Electronic Supplementary Information for：

Synergistic Chemo-Photo Anticancer Therapy by Reversible Diels-Alder Dynamic Covalent Bond Mediated Polyprodrug Amphiphiles and Immunoactivation Investigation

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Experimental Section

Instruments

The ^{1}H nuclear magnetic resonance (NMR) spectra were recorded using a Bruker 600 MHz spectrometer operated in the Fourier Transform mode. Chemical shifts are reported in delta (δ) units and expressed in parts per million (ppm) downfield from tetramethylsilane using the residual proton solvent as an internal standard. Molecular weights and molecular weight distributions were determined using a size exclusion chromatogram (SEC) equipped with a Waters 1515 pump and a Waters 2414 differential refractive index detector (set at 40 °C). A series of three linear Styragel columns (HR0.5, HR2, and HR4; 3.6 × 300 mm) was used at a temperature of 40 °C. The eluent used was THF at a flow rate of 0.3 mL/min. FT-IR spectra were recorded on Perkin-Elmer Spectrum BX FT-IR system using KBr pellets at 25 ℃. UV-vis spectra were performed on UNIC 4802 UV/vis double beam spectrophotometers, quartz cells with 1.0 mm lengths were used in UV-vis measurements. Fluorescence spectra were recorded using a RF-5301/PC (Shimadzu) spectrofluorometer. Dynamic light scattering (DLS) measurements were carried on a Nano-ZS90 Zetasizer of Malvern (UK) instrument, all data were averaged over three times measurements. Transmission electron microscopy (TEM) observations were conducted on a JEM-2100F electron microscope operating at an acceleration voltage of 100 kV. The samples for TEM observation were prepared by casting the corresponding solutions of polymers onto copper mesh grids and drying in air at room temperature. All animal experiments were approved by the Local Ethics Committee for Animal Care and Use at Anhui Medical University, the number LLSC20210651 and the protocols were carried out in accordance with the approved guideline.

Materials

2-(Isocyanatomethyl)furan, methoxypolyethylene glycol (mPEG) (M_n = 2000 Da), tosyl chloride (TsCl), sodium azide (NaN_3), triphosgene, maleic anhydride, propargylamine, bis(2-hydroxyethyl) disulfide (BHD), 2-bromoisobutyryl bromide, camptothecin (CPT), 4-dimethyaminopyridine (DMAP), cuprous bromide (CuBr), and *N,N,N′,N″,N″*-pentamethyl diethylenetriamine (PMDETA) were purchased from

Aladdin and used as received without further purification. Annexin V-FITC apoptosis detection kit was purchased from Shanghai Beyotime Biotechnology Co., Ltd. Cell viability (live dead cell staining) assay kit was purchased from Jiangsu KeyGEN Biotechnology Co., Ltd. Compound 1 was synthesized according to previously reported literature.[1] All solvents were obtained from Sinopharm. Co. Ltd. and purified by the standard procedures before use. Tetrahydrofuran (THF) was further dried over sodium benzophenone ketyl and distilled onto $LiAlH_4$ under nitrogen just before use. Dichloromethane (DCM), chloroform ($CHCl_3$) and dimethylformamide (DMF) were distilled over CaH_2. Water was deionized with a Milli-Q SP reagent water system (Millipore) to a specific resistivity of 18.0 MΩ cm.

Methods

Synthesis of FPBA. Typically, into a 100 mL round-bottom flask, 2-(isocyanatomethyl)furan (1.95 g, 15.84 mmol), compound 1 (3.45 g, 15.75 mmol), anhydrous TEA (0.08 g, 0.8 mmol), and anhydrous THF (50 mL) were charged. The mixture was protected under N_2 atmosphere, and then the reaction was stirred at 50 °C overnight. After the solvent was removed by evaporation under reduced pressure, the crude products were purified by column chromatography with petroleum ether/ethyl acetate (v/v = 3/1) as eluent, affording FPBA (3.24 g, yield: 60%) as a yellow oil.

Synthesis of PEG-OTS. This compound was prepared according to previously reported literature.[2] Typically, into a 100 mL round-bottom flask, mPEG (10.0 g, 5.0 mmol), TEA (1.5 g, 15.0 mmol), and anhydrous DCM (25 mL) were charged. The mixture was protected under N_2 atmosphere and stirred in ice bath for 10 min. Then TsCl (2.86 g, 15.0 mmol) was dissolved in 8 mL DCM and added into the mixture slowly in ice bath for another 20 min. After that, the mixture was stirred at room temperature for another 24h. The dichloromethane solution was washed successively by water (50 mL × 2), brine (50 mL × 2), 1 M HCL (50 mL × 1), saturated sodium bicarbonate solution (50 mL × 1), water (50 mL × 2) and dried over anhydrous magnesium sulfate, and filtered. The crude products were precipitated with cold ether for three times and dried in a vacuum oven. PEG-OTS (9.86 g, yield: 90%; end group substitution efficiency was 99.9%) was obtained as a white powder.

Synthesis of PEG-N_3. This compound was prepared according to previously reported literature.[2] PEG-OTS (4.0 g, 1.83 mmol), NaN_3 (1.19 g, 18.3 mmol), and DMF (20 mL) were charged into a 50 mL round-bottom flask. The reaction was stirred at room temperature for 24h. Then DMF was removed *via* vacuum evaporation, and 15 mL toluene was added with subsequently filtration to remove undissolved solid. Then toluene solution was removed *via* vacuum evaporation. The crude products were precipitated with cold ether for three times and dried in a vacuum oven**.** PEG-N_3 (3.45 g, yield: 92.5%; end group substitution efficiency was 99.9%) was obtained as a white powder.

