TLC should show complete consumption of 2,3-dibromomaleimide.

GSH-induced degradation of $(CPT)_2$-Mal-PEG_{1k}

(1) **Fluorescence measurements**: $(CPT)_2$-Mal-PEG_{1k} in methanol (50 μg/mL, 2.5 mL) was added to a cuvette. GSH was added with a final concentration of 5 mM. The mixture was

incubated at 37°C (100 rpm). At selected time points, the solution was sent for fluorescence measurement on a fluorescence spectrometer.

(2) **Determination of CPT release kinetics by HPLC**: $(CPT)_2$-Mal-PEG_{1k} in methanol (50 μg/mL, 1 mL) was added to a HPLC vial. GSH was added with a final concentration of 5 mM and the mixture was incubated at 37°C (100 rpm). At selected time points, the sample was run on HPLC. A mixture of acetonitrile and water (containing 0.1% TFA) at a volume ratio of 2:3 was used as the mobile phase. A fluorescence detector (excitation wavelength: 370 nm, emission wavelength: 430 nm) and a PDA detector (190-800 nm) were used. The flow rate was set at 1.5 mL/min. Released CPT was quantified by the standard curve of free CPT.

GSH-induced degradation of $(CPT)_2$-Mal-PEG_{1k} NPs

(1) **Fluorescence and DLS measurements**: $(CPT)_2$-Mal-PEG_{1k} NPs in PBS (100 μg/mL, 2.5 mL) in a cuvette was incubated with or without the presence of GSH (5 mM) at 37°C (100 rpm). At selected time points, the solution was sent for fluorescence measurement on a fluorescence spectrometer. After the fluorescence measurement, the same solution was measured by DLS to determine the size and the count rate of NPs.

(2) **Determination of CPT release kinetics by HPLC**: $(CPT)_2$-Mal-PEG_{1k} NPs were dispersed in PBS (100 μg/mL, 2 mL, pH = 7.4) and transferred into a dialysis bag. The dialysis bag was then immersed in PBS (40 mL) with or without GSH (1 mM or 5 mM) and incubated at 37°C (100 rpm). At selected time points, 1 mL aliquot of the dialysis medium was withdrawn and brought to pH = 2 with phosphoric acid (85%, 10 μL) prior to HPLC measurement and the same amount of fresh medium was added. A mixture of acetonitrile and water (containing 0.1% TFA) at a volume ratio of 2:3 was used as the mobile phase for HPLC measurements. A fluorescence

detector (excitation wavelength: 370 nm, emission wavelength: 430 nm) and a PDA detector (190-800 nm) were used. The flow rate was set at 1.5 mL/min. Released CPT was quantified by the standard curve of free CPT.

MTT study. *In vitro* cytotoxicity of $(CPT)_2$-Mal-PEG_{1k} NPs and free CPT was measured by MTT assay. LS174T colon cancer cells were seeded in a 96-well plate at an initial density of 4×10^3 cells/well, allowed to attach for 24 h and treated with free CPT or NPs at various CPT concentrations for 48 h at 37°C. Cells without NP or free CPT treatment were used as control. Free CPT of various concentrations were first dissolved in DMSO and then diluted with a 10-fold volume of PBS prior to adding to the cells. The MTT assay was performed by following the standard procedures.

Influence of GSH-OEt on the cytotoxicity of $(CPT)_2$-Mal-PEG_{1k} NPs and free CPT against LS174T cells. LS174T cells were seeded in a 96-well plate with an initial density of 4×10^3 cells/well and allowed to attach for 24 h. GSH-OEt with a final concentration of 10 mM was added and incubated with the cells for 4 h. Then the medium was replaced with fresh medium, followed by addition of $(CPT)_2$-Mal-PEG_{1k} NPs or free CPT at various CPT concentrations. Cells were further incubated for 48 h. Cell viability was determined by the MTT assay.

TEM measurements. TEM samples were prepared on 200 mesh carbon film supported copper grids. One drop of the NP solution (~ 10 μL) (0.25-0.5 mg/mL) was placed on the grid and allowed to stand for 10 min. Filter paper was then used to remove the residual solution. The resulting sample was imaged using JEOL 2100 Cryo TEM at 200 kV.

