Supporting Information

Dual stimulus-triggered bioorthogonal nanosystem for spatiotemporally controlled prodrug activation and near-infrared fluorescence imaging

Experimental Section

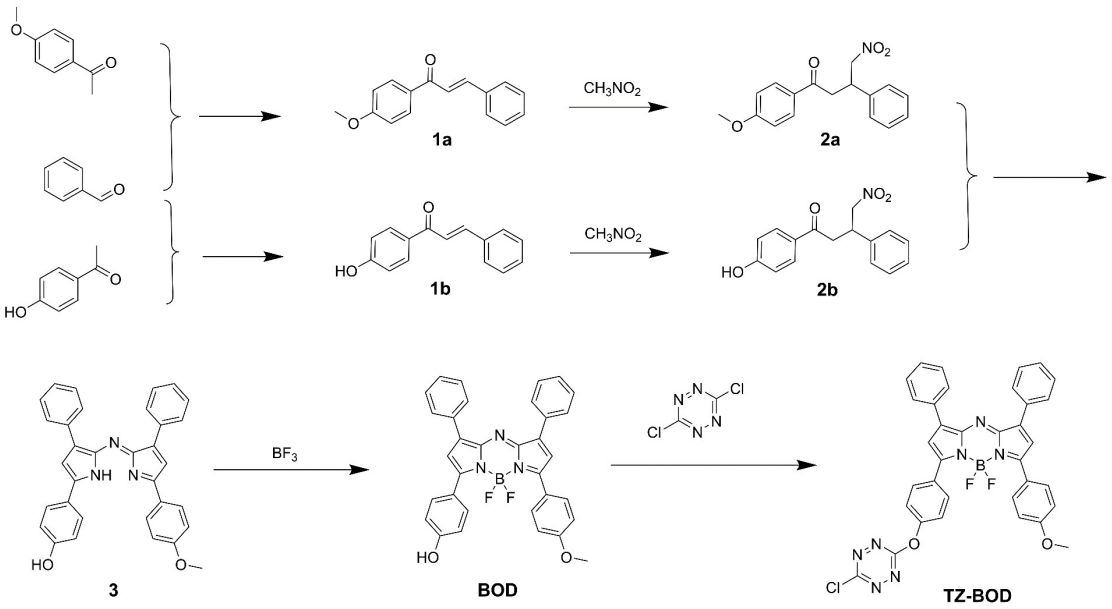
Materials and Methods

1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI), *N, N'*-dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS), 4-cyano-4-(thiobenzoylthio) pentanoic acid (CPDB), azodiisobutyronitrile (AIBN), *N, N*-diisopropylethylamine (DIPEA), 4-dimethylaminopyridine (DMAP) and trimethylamine (TEA) were purchased from Aladdin Industrial, Inc. (Shanghai, China). Dichloromethane (DCM), methanol (MeOH), n-hexane (Hex), ethyl acetate (EA), 1, 2-dimethoxyethane (DME), *N, N*-dimethylformamide (DMF), tetrahydrofuran (THF) and dimethyl sulfoxide (DMSO) were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). All other chemicals used in the experiments were purchased from Energy Chemical (Shanghai, China) and used without further purification. RPMI 1640, trypsin-EDTA and penicillin-streptomycin were obtained from Gibco BRL (Eggenstein, Germany). Fetal bovine serum (FBS) was obtained from ExCell Biology, Inc (Shanghai, China). Hoechst 33342 was purchased from Life Technologies. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich.

Characterization

Nuclear magnetic resonance (NMR) spectra were measured on a Bruker ARX 400 NMR spectrometer (Bruker, Billerica, MA). Deuterated chloroform or deuterated dimethyl sulfoxide (DMSO) was used as the solvent for NMR measurements. An external standard of a known amount of sodium benzenesulfonate was added when necessary. Particle size and size distribution were carried out in an aqueous solution using a Malvern ZS90 dynamic light scattering instrument (Malvern Instruments Ltd., England) with a He-Ne laser (633 nm) and 90° collecting optics. The data were analyzed using Malvern Dispersion Technology Software 7.0.0. The absorption spectra were measured on a UV-3802 (UNICO, Shanghai, China) spectrophotometer. Confocal images were acquired by confocal microscope (CLSM, Nikon Ti-E A1, Japan). The polymer dispersity index (PDI) was determined by gel permeation chromatography (GPC) measurements on a tetrahydrofuran GPC system. pH was measured by a Mettler Toledo FE 20K pH meter. Fluorescence (FL) spectra were measured on a Shimadzu RF-6000 spectrofluorometer (Shimadzu UV-2600, Japan). Absorbance and fluorescence intensity was measured by a multifunctional micropore detection board analysis system (Biotek Cytation5, BioTek, United States).

Synthesis of TZ-BOD



Scheme S1. Synthetic procedures of TZ-BOD.

Synthesis of 1a. 4'-Methoxyacetophenone (2.00 g, 13.30 mmol) and benzaldehyde (1.41 g, 13.30 mmol) were dissolved in absolute ethanol (10 mL). 10 mL of aqueous potassium hydroxide (KOH) solution (2.24 g, 39.90 mmol) was added dropwise at 0 ℃. The reaction was stirred for 12 h at room temperature. The resulting white precipitate was collected by filtration to afford product **1a** (3.20 g, 85 %).

Synthesis of 2a. A solution of 1a (2.00 g, 8.40 mmol), nitromethane (10.25 mL, 168.00 mmol) and KOH (0.09 g, 1.70 mmol) in ethanol (10 mL) was heated at 60 ºC under reflux for 12 h. After cooling to room temperature, the solvent was removed in a vacuum, and the oily residue was partitioned between EA (100 mL) and H_2O (50 mL). The organic layer was separated, dried over sodium sulfate, and evaporated under reduced pressure. The compound was concentrated to afford product **2a** as a white solid (2.50 g, 82%), which was used without further purification.

Synthesis of 1b. 4'-Hydroxyacetophenone (2.00 g, 14.70 mmol) and benzaldehyde (1.56 g, 14.70

mmol) were dissolved in absolute ethanol (10 mL). 10 mL of aqueous KOH solution (2.47 g, 44.1 mmol) was added dropwise at 0 ℃. The reaction was stirred for 12 h at room temperature. The solution/suspension was poured into 1 M HCl (10 mL) and further concentrated HCl was added until the solution was acidic (pH = 1.0). The resulting precipitate was collected by filtration to afford product **1b** as a yellow solid (2.70 g, 77%).

Synthesis of 2b. A solution of 1b (2.00 g, 8.90 mmol), nitromethane (11.04g, 178.00 mmol) and KOH (0.60 g, 10.68 mmol) in ethanol (10 mL) was heated at 60 ºC under reflux for 12 h. After cooling to room temperature, the solvent was removed in a vacuum, and the oily residue was partitioned between EA (100 mL) and H_2O (50 mL). The organic layer was separated, dried over sodium sulfate, and evaporated under reduced pressure. The compound was concentrated to afford product **2b** as a white solid (2.40 g, 73%), which was used without further purification.

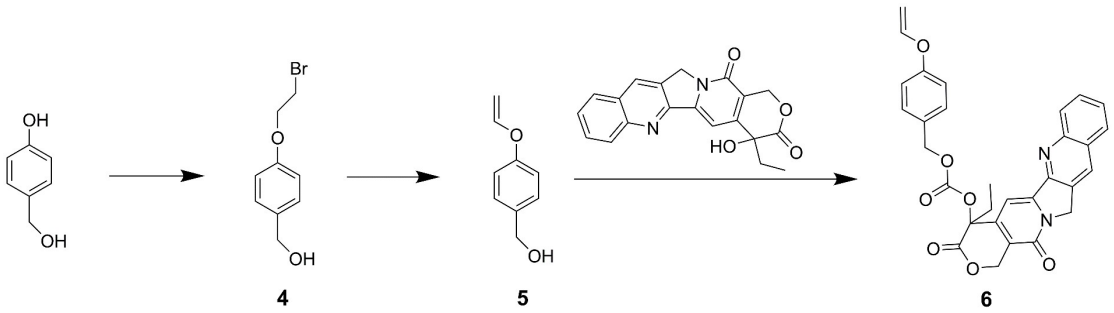
Synthesis of 3. Compound 2a (1.00 g, 3.30 mmol), compound 2b (0.94 g, 3.30 mmol) and ammonium acetate (8.89 g, 115.00 mmol) in butanol (50 mL) were heated to 120 ℃ under reflux for 24 h. The reaction was cooled to room temperature, concentrated under reduced pressure, suspended in brine, and extracted with DCM (3 × 100 mL). The combined organic layers were dried over Na_2SO_4 and purified by silica gel column chromatography (DCM: MeOH = 50: 1) to concentrated to afford product **3** as a blue-black solid (0.49 g, 30%).