Synthesis of Fu(-COOH)-PEG. This compound was synthesized *via* copper(I) catalyzed azide-alkyne cycloaddition click reaction. Typically, FPBA (0.34 g, 1.0 mmol), PEG-N_3 (1.0 g, 0.49 mmol), PMDETA (0.33 g, 1.94 mmol), and anhydrous DMF were charged into a glass ampoule equipped with a magnetic stirring bar. The mixture was degassed by three freeze-pump-thaw cycles, and then CuBr (0.21 g, 1.46 mmol) was introduced under nitrogen atmosphere. The reaction was allowed to stir for 24 h at 35 ℃ and afterwards terminated by exposition to air. The solution was then passed through neutral alumina column using DCM as the eluent to remove copper catalysts. After removing most of the solvents on a rotary evaporator, the crude products were precipitated with cold ether for three times and dried in a vacuum oven**.** Fu(-COOH)-PEG (1.12 g, yield: 83%) was obtained as a white solid.

Synthesis of Compound 2. This compound was prepared according to previously reported literature.[3] Typically, into a 500 mL round-bottom flask, BHD (2.0 g, 12.97 mmol), triethylamine (1.09 g, 10.8 mmol), and anhydrous THF (60 mL) were charged. The mixture was stirred in an ice bath for 10 min. Then, 2-bromoisobutyryl bromide (3.97 g, 17.27 mmol) was dissolved in dry THF (80 mL) and added dropwise, and then the mixture was stirred at room temperature for another 24 h. After removing triethylamine hydrochloride by filtration, the solution was concentrated under reduced pressure. The crude product was further purified by silica gel column chromatography using petroleum ether/ethyl acetate (v/v = 3/1) as eluent. After removing all the solvents, Compound 2 (1.57 g, yield: 40%) was obtained as a transparent viscous liquid.

Synthesis of Compound 3. This compound was prepared according to previously reported literature.[3] Typically, compound 2 (1.0 g, 3.3 mmol) and NaN_3 (1.07 g, 16.5 mmol) were added into a reaction flask containing 10 mL DMF, and then the reaction was stirred at 40 ℃ for 48 h. Unreacted NaN_3 was removed by filtration. Solvent was evaporated by a rotatory evaporator. The crude product was purified by silica gel column chromatography using petroleum ether/ethyl acetate (v/v = 3/1) as eluent. After removing all the solvents, Compound 3 (0.56 g, yield: 64%) was obtained as a transparent viscous liquid.

Synthesis of CPT-N_3. This compound was prepared according to previously reported literature.[3] Firstly, into a 100 mL round-bottom flask, CPT (0.39 g, 1.12 mmol), DMAP (0.41 g, 3.36 mmol), and 50 mL dry DCM were charged. The mixture was stirred in ice bath for 10 min. Triphosgene (0.17 g, 0.57 mmol) was dissolved in dry DCM (10 mL) and added slowly to the mixture in an ice bath for another 15 min. After addition, the reaction was stirred at room temperature for another 2 hours. Then compound 3 (0.45 g, 1.70 mmol) was dissolved in dry THF (5 mL) and added dropwise. The reaction mixture was further performed at room temperature overnight. After filtrati$_o$n, the reaction mixture was extracted with 0.1 M HCl (50 mL × 3), saturated brine (50 mL × 3), and deionized water (50 mL × 3), respectively. The organic phase was collected and concentrated under reduced pressure. The crude product was purified by silica gel column chromatography using dichloromethane/ethyl acetate (v/v = 4/1) as eluent. After removing all the solvents, CPT-N_3 (0.36 g, yield: 50%) was obtained as a pale-yellow powder.

Synthesis of Compound 4 and Compound 5. This compound was prepared according to previously reported literature.[4] Into a 100 mL round-bottom flask, maleic anhydride (1.78 g, 18.2 mmol), and 17 mL dry acetone were charged. The mixture was stirred at 60 ℃ until the solid was dissolved. Propargylamine (1.0 g, 18.2 mmol) was dissolved in dry acetone (10 mL) and added slowly to the mixture for 10 min, and then mixture was stirred for another 1h. After removing all the solvents, the mid product was recrystallized from ether/methanol (v/v = 4:1) for three times. Compound 4 (2.16 g,

yield: 78%) was obtained as a white crystal after filtration. Into another round-bottom flask, the mid product (2.0 g, 13.3 mmol) and xylene (70 mL) were charged. The mixture was stirred at 145 ℃ overnight. Filtrating to remove solid by-products, after removing all the solvents, the crude product was purified by silica gel column chromatography using petroleum ether/ethyl acetate (v/v = 6/1) as eluent. Compound 5 (0.19 g, yield: 10%) was obtained as a yellow solid after drying.

Synthesis of MI(-SS-)-CPT. Typically, into a 10 mL round-bottom flask, CPT-N_3 (1.0 g, 1.56 mmol), compound 5 (0.42 g, 3.11 mmol), PMDETA (0.14 g, 0.80 mmol), and anhydrous $CHCl_3$ (7 mL) were charged. The mixture was degassed by three freeze-pump-thaw cycles, and then CuBr (0.11 g, 0.77 mmol) was introduced under nitrogen atmosphere. The reaction was allowed to stir for 24 h at 35 ℃ and afterwards terminated by exposure to air. The solution was then passed through neutral alumina column using DCM as the eluent to remove copper catalysts under nitrogen atmosphere. The crude product was purified by silica gel column chromatography using dichloromethane/ethyl acetate (v/v = 1/6) as eluent. After removing all the solvents, MI(-SS-)-CPT (0.65 g, yield: 46%) was obtained as a pale-yellow powder.

Synthesis of PEG(-COOH)Fu/MI(-SS-)CPT. Typically, MI(-SS-)-CPT (0.51g, 0.66 mmol), Fu(-COOH)-PEG (0.53 g, 0.22 mmol) and 3 mL anhydrous $CHCl_3$ were charged into 10 mL round-bottomed flask. The mixture was reacted at 60 ℃ for 24 h under nitrogen atmosphere. After removing all the solvents, the crude products were precipitated with cold ether for three times. PEG(-COOH)Fu/MI(-SS-)CPT (0.62 g, yield: 89%) was obtained as white solid.