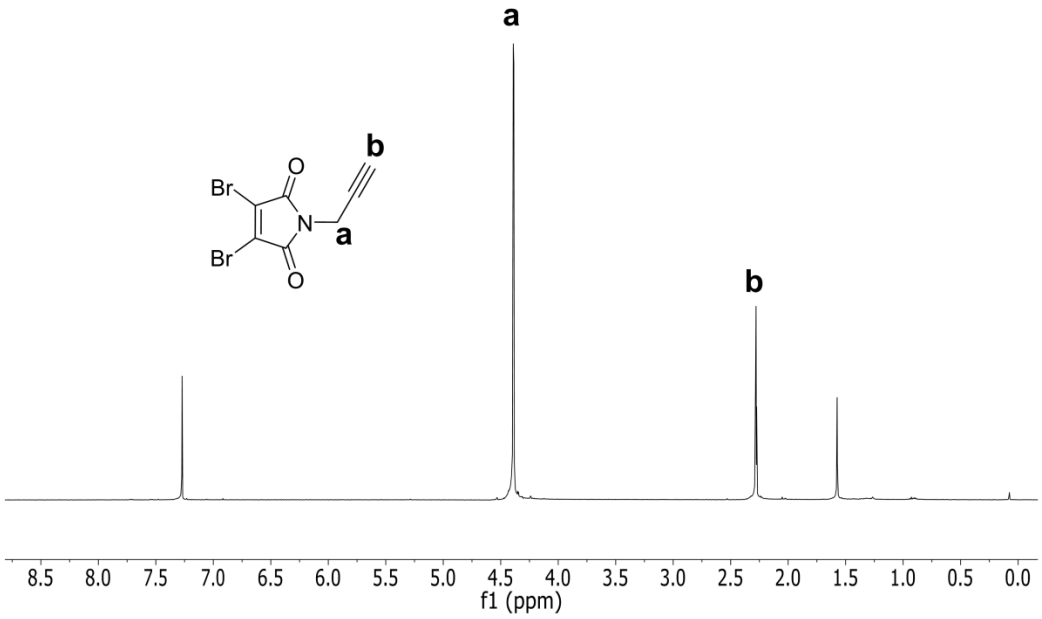


Figure S1. ^{1}H NMR spectrum of *N*-propargyl-2,3-dibromomaleimide in $CDCl_3$.