Synthesis of BOD. Compound 3 (400 mg, 0.81 mmol) and DIPEA (1.58 mL, 8.1 mmol) were dissolved in dry DCM (50 mL) and then cooled to 0 °C in an ice bath and treated dropwise with boron trifluoride diethyl etherate (1.54 mL, 30 mmol). The reaction was stirred at room temperature for 24 h under a nitrogen atmosphere, quenched with H_2O (20 mL), and extracted with DCM (3 × 100 mL). The combined organic layers were concentrated and purified by silica gel column

chromatography (DCM: MeOH = 100: 1) to afford product **BOD** as a red metallic solid (198 mg, 45%).

Synthesis of TZ-BOD. Compound BOD (100 mg, 0.18 mmol) and 3,6-dichloro-1,2,4,5-tetrazine (30 mg, 0.20 mmol) were dissolved in dry DCM (20 mL) under N_2 and the solution cooled to 0 °C. DIPEA (48 μL, 0.27 mmol) was slowly added into the solution at 0 °C; then, the reaction was warmed to room temperature and stirred for an additional 1 h. The solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography (DCM: EA = 4: 1) to give the product **TZ-BOD** as a metallic red solid (71 mg, 60%).

Synthesis of VE-CPT



Scheme S2. Synthetic procedures of VE-CPT.

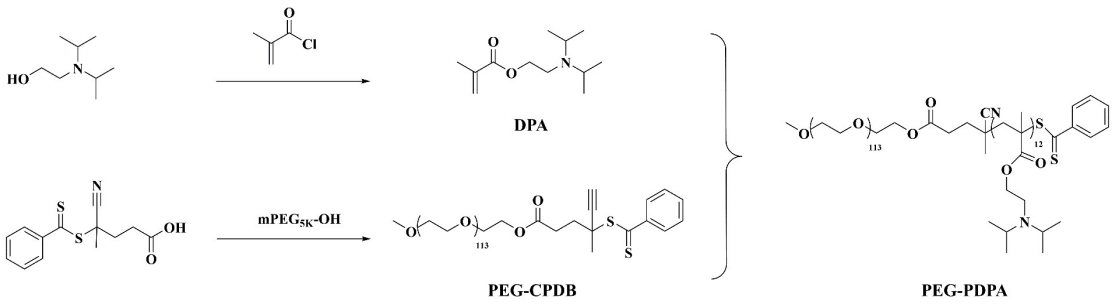
Synthesis of 4. 4-Hydroxybenzyl alcohol (2.50 g, 20.00 mmol) and K_2CO_3 (5.52 g, 40.00 mmol) were dissolved in 50 mL acetone. The mixture was stirred at room temperature for 30 min, and then 1,2-dibromoethane (8.7 mL, 100 mmol) was added under an N_2 atmosphere. The resultant mixture was refluxed at 58 ℃ for another 12 h. After completion of the reaction, the mixture was filtered, and the filtrate was concentrated by a rotary evaporator. The residue was purified by silica gel column chromatography (Hex: EA= 4: 1) to afford **4** as a white solid (3.30 g, 7%).

Synthesis of 5. Compound 4 (2.00 g, 8.66 mmol) and potassium tert-butoxide (6.35 g, 51.96 mmol) were dissolved in 100 mL anhydrous THF and refluxed at 70 ℃ for 4 h. After completion

of the reaction, the mixture solution was cooled to room temperature, and the solvent was evaporated in a vacuum. The residue was purified by silica gel column chromatography (Hex: EA = 5: 1) to obtain **5** as a yellow oil (0.73 g, 56%).

Synthesis of the prodrug 6 (VE-CPT). Camptothecin (522 mg, 1.50 mmol) and DMAP (275 mg, 2.25 mmol) were dissolved in anhydrous dichloromethane (30 mL), and a solution of triphosgene (223 mg, 0.75 mmol) in 5 mL dichloromethane was dropwise at 0 °C. The mixture was stirred at room temperature for 2 h. After removal of unreacted phosgene gas by flushing nitrogen gas, a solution of compound 5 (225 mg, 1.5 mmol) and DIPEA (260 μL, 1.5 mmol) in 5 mL dichloromethane was added to the mixture, which was then stirred for another 12 h at room temperature. After completion of the reaction, the solvent of the reaction mixture was removed in a vacuum. The residue was purified by silica gel column chromatography eluted with DCM to obtain the prodrug **6** as a white solid (393 mg, 50%).

Synthesis of PEG-PDPA



Scheme S3. Synthetic procedures of PEG-PDPA.

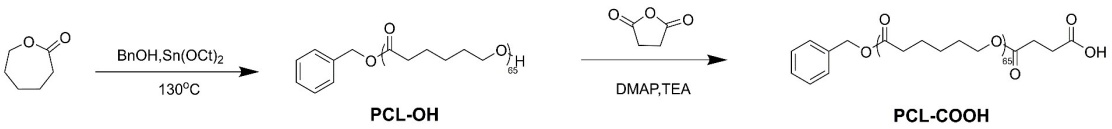
Synthesis of DPA. Diisopropyl ethanolamine (10 mL, 56.90 mmol) and TEA (12 mL, 85.30 mmol) were added into a 250 mL-round bottom containing 150 mL DCM, and then the mixture was stirred in an ice-water bath for 30 min. A solution of methacryloyl chloride (8.23 mL, 85.8 mmol) in 10 mL of DCM was dropwise added into the mixture *via* a constant pressure funnel. After stirring

at room temperature for 24 h, the resultant mixture was washed with saturated $NaHCO_3$ and NaCl solution three times, respectively. The organic phase was dried with anhydrous Na_2SO_4 for 2 h and concentrated by rotary evaporation. The crude product was purified by silica column chromatography (DCM: Hex = 1: 10) to obtain **DPA** as a faint yellow oily liquid after rotary evaporation (8.85 g, 73%)

Synthesis of PEG-CPDB. Anhydrous methoxypolyethylene glycol (PEG_{5K}-OH) (500 mg, 0.10 mmol), DMAP (6 mg, 0.05 mmol) and CPDB (112 mg, 4.00 mmol) were dissolved in 10 mL anhydrous DCM and stirred at 0 °C for 30 min under an N_2 atmosphere. A solution of DCC (83 mg, 4.00 mmol) in 5 mL anhydrous DCM was added dropwise to the above reaction solution and stirred at room temperature for 48 h. The reaction solution was filtered, and the concentrated filtrate was added dropwise to ether for precipitation three times. A solid pink powder was afforded **PEG-CPDB** as a product after vacuum drying (325 mg, 65%).

Synthesis of PEG-PDPA. PEG_{5k}-CPDB (300 mg, 0.06 mmol), DPA (486 mg, 0.30 mmol) and AIBN (9 mg, 0.06 mmol) were dissolved in 10 mL 1,4-dioxane. After three cycles of freeze-pump-thaw to remove oxygen, the mixture was stirred at 75 °C for 10 h under a nitrogen atmosphere. The reaction solution was concentrated and added dropwise to n-hexane to precipitate three times and then filtered to afford **PEG-PDPA** as a pink solid (120 mg, 40%).