Preparation of blank NPs and IR780@NPs Micelles. Polymeric micelles blank NPs and IR780@NPs were prepared by a cosolvent approach. For preparation of IR780@NPs micelles, PEG(-COOH)Fu/MI(-SS-)CPT (12 mg) and IR780 (2mg) were dissolved in 1.0 mL of DMF. Under vigorous stirring, 9.0 mL of deionized water were added via a syringe pump at a flow rate of 0.1 mL/min. After the addition was completed, the dispersion was left stirring for another 4 h. DMF was then removed by dialysis (MWCO 3.5 kDa) against pure water for 24 h. Fresh water was replaced approximately every 6 h. The obtained dispersion with a characteristic of colloidal

aggregates did not exhibit any macroscopic phase separation upon standing at room temperature for more than one week, suggesting the formation of stable IR780@NPs aggregates. The final micellar dispersion was diluted with phosphate buffer solution (PBS; pH 7.4) for further use. Following the similar procedure, aqueous dispersion of blank NPs was also prepared and served as a control sample.

Photothermal Effect of nanoparticles. For photothermal efficiency measurements, 2.0 mL of polymeric micelles dispersion was charged into a cuvette, the temperature after being subjected to light (808 nm, 1.5 W cm^{-2}) irradiation was directly measured by thermometer probe. For comparison, pure PBS buffer was also tested as control group.

In Vitro Cargo Release Profile. The cargo release from polymeric micelles was recorded by UV spectrum.Briefly, the micelle dispersion was treated by DTT (5 mM) and 808 nm NIR light (1.5 W cm^{-2}) in turn or simultaneously according to the need. At different time intervals, the absorption of CPT was recorded.

Cell Culture and in Vitro Cytotoxicity Assessment. HeLa cells (5×10^3 cells/well) in Dulbecco's modified Eagle's medium (DMEM) complete medium were plated into a 96-well plate and incubated overnight. Then, cells were exposed to different micelles with different concentrations at 37 °C for up to 30 h in DMEM complete medium. Then, cells were rinsed with PBS buffer and DMEM complete medium. Cytotoxicity was assessed by adding 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) for another 4h. Cell injury was directly observed by calcein-AM and propidium idodide (PI) staining for 20 min. The live cells were stained with calcein-AM and dead cells were stained with PI. Cells incubated with blank polymeric micelles were served as positive control.

Cell Uptake. Confocal laser scanning microscopy (CLSM) was employed to investigate the cell uptake. HeLa cells were incubated with blank NPs and IR780@NPs for different times (1 h, 3 h, and 6 h) to allow uptake. HeLa cells were washed with PBS solution before fixing with 4% paraformaldehyde several times, and imaged by CLSM. The red channel was excited at 633 nm and collected between 650 and 700 nm. The blue channel was excited at 405 nm and collected between 410 and 500 nm.

In vivo therapy. All animal experiments were approved by the Local Ethics Committee for Animal Care and Use at Anhui Medical University, the number LLSC20210651 and the protocols were carried out in accordance with the approved guideline. 4T1 tumor model was established by subcutaneous injection of 4T1 cells (1×10^6) on the right back of hind leg positions of the Balb/c-nu mice (~ 20 g). Tumors were allowed to grow to ~ 150 mm^3 before experiment. The mice were divided into four groups and each group included five mice. Each group was injected with 50 μL sample, groups 4 were received an irradiation of 808 nm laser (1.5 W cm^{-2}) for 6 min after 6 h of injection. After various treatments, the lengths (L) and widths (W) of the tumors were measured by a digital caliper every 2 days. The tumor volume was defined as follows: $V= W^2 \times L/2$.

Detection of HMGB1 in Vitro. 4T1 cells were added to the 24-well plate overnight at a density of 3×10^5/ml on culture slides to detect HMGB1. The following micelles were used to expose cells to: PBS, blank NPs, IR780@ NPs (laser off), and IR780 @ NPs (laser on) for up to 2 h in RPMI complete medium. The chosen group was then exposed to an 808 nm laser (1.5 W cm^{-2}) under signal generator control for 6 min after a further 12 hours of incubation. These cells were then fixed in 4% paraformaldehyde and treated with HMGB1 Rabbit Polyclonal Antibody (1:200) (AF0180, Beyotime, Shanghai, China), an anti-rabbit IgG DyLight594-conjugated antibody (1:200). Utilizing DAPI, cell nuclei were stained. A negative fluorescence microscope (Leica, Wetzlar, Germany) was used to capture images of HMGB1.

Leukocyte isolation. The first step was mincing the tumor tissues with a razor blade and digesting them in DMEM at 37 °C for 1 hour with 1 mg/ml collagenase type 1 (Sigma, St. Louis, MO, USA) and 280 U/ml Deoxyribonuclease I (DNase I) (Worthington, Lakewood, NJ, USA). The filtered, 70-m-thick tissues were then pelleted. 40% Percoll (GE Healthcare, Chicago, IL, USA) was used to resuspend the cells before they were placed on top of 70% Percoll. Without stopping, samples were centrifuged for 30 minutes at 1,260 g and 25 °C. After centrifugation, the intermediate layer was collected and washed with 1× PBS used for flow cytometry analysis or sorting.

Flow cytometry assay. For the detection of cell apoptosis, cells were harvested by trypsinization without EDTA at 30 hours after stimulation, stained with FITC-Annexin V and propidium iodide (PI) using the FITC Annexin V Apoptosis Detection Kit I (BB-4101, BestBio), and subsequently examined using a flow cytometer (Beckman Coulter; Brea, CA, USA). Cells were first stained with a cell-viability dye (Biolegend, Biolegend, San Diego, CA, USA), and then with surface antibodies from Biolegend: FITC anti-mouse CD45.2 (clone: 104, Biolegend, San Diego, CA, USA), PE anti-mouse CD3 (clone: 145-2C11, Biolegend), and APC anti-mouse CD4 (clone: 145-2C11, Biolegen (clone:RMP4-5, Biolegend) Anti-mouse CD8a PerCP/Cyanine5.5 (clone:53-6.7, 100734; Biolegend) Pan-NK cells) anti-mouse CD49b PE/Cyanine7 (clone:DX5, 108922; Biolegend) Anti-mouse CD25 PerCP/Cyanine5.5 (clone:3C7, 101912; Biolegend). For intracellular staining of Foxp3, single-cell suspensions were stained with the aforementioned cell-surface antigen antibodies, fixed, and permeabilized using the Foxp3/Transcription Factor Labeling Buffer Set (00-5523-00; eBioscience™, San Diego, CA, USA), and then stained with PE anti-mouse Foxp3 (clone:MF-14; 126404; Biolegend). A flow cytometer (Beckman Coulter) was used to collect the data, and FlowJo software, version 10.6.2 (Tree Star; Ashland, OR, USA) was used to analyze it.