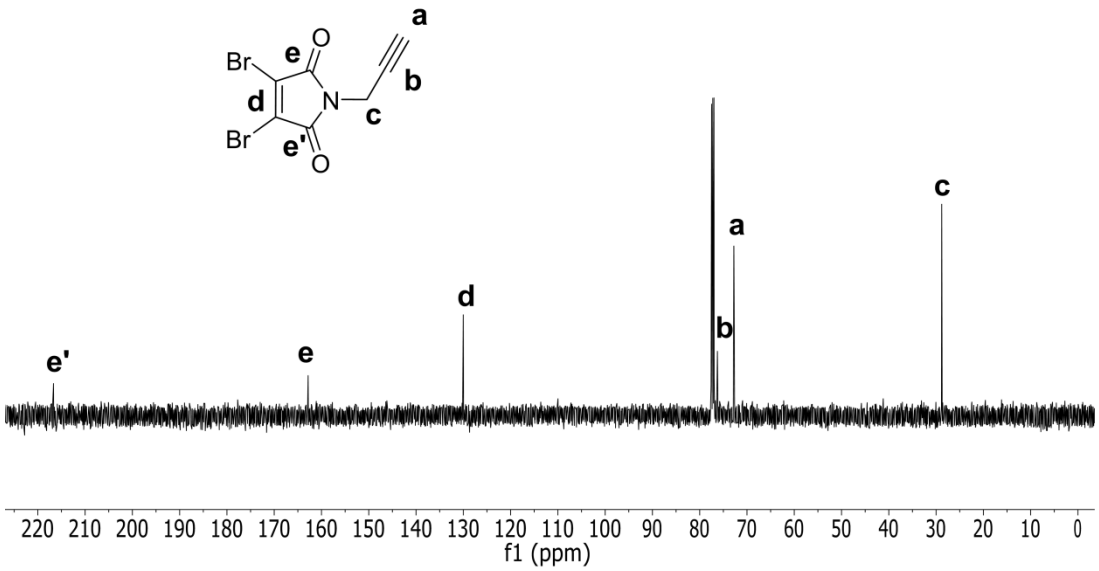


Figure S2. ^{13}C NMR spectrum of *N*-propargyl-2,3-dibromomaleimide in $CDCl_3$.

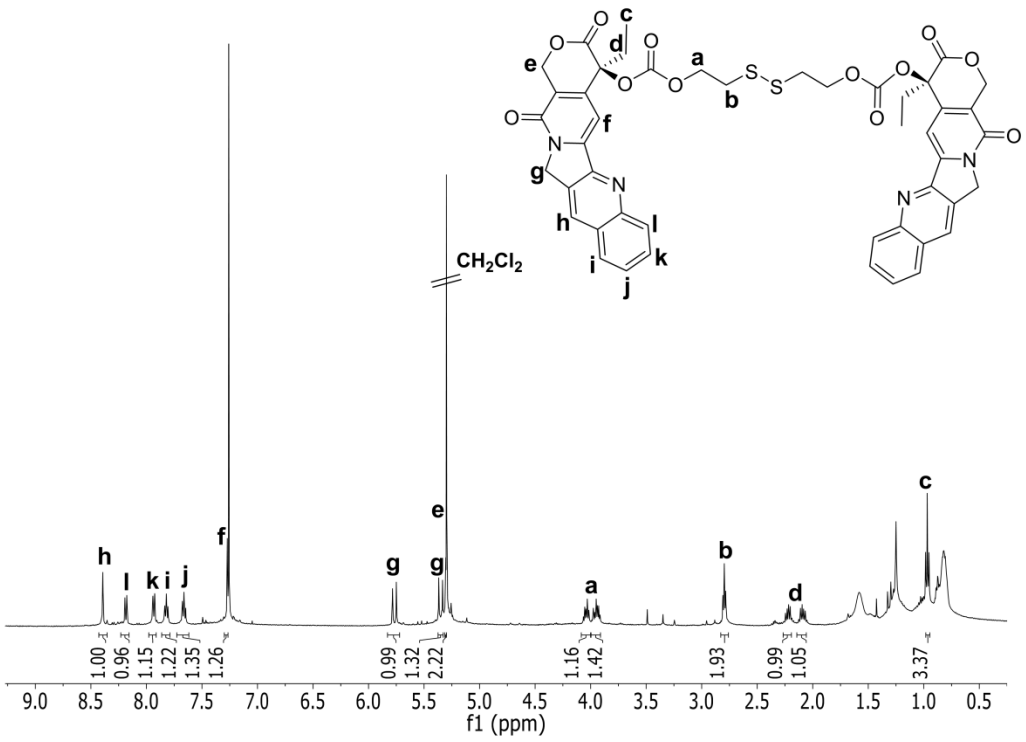


Figure S3. ^{1}H NMR spectrum of CPT-S-S-CPT in $CDCl_3$.