Synthesis of PEG-VC-PCL

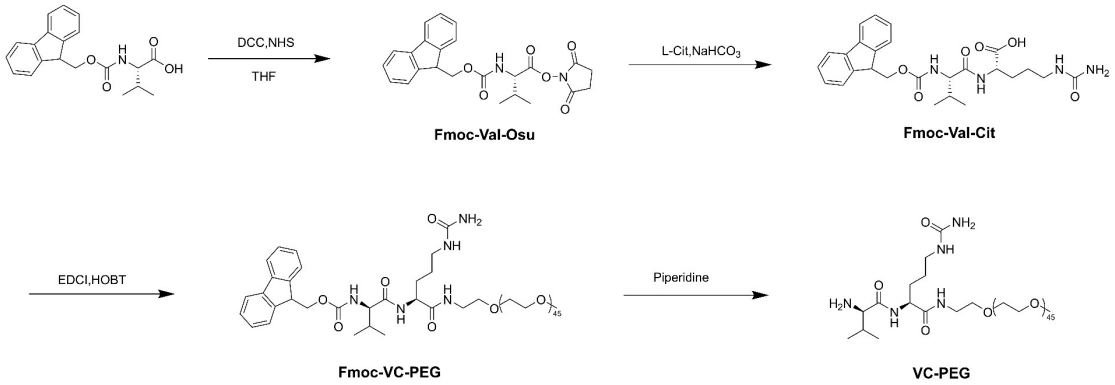


Scheme S4. Synthetic procedures of PCL-COOH.

Synthesis of PCL-OH. In the glove box, 6-hexanolactone (4.00 g, 35.00 mmol) and benzyl

alcohol (54 mg, 0.50 mmol) were heated and stirred at 130 ℃ for 10 min, then a drop of stannous octoate was added dropwise as a catalyst, and the reaction was quenched with liquid nitrogen after stirring for 1 h. Add 10 mL THF to dissolve the reactant, precipitate three times in Hex, and filter to afford **PCL-OH** as a white solid (3.70 g, 93%).

Synthesis of PCL-COOH. To a solution of compound 2 (1.00 g, 0.15 mmol), succinic anhydride (100 mg, 1.00 mmol) and DMAP (122 mg, 1.00 mmol) in 1,4-dioxane (30 ml), trimethylamine (140 μL, 1 mmol) was added dropwise and stirred at room temperature for 24h. The reaction solution was concentrated to 10 mL, added dropwise to 100 mL of 40 °C water, and extracted with dichloromethane. After removing dichloromethane, it was recrystallized in methanol and filtered to afford the product **PCL-COOH** as a white solid (0.70 g, 70%).



Scheme S5. Synthetic procedures of VC-PEG.

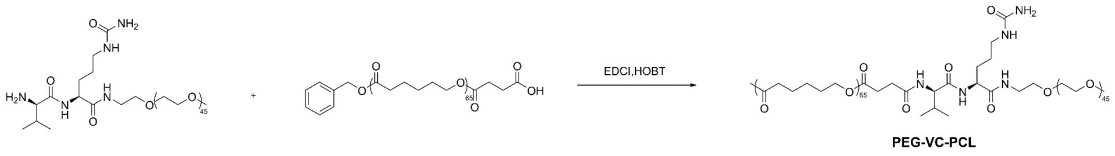
Synthesis of Fmoc-Val-Osu. To the solution of Fmoc-Val-OH (10.00 g, 0.03 mol) and NHS (3.39 g, 0.03 mol) in anhydrous THF (200 mL) was added DCC (6.08 g, 0.03 mol) at 0 °C. The mixture was stirred at room temperature for 16 h. Then, the precipitation was removed by filtration under reduced pressure. Finally, the solvent was evaporated to dryness to give **Fmoc-Val-NHS** as a light-yellow solid (11.26g, 86%), which was used without further purification.

Synthesis of Fmoc-Val-Cit. L-citrulline (3.80 g, 0.03 mol) and $NaHCO_3$ (1.80 g, 3.24 mmol)

were dissolved in 60 mL water. Then, Fmoc-Val-NHS (9.00 g, 0.02 mol) in a mixture of DME (60 mL) and THF (39 mL) was added. After stirring for 16 h, the aqueous citric acid solution 103 mL (15%) was added under continuous stirring. The reaction mixture was extracted with 10% 2-propanol/ethyl acetate (3 × 100 mL), and the organic layers were washed with water (2 × 100 mL) and dried with anhydrous $MgSO_4$. Finally, the ether layer was evaporated to dryness to afford **Fmoc-Val-Cit** as a white powder (10.70 g, 84%).

Synthesis of Fmoc-VC-PEG. Fmoc-Val-Cit (4.00 g, 8.00 mmol), EDCI (1.54 g, 8.00 mmol) and HOBT (1.08 g, 8.00 mmol) were dissolved in THF. The mixture was stirred at room temperature for 2 h. Then methoxypoly (ethylene glycol) amine ($mPEG_{2k}$-NH_2) (4.00 g, 2.00 mmol) was added, and the solution was stirred for another 24 h. When the reaction was completed, the solvent was removed under reduced pressure. The crude product was redissolved in dichloromethane and precipitated by dropwise addition to ether. The crude product was purified by gel column (1% cross-linked: separation of MW 600-14,000 lipophilic polymers) using DMF as eluents and dried under vacuum to obtain **Fmoc-VC-PEG** as a pale yellow solid (3.60 g, 72%).

Synthesis of VC-PEG. To a solution of compound Fmoc-VC-PEG (2.50 g, 1.00 mmol) in DMF (20 mL) was added anhydrous piperidine (300 μL), the reaction mixture was stirred for 2 h at room temperature. Finally, the crude product was precipitate three times in ethyl ether (200 mL) and filter to afford **VC-PEG** as a pale yellow solid after freeze-drying (2.09g, 92%).



Scheme S6. Synthetic procedures of PEG-VC-PCL.

Synthesis of PEG-VC-PVL. PCL-COOH (2.00 g, 0.40 mmol), EDCI (77 mg, 0.40 mmol) and

HOBT (54 mg, 0.40 mmol) were dissolved in DMF. The mixture was stirred for 4 h at room temperature. Then VC-PEG (1.00 g, 0.40 mmol) was added to the reaction solution and stirred for another 24 h at room temperature. When the reaction was completed, the reaction solution was concentrated to 5 mL and then purified by a gel column using DMF as eluents and dried under vacuum to obtain **PEG-VC-PCL** as a pale yellow solid (1.27g, 35%).

Preparation nanoparticles of pH@VE-CPT and CTB@TZ-BOD

Dissolve VE-CPT (10 mg) + PEG-PDPA (40 mg) or TZ-BOD (10 mg) + PEG-VC-PCL (40 mg) in 1 mL of DMSO and then dropwise added to 9 mL of ultra-pure water. The mixture was stirred at room temperature for 3 h. And then transferred into the dialysis membrane tubing (MWCO 3500 Da) and dialyzed against water for 24 h. The VE-CPT and TZ-BOD concentration was determined by a microplate system (Molecular Devices) at 373 nm and 680 nm by UV absorption.

Characterization of nanoparticles

The changes of nanoparticle hydrodynamic diameters (Rh) in different media were investigated by a Malvern ZS90 dynamic light scattering instrument (DLS) to verify the responsiveness of nanoparticles. Specifically, nanoparticles (1 mg mL^{-1}) were mixed with different media and incubated at 37 °C in a constant temperature water bath oscillator.

Study on the stability of VE-CPT under physiological conditions

The content of VE-CPT under physiological conditions was performed by incubating VE-CPT solution (100 μg mL^{-1}) with a RPMI 1640 culture medium for 7 days, followed by the analysis of HPLC (UV/Vis detector, λ = 365 nm).

Study on the release of VE-CPT from nanoparticles

The release profile of VE-CPT from nanoparticles was determined by the dialysis method. Nanoparticles were dissolved in phosphate-buffered saline (PBS pH 7.4) (2 mg mL^{-1}, 1 mL) and transferred into a dialysis bag (MWCO 3500 Da), followed by incubation with 25 mL buffer solution under various conditions (with pH 5.4 or pH 7.4 PBS) in a shaking water bath at 37 °C. At regular intervals, collected 1 mL of buffer solution from the outside of the dialysis bag and replaced with 1 mL of fresh buffer solution. The VE-CPT concentration was determined by a microplate system (Molecular Devices) at 373 nm by UV absorption.