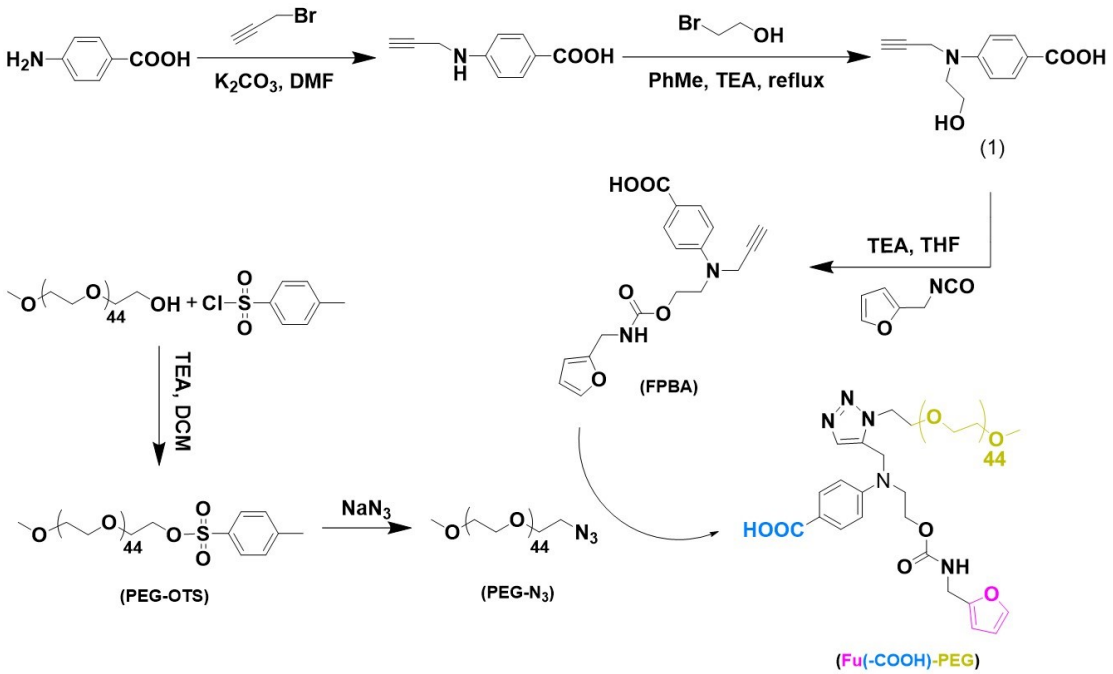
References

(1) Y. Chen, Z. H. Zhang, X. Han, J. Yin and Z. Q. Wu, *Macromolecules.* 2016, **49**, 7718.

(2) J. Li, Z. E. Hu, X. L. Yang, W. X. Wu, X. Xing, B. Gu, Y. H. Liu, N. Wang and X. Q. Yu, *Biomater. Sci.* 2019, **7**, 3277.

(3) J. Li, Z. E. Hu, X. L. Yang, W. X. Wu, X. Xing, B. Gu, Y. H. Liu, N. Wang and X. Q. Yu, *ACS Appl. Bio Mater.* 2020, **3**, 7382.

(4) E. W. McConnell, A. L. Smythers and L. M. Hick, *J. Am. Soc. Mass Spectrom.* 2020, **31**, 1697.



Scheme S1. Synthetic routes employed for the preparation of Fu(-COOH)-PEG.

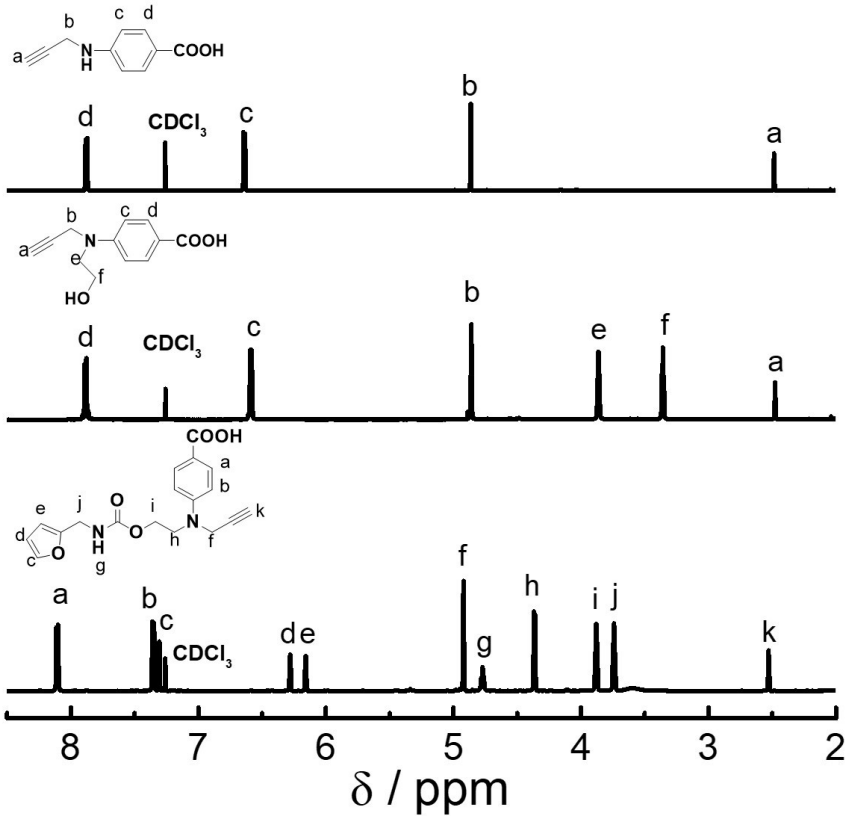


Figure S1. ^{1}H NMR spectra obtained for compound 1, FPBA, and intermediate product using $CDCl_3$ as solvent at 25 ℃.