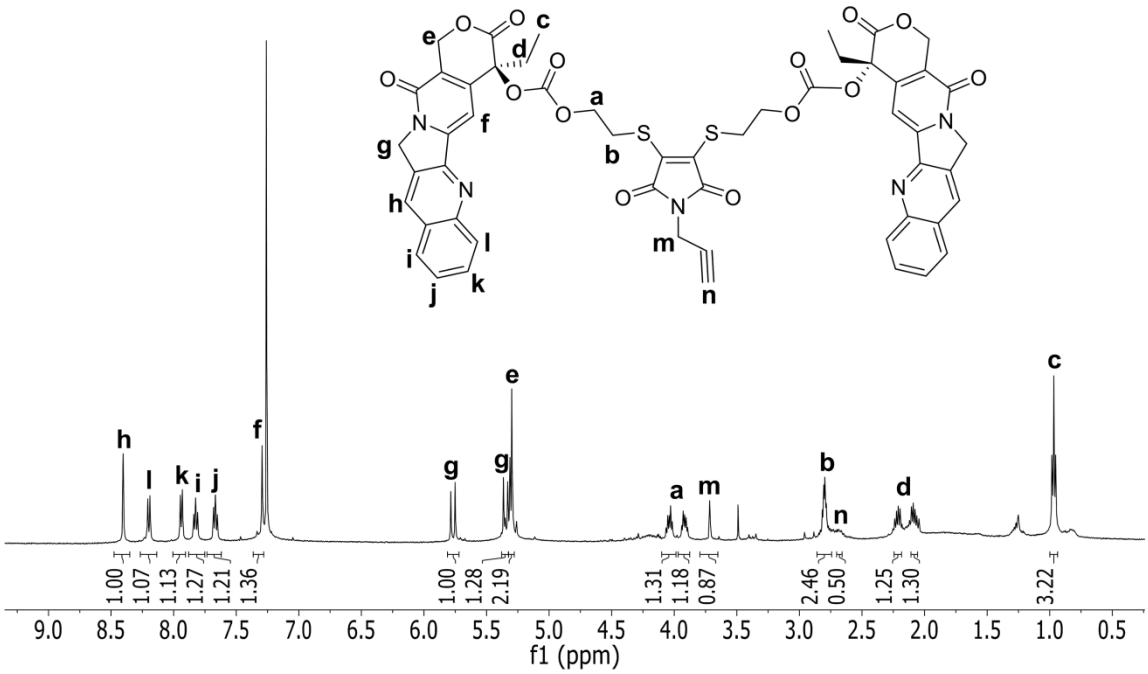


Figure S4. ^{1}H NMR spectrum of $(CPT)_2$-Mal-alkyne in $CDCl_3$.

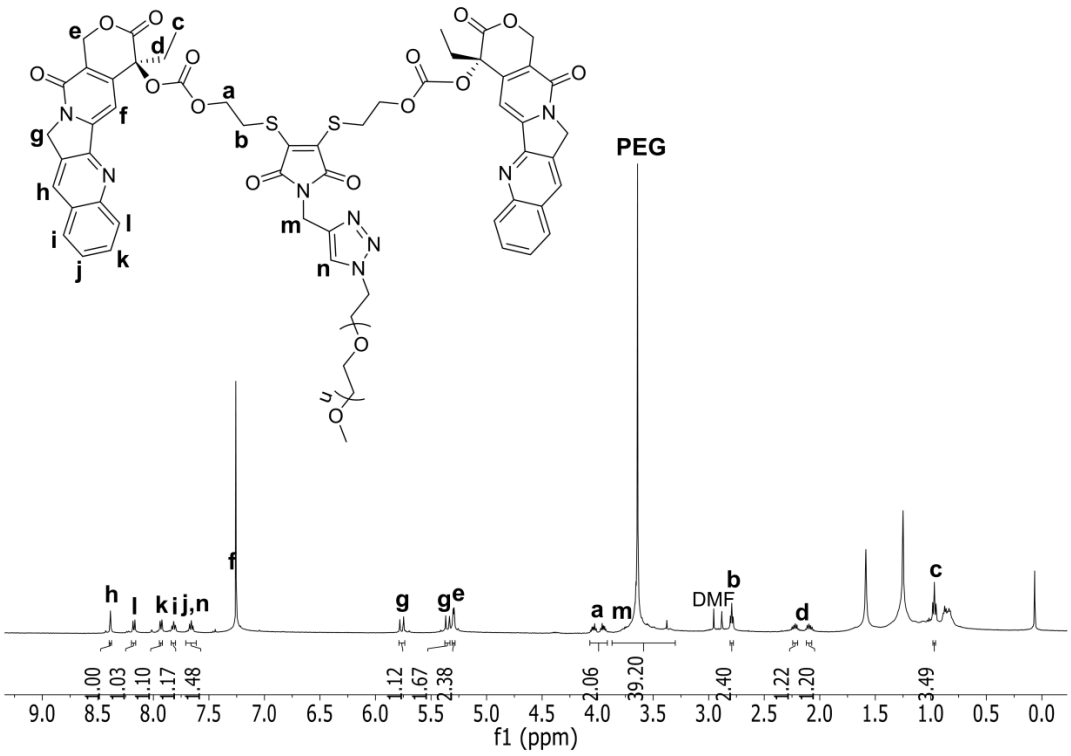


Figure S5. ^{1}H NMR spectrum of $(CPT)_2$-Mal-PEG_{1k} in $CDCl_3$.