Fluorescence activation in solutions

The evaluation of bioorthogonal reaction by fluorescence measurement was performed in pH 7.4 PBS solution at 37 °C. A TZ-BOD solution (10 μM) in pH 7.4 THF/PBS (30/70, v/v) was incubated with VE-CPT solution (20 μM) in pH 7.4 THF/PBS (30/70, v/v), and fluorescence spectra at different incubation times were obtained. In addition, fluorescence spectra of TZ-BOD solutions (10 μM) in pH 7.4 THF/PBS (30/70, v/v) treated with various concentrations of VE-CPT solutions were also recorded.

The prodrug activation verified by HPLC in solutions

The qualitative evaluation the bioorthogonal reaction by HPLC was performed by incubating VE-CPT solution (10 μM) in acetonitrile with that of TZ-BOD (20 μM) for 3 h, followed by the analysis of HPLC (FLR detector, $\lambda_{ex}/\lambda_{em}$ = 365/430 nm).

Cell culture

Mouse breast cancer cell line 4T1 cells were purchased from American Type Culture Collection (Manassas, VA, USA). 4T1 cells were cultured in RPMI 1640 medium (37 °C, 5% CO_2), supplemented with 10% fetal bovine serum and 1% penicillin (100 IU mL^{-1}), streptomycin (100 μg mL^{-1}).

Cytotoxicity assay

4T1 cells were seeded in 96-well culture plates (1×10^4 cells/well) and incubated for 12 h. The cells were then treated with CPT, VE-CPT, VE-CPT+TZ-BOD, pH@VE-CPT, CTB@TZ-BOD, and pH@VE-CPT + CTB@TZ-BOD at the desired concentrations. The cell viability was measured by the standard commercial methyl thiazolyl tetrazolium (MTT) assay after 48 h of cell culture.

Intracellular fluorescence activation

4T1 cells were seeded in 24-well culture plates (1.5×10^4 cells/well) and incubated for 12 h. Afterward, the medium was discarded and the cells were incubated with fresh medium containing BOD (4 μM), TZ-BOD (4 μM), VE-CPT + TZ-BD (containing VE-CPT 8 μm, BOD 4 μM), CTB@TZ-BOD (containing TZ-BOD 4 μM), pH@VE-CPT + CTB@TZ-BOD (containing VE-CPT 8 μM, TZ-BOD 4 μM), pH@VE-CPT/ CTB@TZ-BOD + CA-074-Me (containing CA-074-Me 10 μM), pH@VE-CPT/ CTB@TZ-BOD + Baf-A1 (containing Baf-A1 1 μM pretreated for 1 h then added pH@VE-CPT/ CTB@TZ-BOD) and pH@VE-CPT/ CTB@TZ-BOD + CA-074-Me + Baf-A1(containing CA-074-Me 10 μM, Baf-A1 1 μM, likewise pretreated by Baf-A1 for 1 h). After 4 h, the cells were then washed with PBS and stained with Hoechst 33342, studied by confocal laser scanning microscope (CLSM).

For flow cytometry study, 4T1 cells were seeded in 24-well culture plates (1×10^4 cells/well) and incubated for 12 h. Afterward, the medium was discarded and the cells were incubated with fresh medium containing different formulations. After 4 h, the cells were collected and washed with PBS three times, then analyzed by flow cytometry.

Intracellular prodrug activation verified by HPLC

4T1 cells were seeded in 12-well culture plates (1×10^5 cells/well) and incubated for 24 h. Afterward, the medium was removed and the cells were incubated with fresh medium containing CPT (10 μM), VE-CPT(10 μM), VE-CPT (10 μM) + TZ-BOD (20 μM), pH@VE-CPT (containing VE-CPT 10 μM), pH@VE-CPT + CTB@TZ-BOD (containing VE-CPT 10 μM, TZ-BOD 20 μM), pH@VE-CPT + CTB@TZ-BOD (containing VE-CPT 10 μM, TZ-BOD 20 μM) + CA-074-Me(10 μM), pH@VE-CPT+ CTB@TZ-BOD (containing VE-CPT 10 μM, TZ-BOD 20 μM) + Baf-A1 (1 μM, pretreated for 2 h)and pH@VE-CPT + CTB@TZ-BOD (containing VE-CPT 10 μM, TZ-BOD 20 μM) + CA-074-Me (10 μM)+Baf-A1 (1 μM, pretreated for 2 h) for 8 h. Then the cells were washed with PBS for two times and added 200 μL deionized water every well. After three times of freeze-thaw cycles, 150 μL lysis cell were taken and 150 μL acetonitrile was added to each tube for 30 min ultrasound. The mixtures were centrifuged for 10 min at 1000 g. The supernatant was collected and analyzed by HPLC (FLR detector, $\lambda_{ex}/\lambda_{em}$ = 365/430 nm).

Cell apoptosis analysis detection

4T1 cells were seeded in 12 well culture plates (1×10^5 cells/well) and incubated for 24 h. Afterward, the medium was removed and the cells were incubated with fresh medium containing CPT (10 μM), VE-CPT (10 μM), TZ-BOD (20 μM), VE-CPT (10 μM) + TZ-BOD (20 μM),

pH@VE-CPT (containing VE-CPT 10 μM), pH@VE-CPT + CTB@TZ-BOD (containing VE-CPT 10 μM, TZ-BOD 20 μM), pH@VE-CPT + CTB@TZ-BOD (containing VE-CPT 10 μM, TZ-BOD 20 μM)+CA-074-Me (10 μM), pH@VE-CPT + CTB@TZ-BOD (containing VE-CPT 10 μM, TZ-BOD 20 μM)+Baf-A1(1 μM, pretreated for 2 h) and pH@VE-CPT + CTB@TZ-BOD (containing VE-CPT 10 μM, TZ-BOD 20 μM) + CA-074-Me (10 μM)+Baf-A1(1 μM, pretreated for 2 h) for 12 h. Then cells were stained with Annexin V-FITC and Propidium Iodide (PI) according to the instructions of the kit and then analyzed by flow cytometry.

Live/Dead Cell Staining Assay

4T1 cells were seeded in 24 well culture plates (1×10^5 cells/well) and incubated for 24 h. After incubation, the culture medium was refreshed, and the cells were treated with CPT (50 μM), VE-CPT (50 μM), TZ-BOD (100 μM), VE-CPT (50 μM) + TZ-BOD (100 μM), pH@VE-CPT (containing VE-CPT 50 μM), pH@VE-CPT + CTB@TZ-BOD (containing VE-CPT 50 μM, TZ-BOD 100 μM), pH@VE-CPT + CTB@TZ-BOD(containing VE-CPT 50 μM, TZ-BOD 100 μM) + CA-074-Me (10 μM), pH@VE-CPT + CTB@TZ-BOD (containing VE-CPT 50 μM, TZ-BOD 100 μM) + Baf-A1 (1 μM, pretreated for 2 h) and pH@VE-CPT + CTB@TZ-BOD (containing VE-CPT 50 μM, TZ-BOD 100 μM) + CA-074-Me (10 μM) + Baf-A1 (1 μM, pretreated for 2 h) for 12 h. Subsequently, all of the groups were stained with calcein-AM (2 μM) and PI solutions (8 μM) in phosphate buffer solution and cultivated for 30 min at room temperature to differentiate the live and dead cells. Finally, the cells were washed three times with cold PBS and observed on the fluorescence microscope.

Animals and tumor models

Female BALB/c mice (20 ± 2 g, 6 weeks old) were purchased from Hunan SJA Laboratory Animal Co., Ltd (Hunan, China). 4T1 cells (1×10^6) were injected into the right mammary fat pads to establish an orthotopic 4T1 tumor model. At the end of experiments, all mice were killed by CO_2 inhalation. All animal experiments approved by the Ethics Committee of the South China University of Technology followed the guidelines of "Guangdong Province Experimental Animal Policies and Regulations" and "National Experimental Animal Management Regulations".