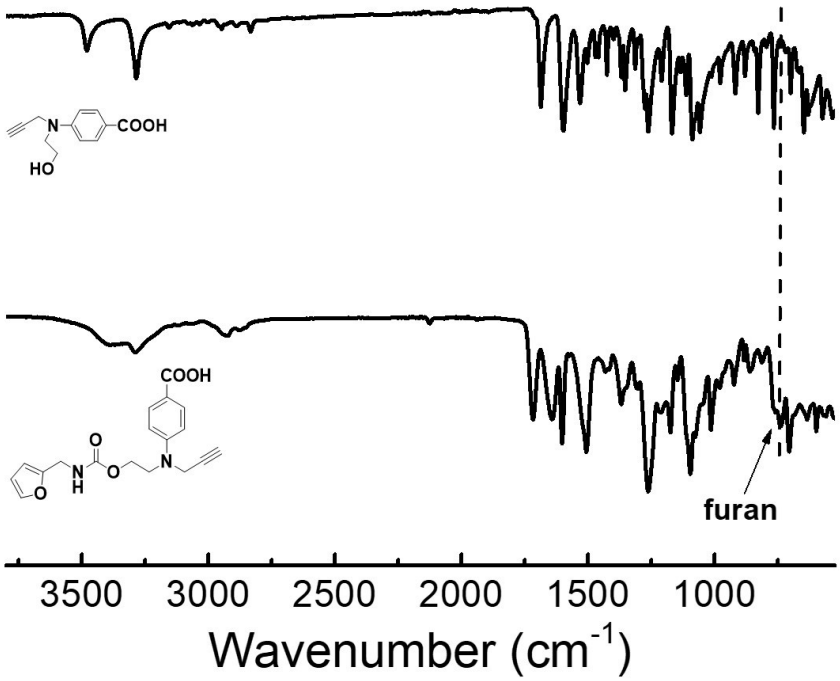


Figure S2. FT-IR spectra obtained for compound 1 and FPBA at 25 ℃ using KBr pellets.

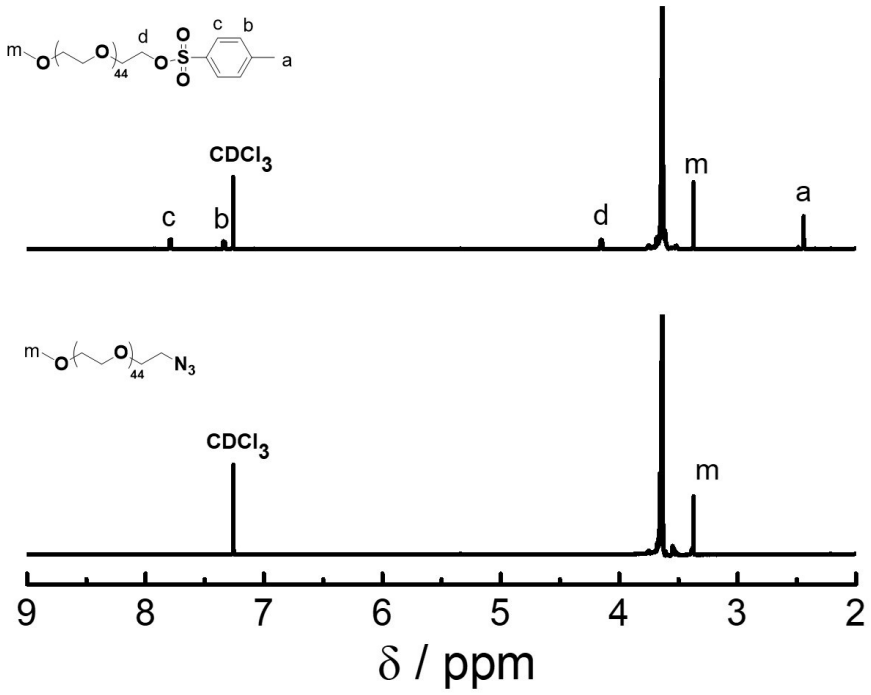


Figure S3. ^{1}H NMR spectra obtained for PEG-OTS and PEG-N_3 using $CDCl_3$ as solvent at 25 °C.