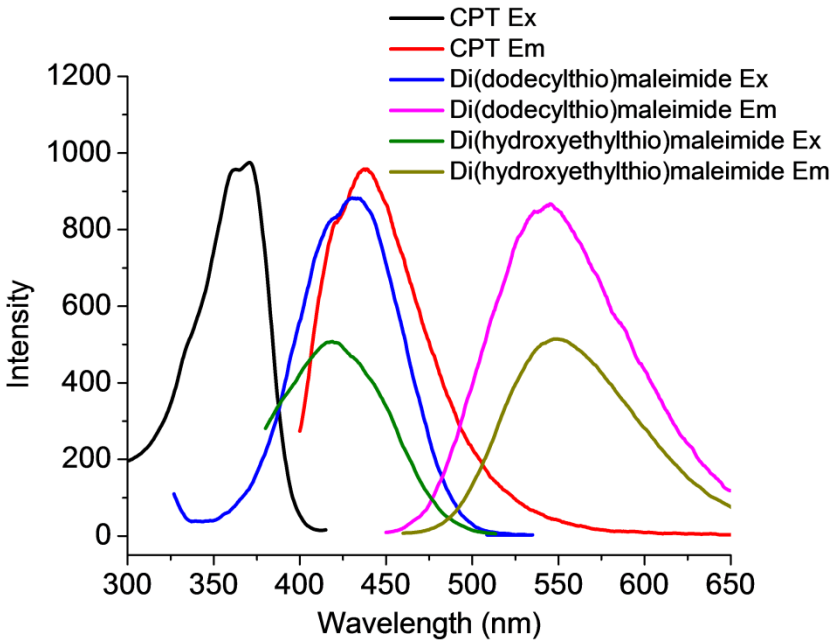


Figure S6. Fluorescence spectra of free CPT and two representative 2,3-dithiomaleimides: 2,3-di(dodecylthio)maleimide and 2,3-di(hydroxylethylthio)maleimide. Methanol was used as the solvent.

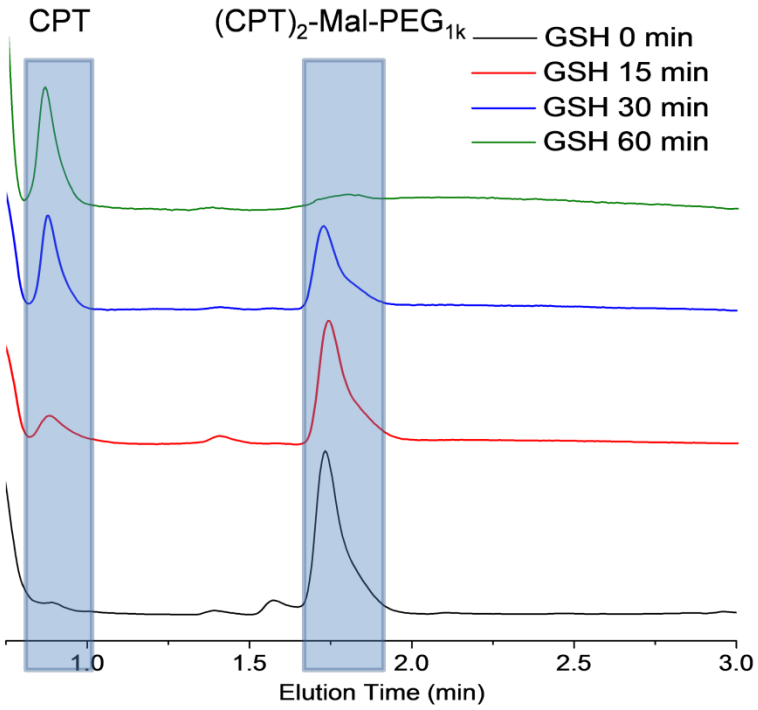


Figure S7. HPLC profiles of $(CPT)_2$-Mal-PEG_{1k} after treated with 5 mM GSH for 0, 15, 30, and 60 min, respectively. The absorbance wavelength was set at 370 nm.