Fluorescence activation *in vivo*

When the tumor volume reached 100 mm^3, the tumor-bearing mice were randomly divided into two groups (n = 3) and intravenously injected with CTB@TZ-BOD and pH@VE-CPT/CTB@TZ-BOD (VE-CPT: 20 μM, TZ-BOD: 10 μM). The mice were anesthetized and the fluorescence images of mice were observed at determined time points (6, 12, 24 h) by using an *in vivo* Xtreme (Bruker, German) instrument. After the imaging experiments, the mice were sacrificed humanely and their tumors and major organs (heart, liver, spleen, lung, and kidney) were dissected for *ex vivo* imaging.

Tumor growth inhibition

When the tumor volume reached 100 mm^3, 4T1 tumor-bearing mice were randomly divided into five groups (n = 5). The mice were treated with PBS, CPT, pH@VE-CPT, CTB@TZ-BOD and pH@VE-CPT/CTB@TZ-BOD (5 mg kg^{-1} CPT, 20 mg kg^{-1} TZ-BOD equivalent) by intravenous injection once every two days, four times in all. The tumor volumes and body weight were measured every other day. Using this formula to calculate the volume of tumor: $V = L \times W^2/2$ (L, the longest dimension; W, the shortest dimension).

Fluorescence activation in tumor sections

The mice were sacrificed by cervical vertebra dislocation at 24 h post-injection (n = 3). Tumors were excised and embedded in optimum-cutting temperature (OCT) tissue compound (Sakura, Tokyo, Japan), sectioned into 7 μm slices. After stained nucleus with Hoechst 33342, the signals of DZ-BOD fluorescence probe and Hoechst 33342 were observed by CLSM.

Immunohistochemical analysis

The tumors were harvested, 4% paraformaldehyde fixed, paraffin embedded, sliced and stained with hematoxylin and eosin (H&E). Tumor sections were also stained with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) apoptosis detection kit assay then observed by CLSM.

CPT and VE-CPT content in tumor

The mice were sacrificed by cervical vertebra dislocation after an administration in 4T1 tumor-bearing mice for 24 h (n = 3), the tumors were collected, washed with saline. The collected tumors were homogenized in KH_2PO_4 solution (2×10^{-2}M, pH 2.8) with an Ultra-Turrax T18 Homogenizer (IKA). Tumors homogenate were exposed to 5 M HCl (200 μL) at 50 ℃ for 1 h. After cooling to room temperature, 1 M sodium hydroxide (200 μL) was added. The mixture was subsequently extracted with chloroform/acetonitrile (4: 1, v/v, 1 mL) by vortex mixing for 3 min. After centrifugation (10000 g, 15 min), the organic phase was separated and evaporated to dryness under a stream of nitrogen. The residue was dissolved in acetonitrile (400 μL), centrifugation at 10,000 g for 15 min; then, the supernatant was collected for HPLC analysis (FLR detector, $\lambda_{ex}/\lambda_{em}$ = 365/430

nm).

Statistical analysis

All the obtained data were expressed as mean ± standard deviation. Statistical significance of the treatment groups was evaluated using unpaired two-tailed Student's t-test. ns, non-significant. *P < 0.05, **P < 0.01, and ***P < 0.001 were considered statistically significant in analyses.

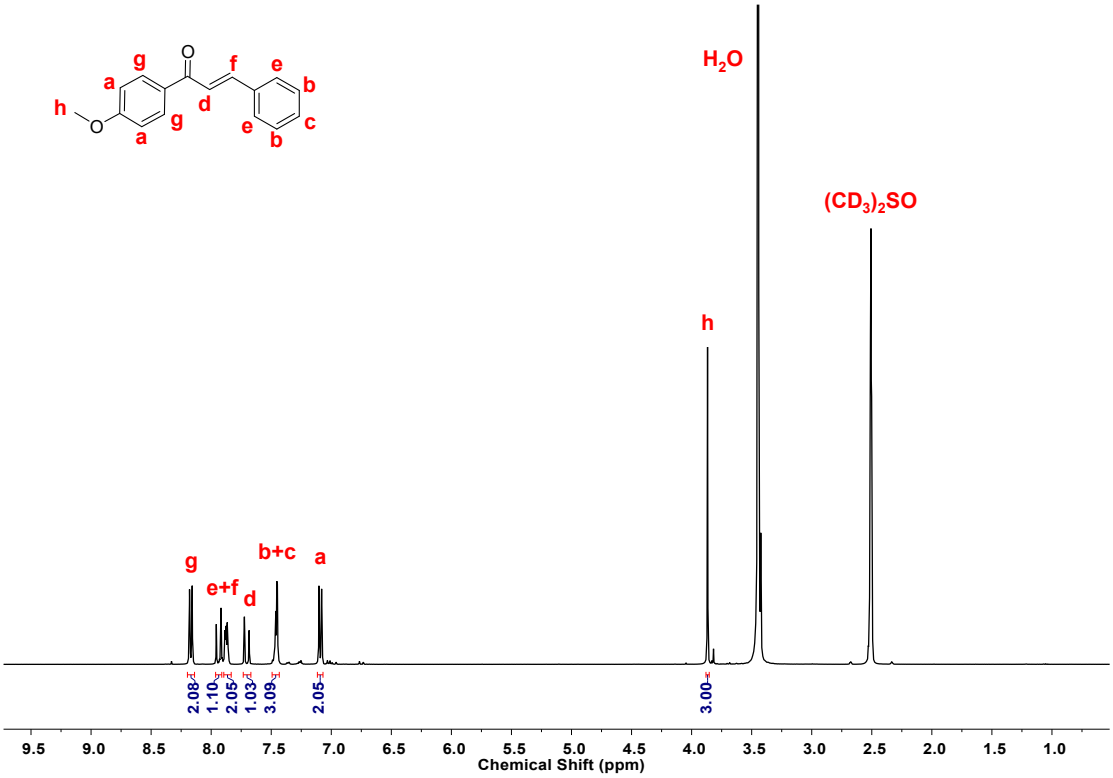


Figure S1. ^{1}H NMR spectrum of 1a in DMSO-d_6.

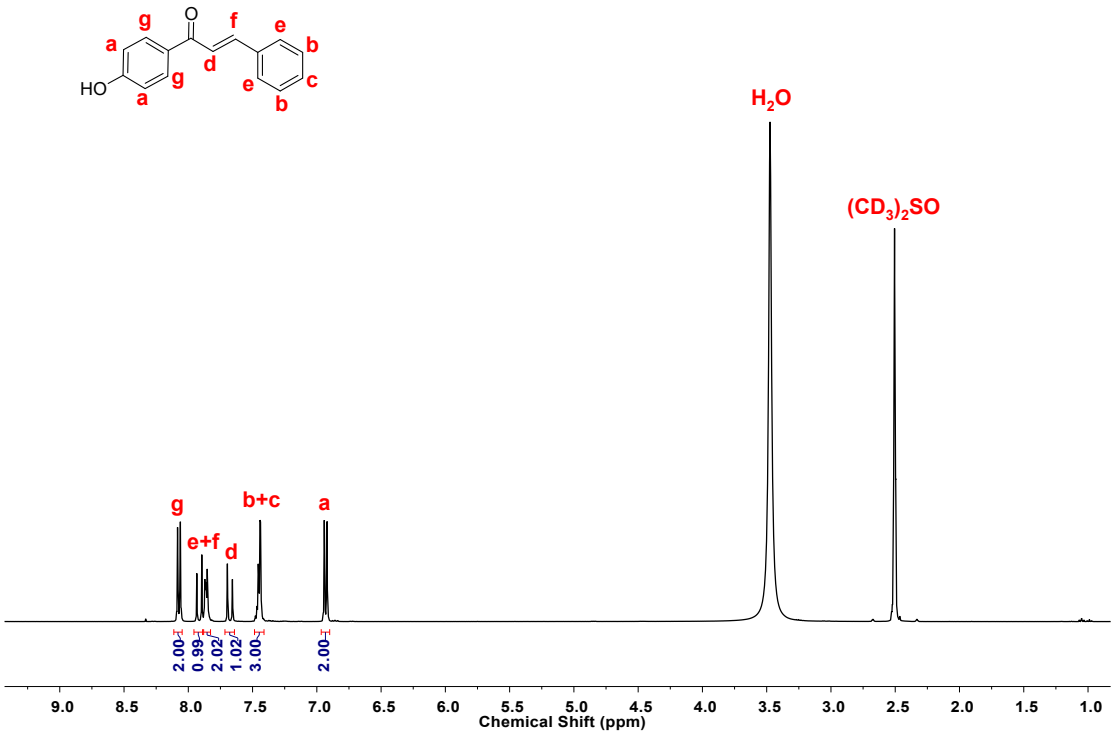


Figure S2. [1]H NMR spectrum of 1b in DMSO-d_6.