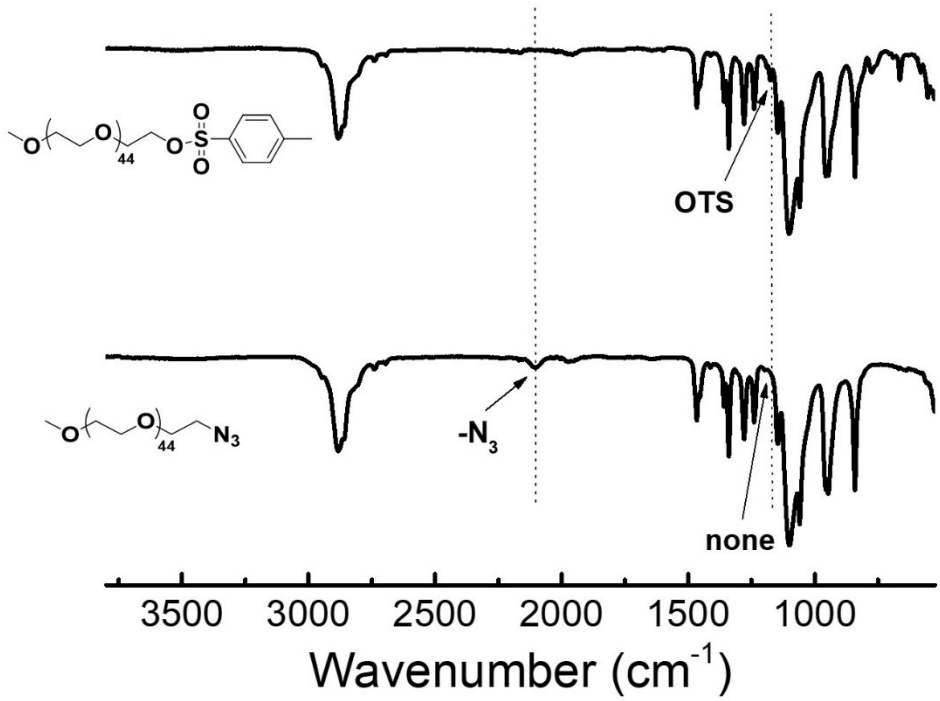
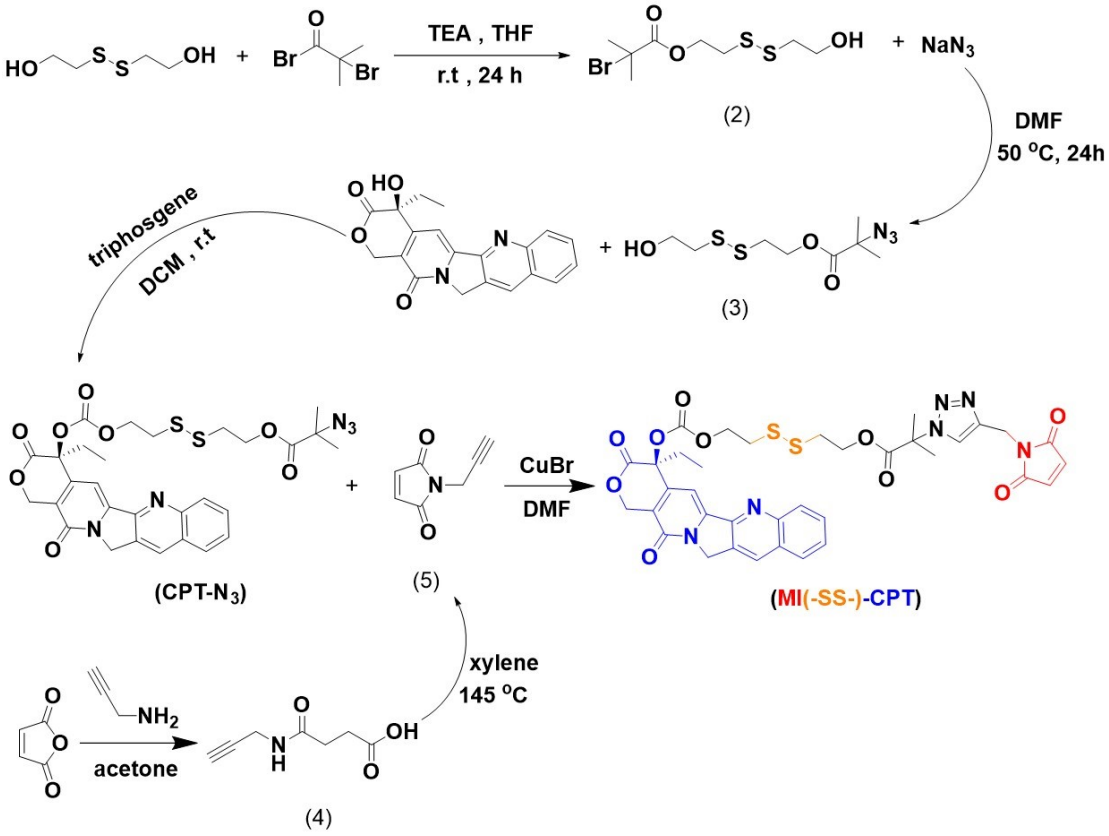


Figure S4. FT-IR spectra obtained for PEG-OTS and PEG-N_3 at 25 °C using KBr pellets.



Scheme S2. Synthetic routes employed for the preparation of MI(-SS-)-CPT.

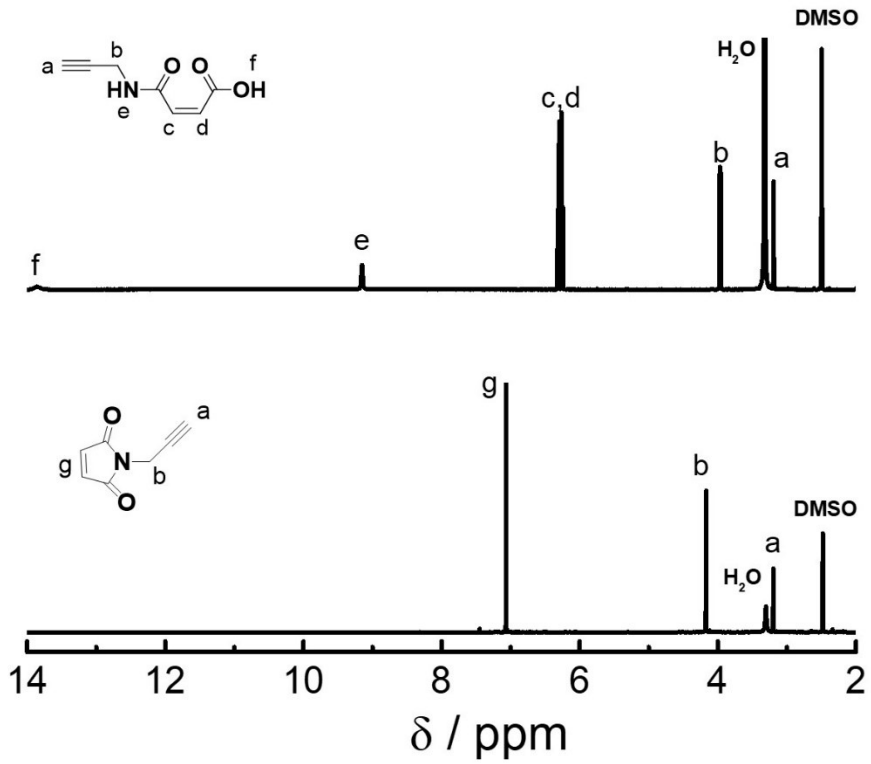


Figure S5. ^{1}H NMR spectra obtained for compound 4 and compound 5 using DMSO-d_6 as solvent at 25 ℃.