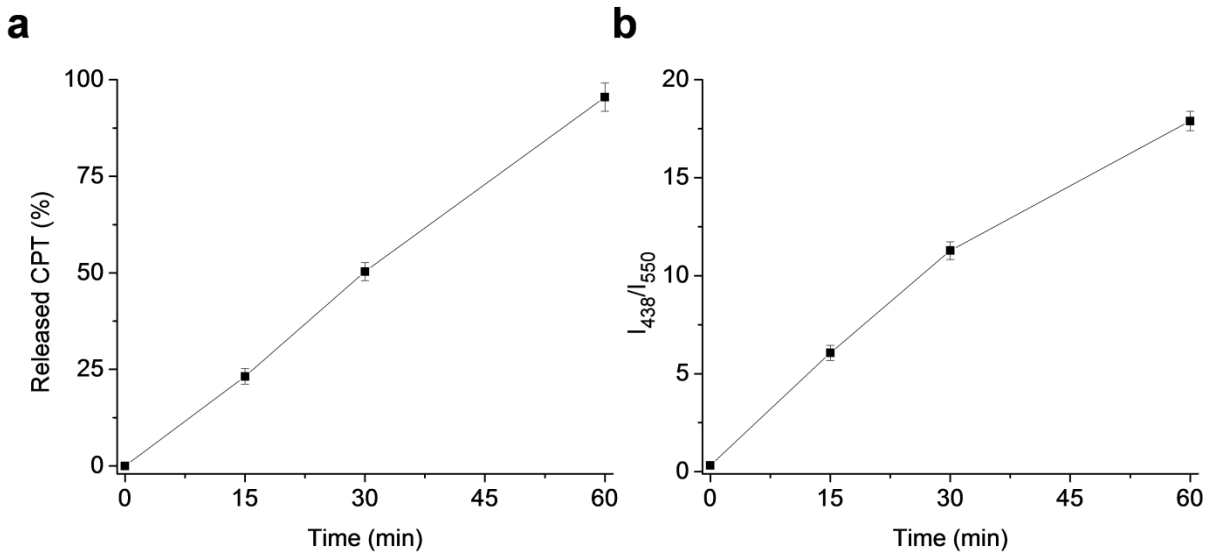


Figure S8. (a) CPT release profiles and (b) Increase of I_{438}/I_{550} of $(CPT)_2$-Mal-PEG_{1k} in methanol over time in the presence of 5mM GSH.

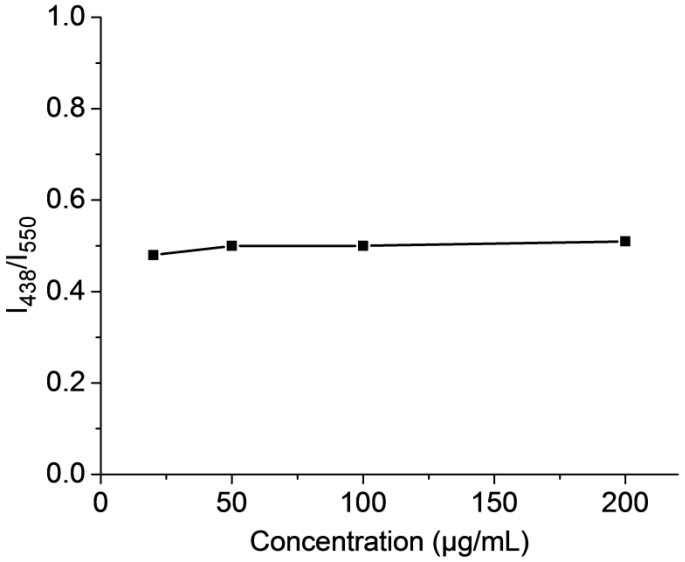


Figure S9. Change of I_{438}/I_{550} of CPT_2-Mal-PEG_{1k} NPs over concentration. CPT_2-Mal-PEG_{1k} NPs with different final concentrations were dispersed in PBS for fluorescence measurements.