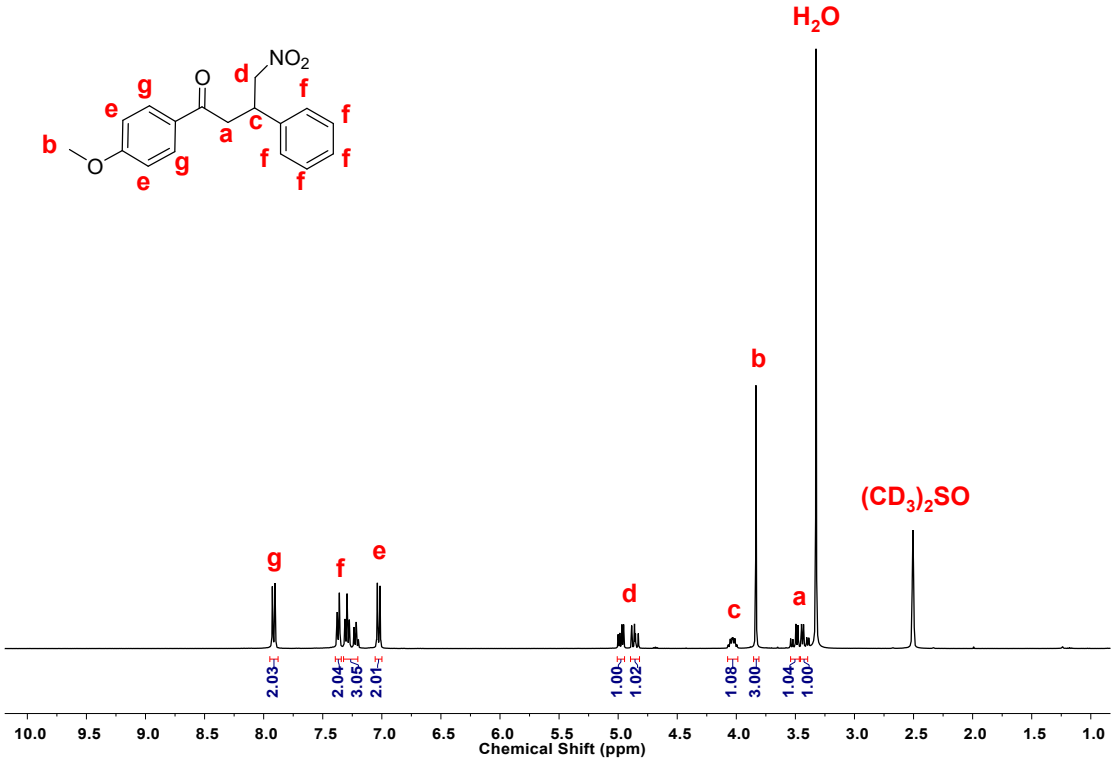


Figure S3. [1]H NMR spectrum of 2a in DMSO-d_6.

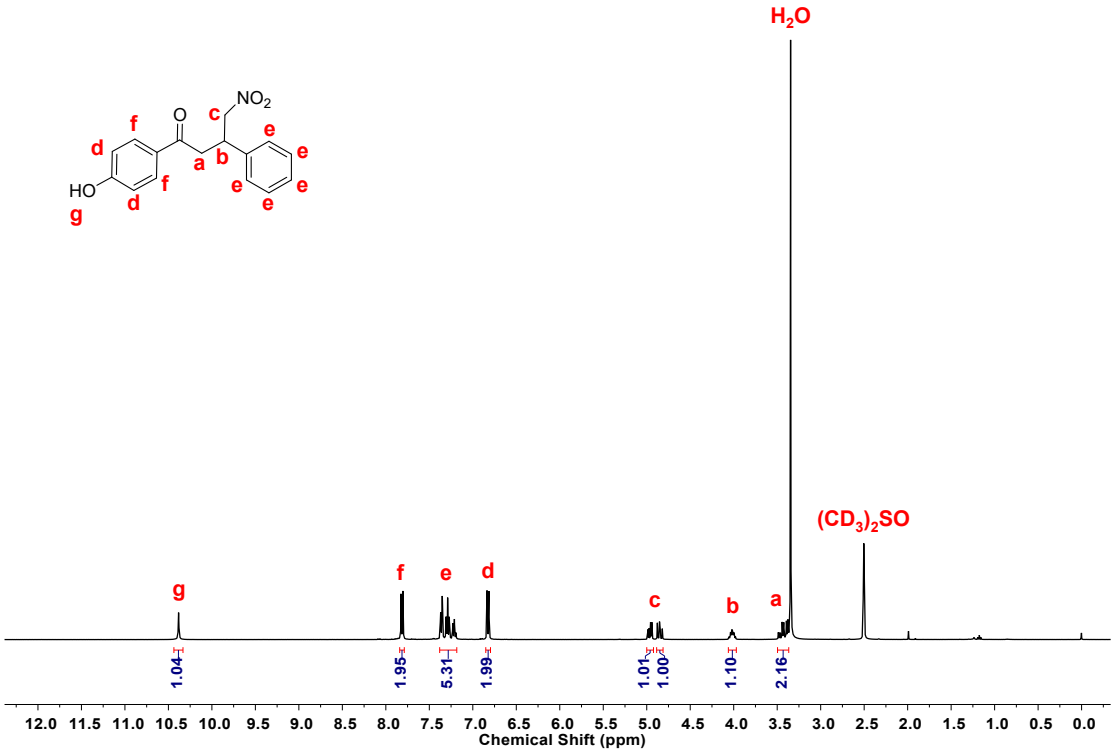


Figure S4. [1]H NMR spectrum of 2b in DMSO-d_6.

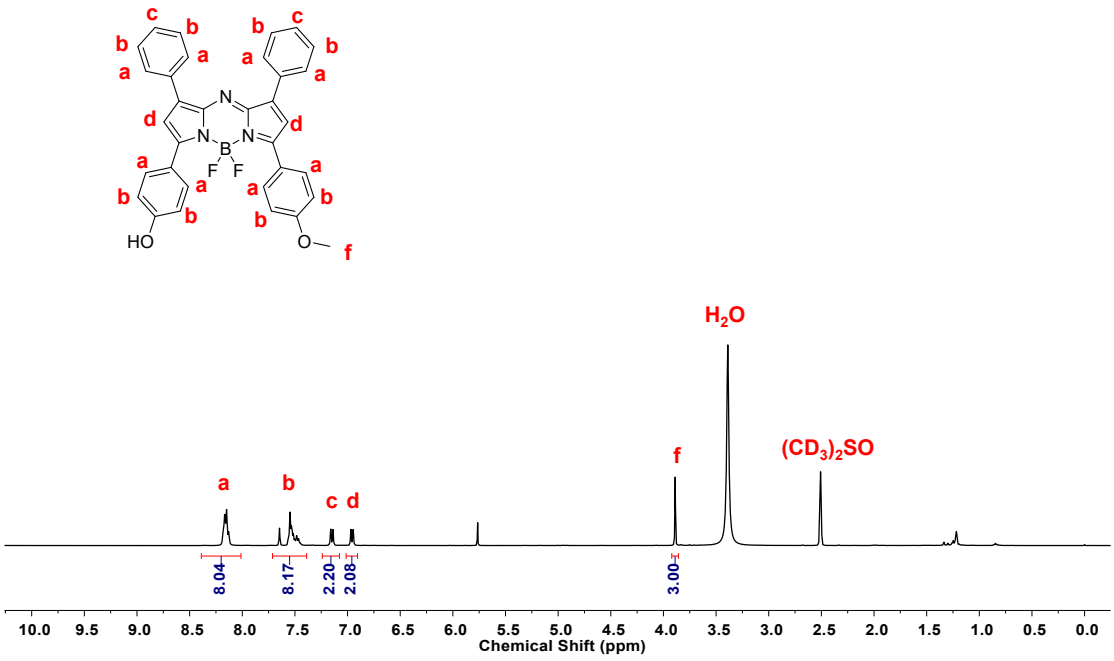


Figure S5. [1]H NMR spectrum of BOD in DMSO-d_6.