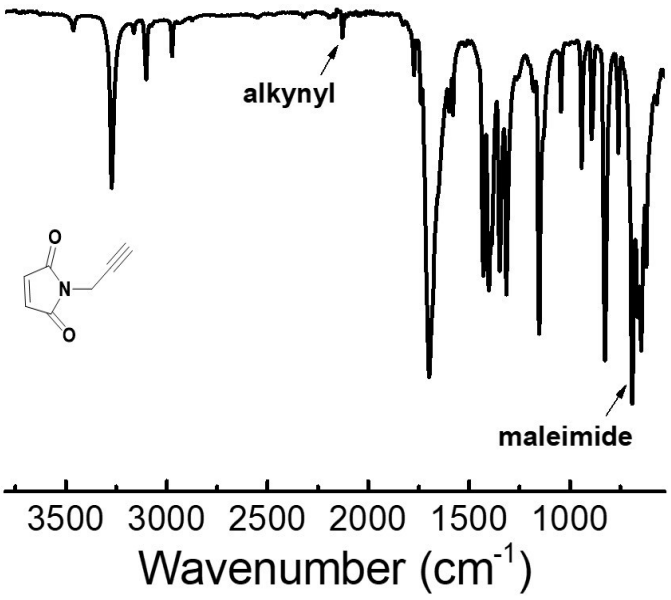


Figure S6. FT-IR spectrum obtained for compound 5 at 25 ℃ using KBr pellets.

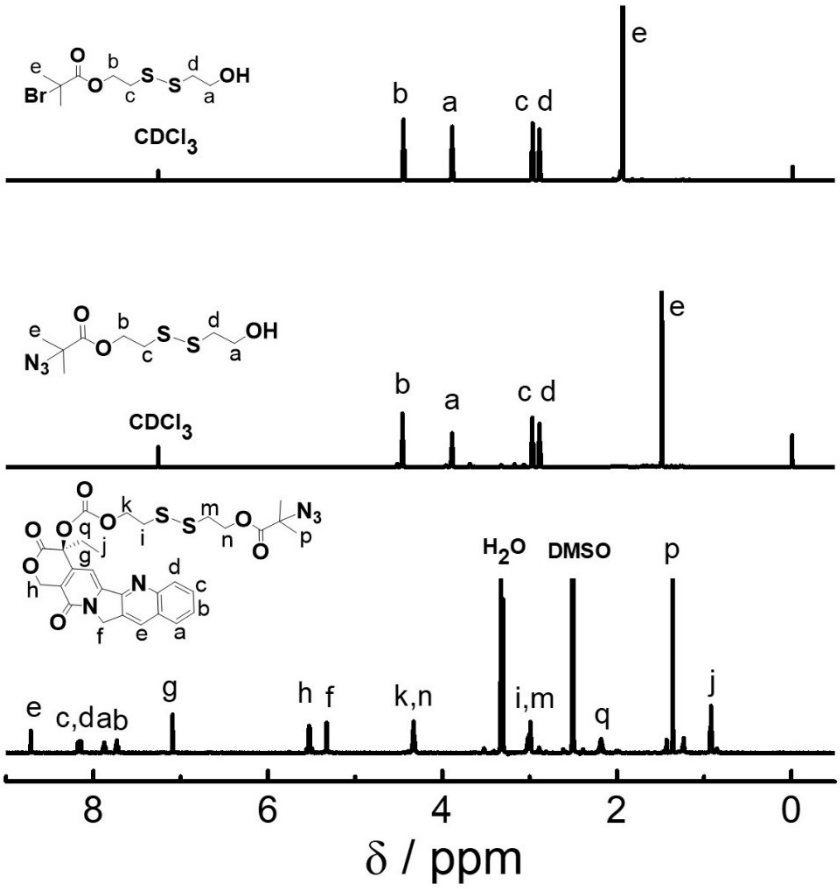


Figure S7. ^{1}H NMR spectra obtained for compound 2, compound 3, and CPT-N_3 using $CDCl_3$ or DMSO-d_6 as solvent at 25 ℃.

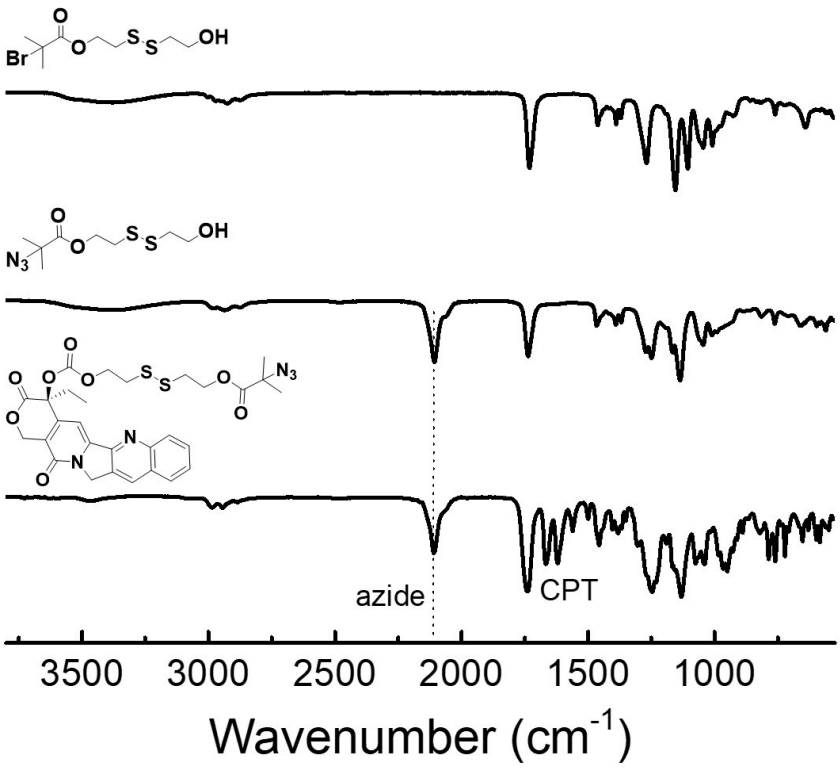


Figure S8. FT-IR spectra obtained for compound 2, compound 3, and CPT-N_3 at 25 ℃ using KBr pellets.