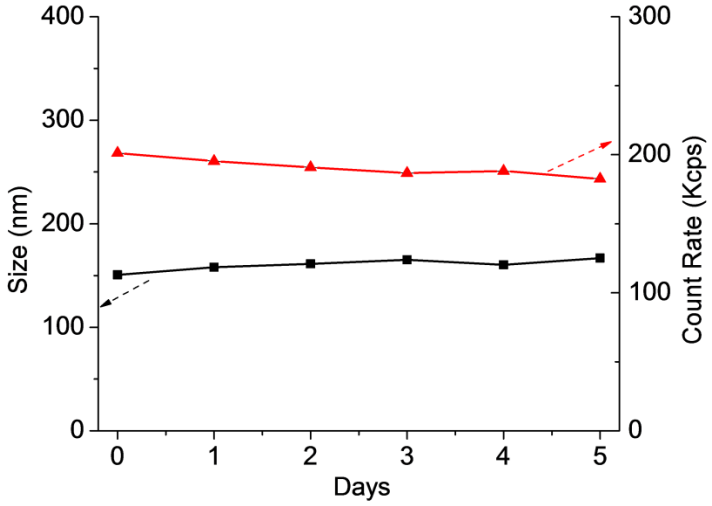


Figure S10. Changes of size (black line) and count rate (red line) of $(CPT)_2$-Mal-PEG_{1k} NPs over time in the presence of 5 mM GSH.

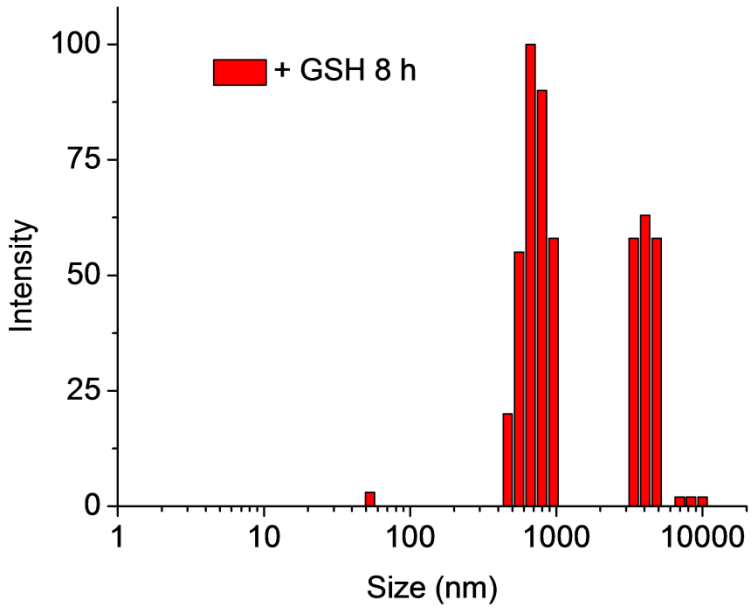


Figure S11. DLS characterization of $(CPT)_2$-Mal-PEG_{1k} NPs after treated with 5 mM GSH for 8 h.