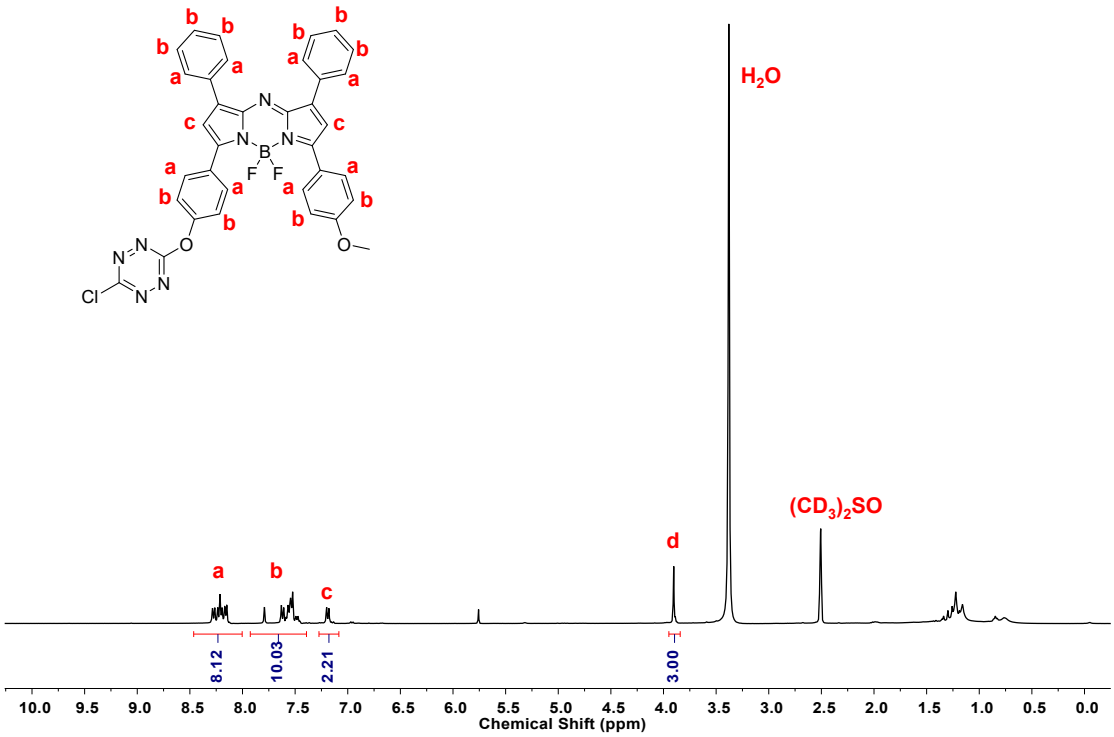


Figure S6. ^{1}H NMR spectrum of TZ-BOD in DMSO-d_6.

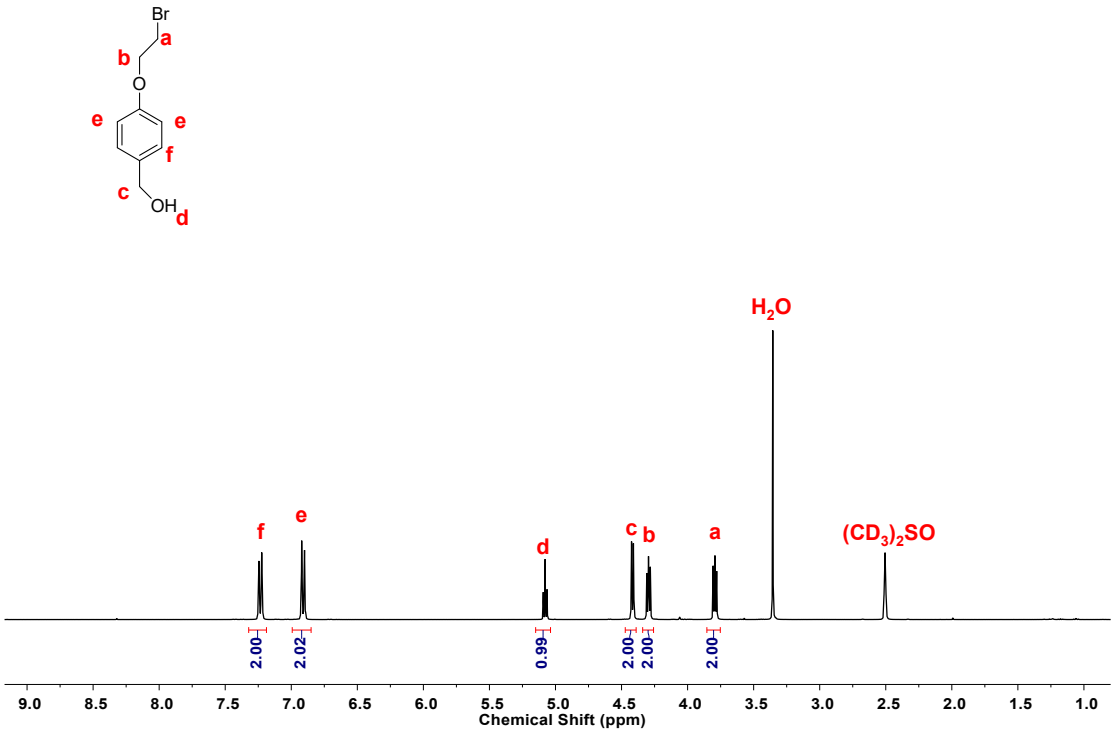


Figure S7. ^{1}H NMR spectrum of 4 in DMSO-d_6.

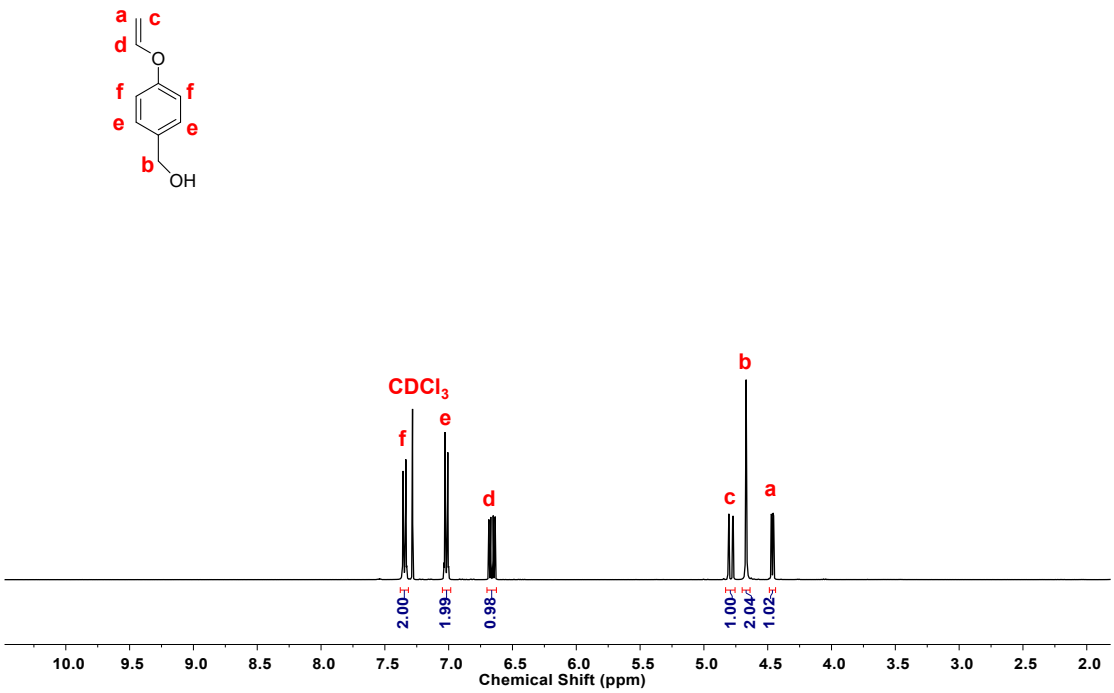


Figure S8. ^{1}H NMR spectrum of 5 in $CDCl_3$.