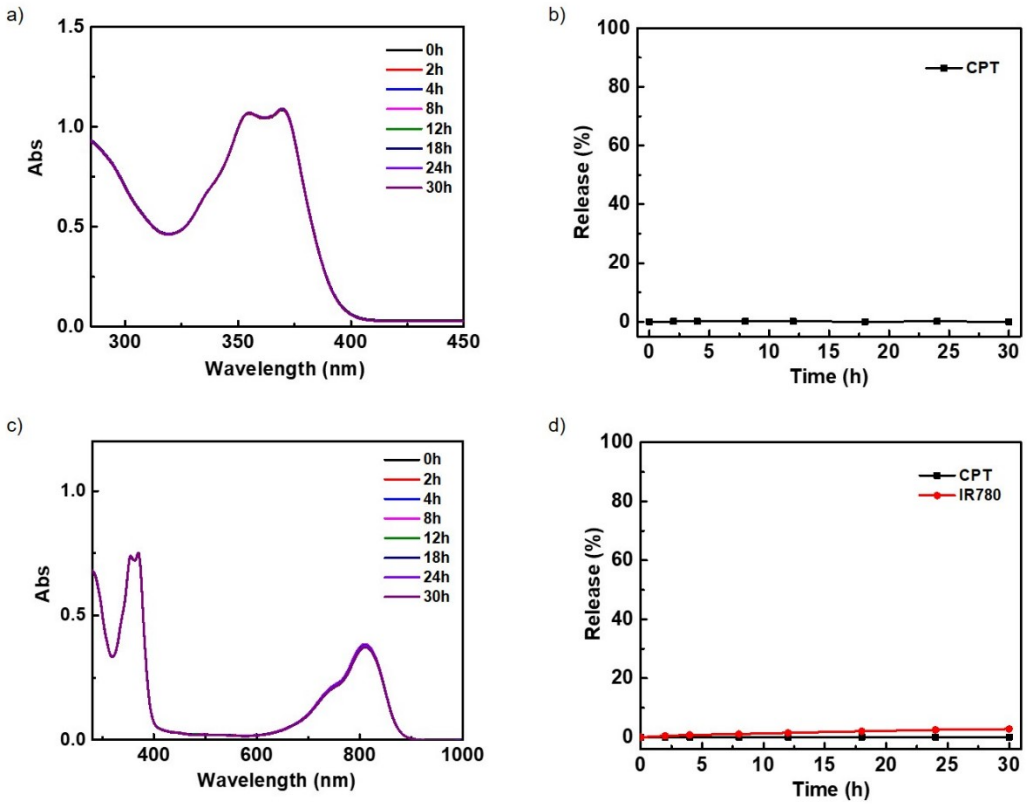


Figure S9. Time dependent UV-vis spectra recorded for (a) blank NPs and (c) IR780@NPs in mimicked physiological conditions (pH 7.4) without DTT. CPT release profile from blank NPs (b) and IR780@NPs (d) at different times.

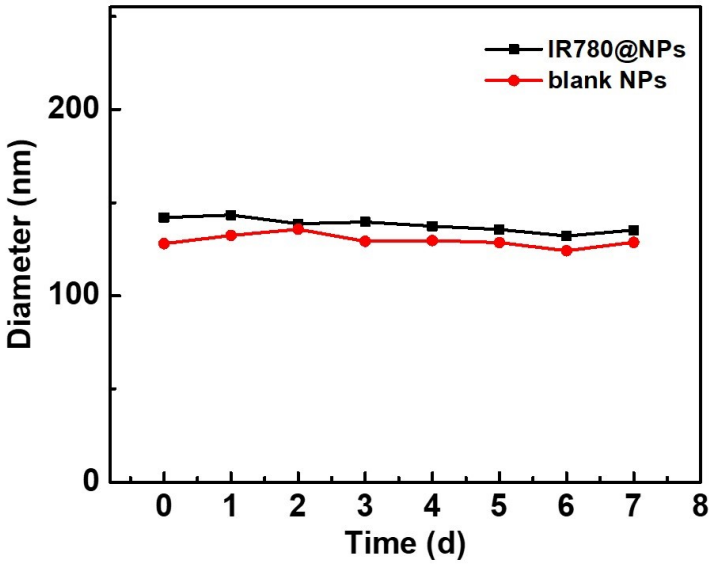


Figure S10. The time dependent size change of blank NPs and IR780@NPs in PBS buffer (pH 7.4) at 37 ℃.

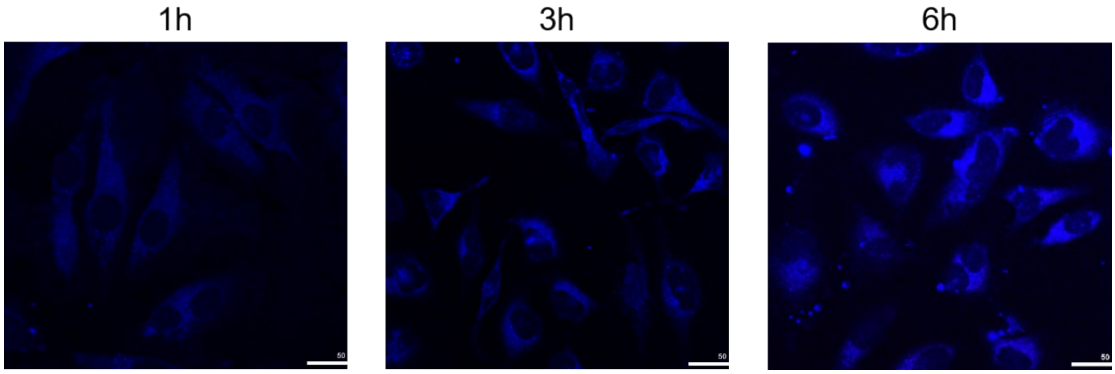


Figure S11. CLSM images of Hela cells after coincubation with blank NPs for different times (1-6 h). The scale bars correspond to 50 μm in images.