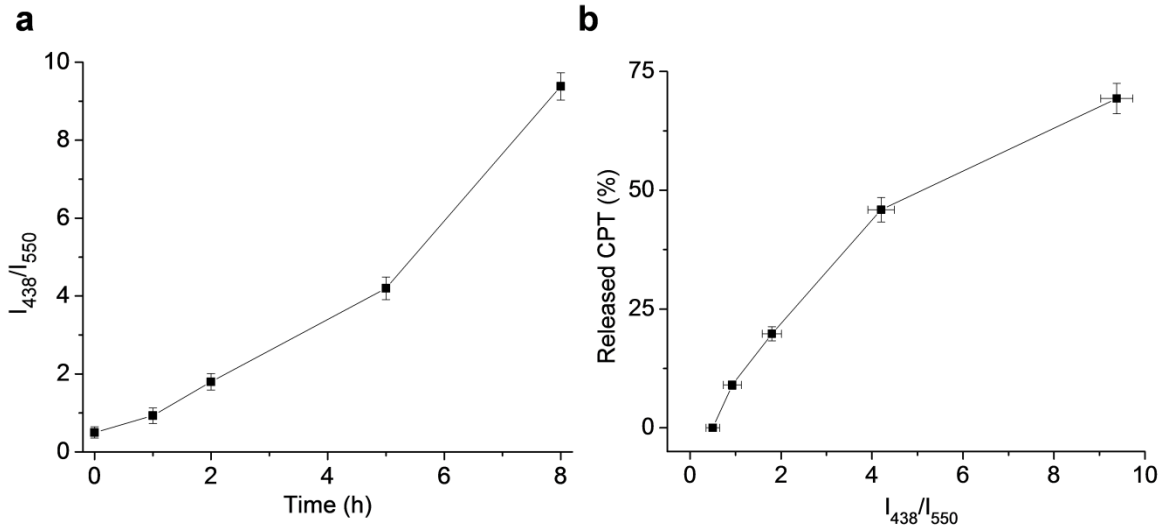


Figure S12. (a) Increase of I_{438}/I_{550} of $(CPT)_2$-Mal-PEG_{1k} NPs over time in the presence of 5 mM GSH. (b) Correlation of I_{438}/I_{550} to the percentage of released CPT from $(CPT)_2$-Mal-PEG_{1k} NPs in the presence of 5 mM GSH.

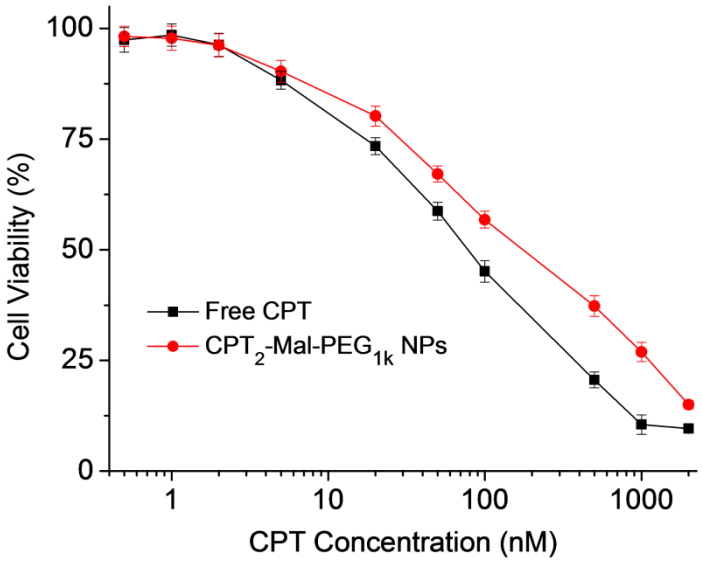
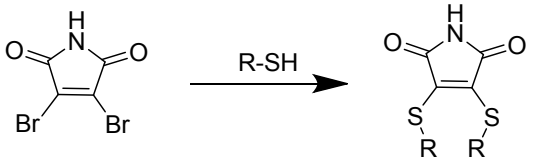


Figure S13. Viability of LS174T colon cancer cells after treated with free CPT or $(CPT)_2$-Mal-PEG_{1k} NPs at various CPT concentrations for 48 h.

Table S1. Maximum Excitation and Emission Wavelengths of Dithiomaleimides in Methanol.



R-SH	Maximum Excitation Wavelength (nm)	Maximum Emission Wavelength (nm)
$CH_3(CH_2)_{11}$-SH	465	545
HS-CH_2CH_2-OH	450	540
HS-CH_2CH_2-COOH	430	550
DTT	420	520
GSH	440	520
Phenyl-SH	415	520