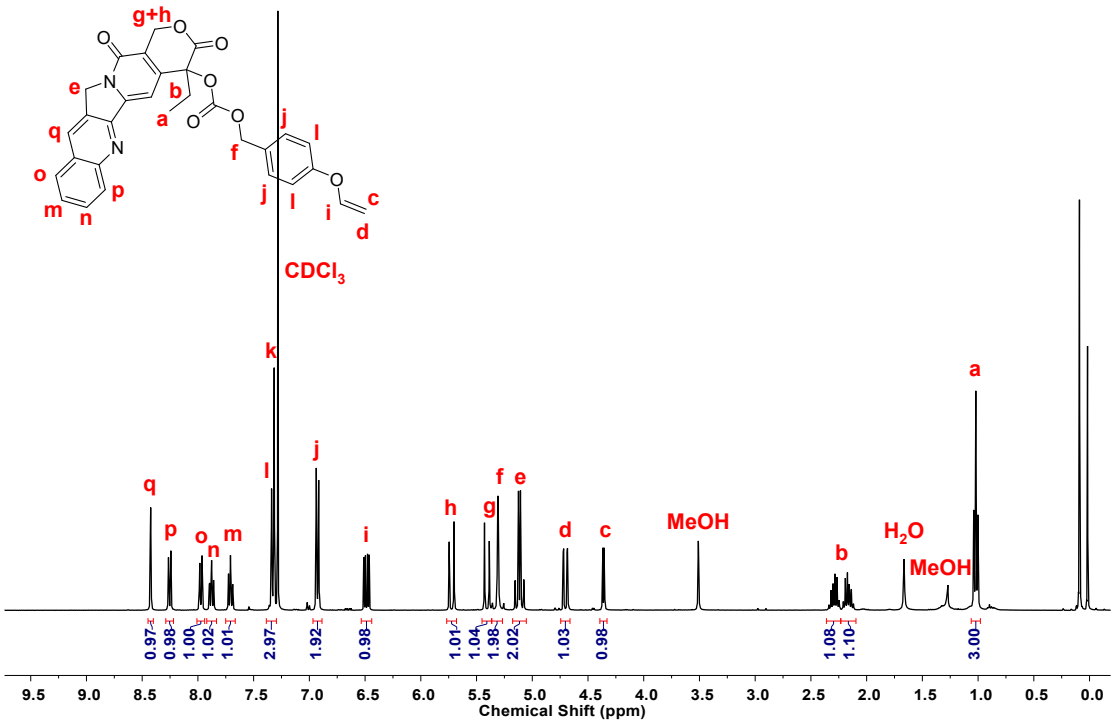


Figure S9. ^{1}H NMR spectrum of VE-CPT in $CDCl_3$.

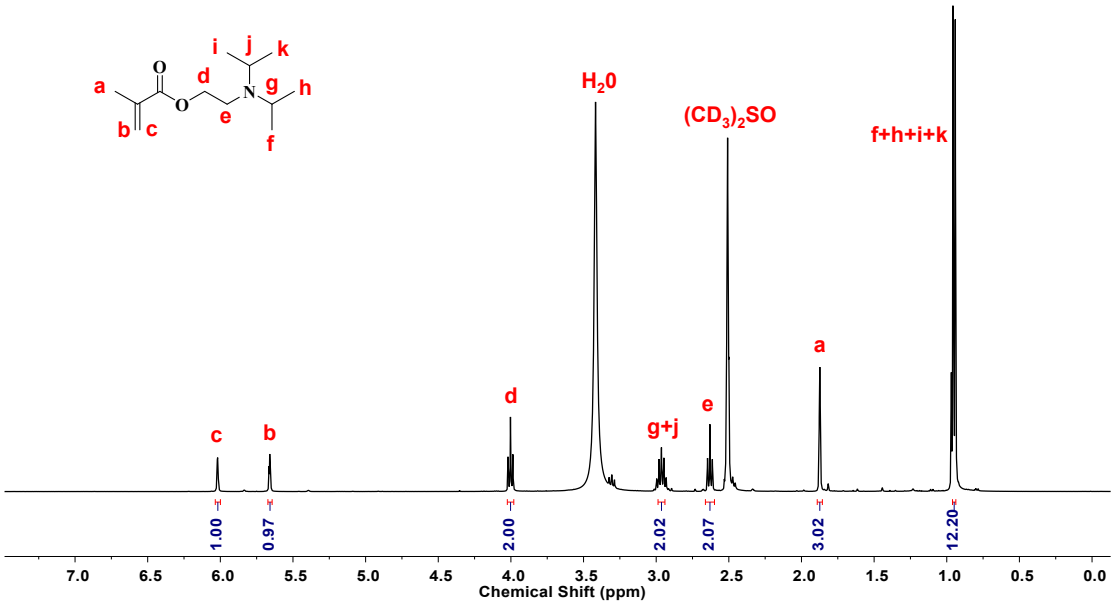


Figure S10. ^{1}H NMR spectrum of DPA in DMSO-d_6.

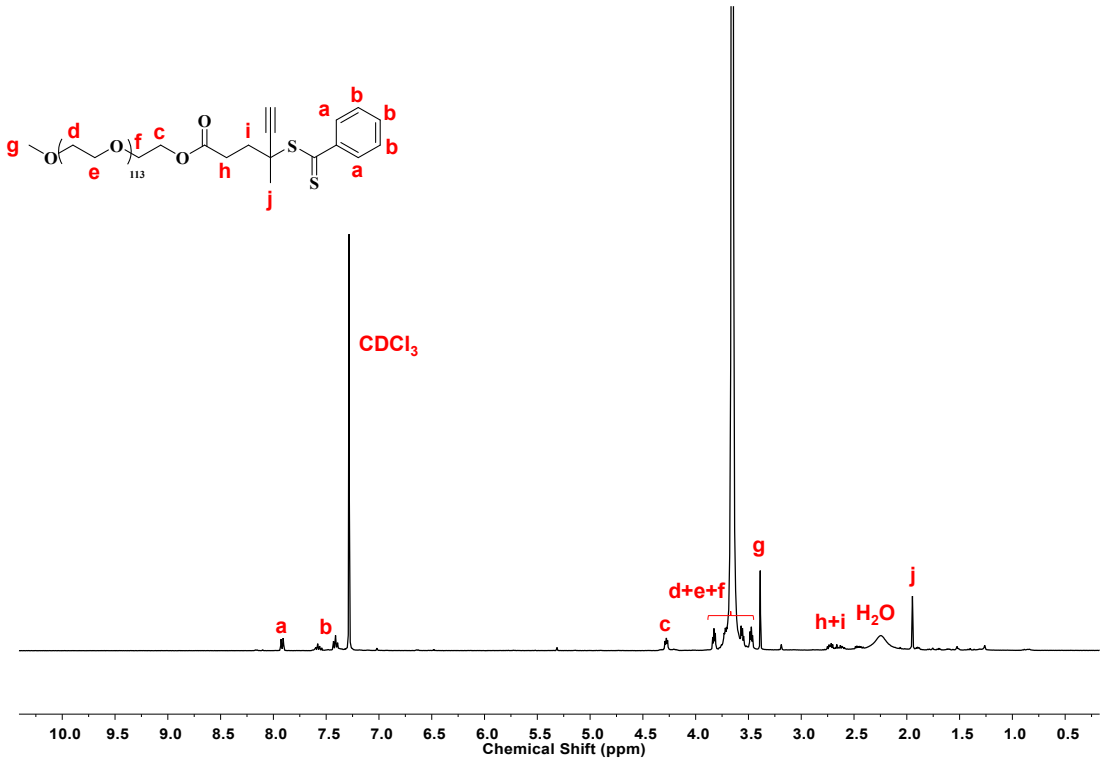


Figure S11. ^{1}H NMR spectrum of PEG-CPDB in $CDCl_3$.

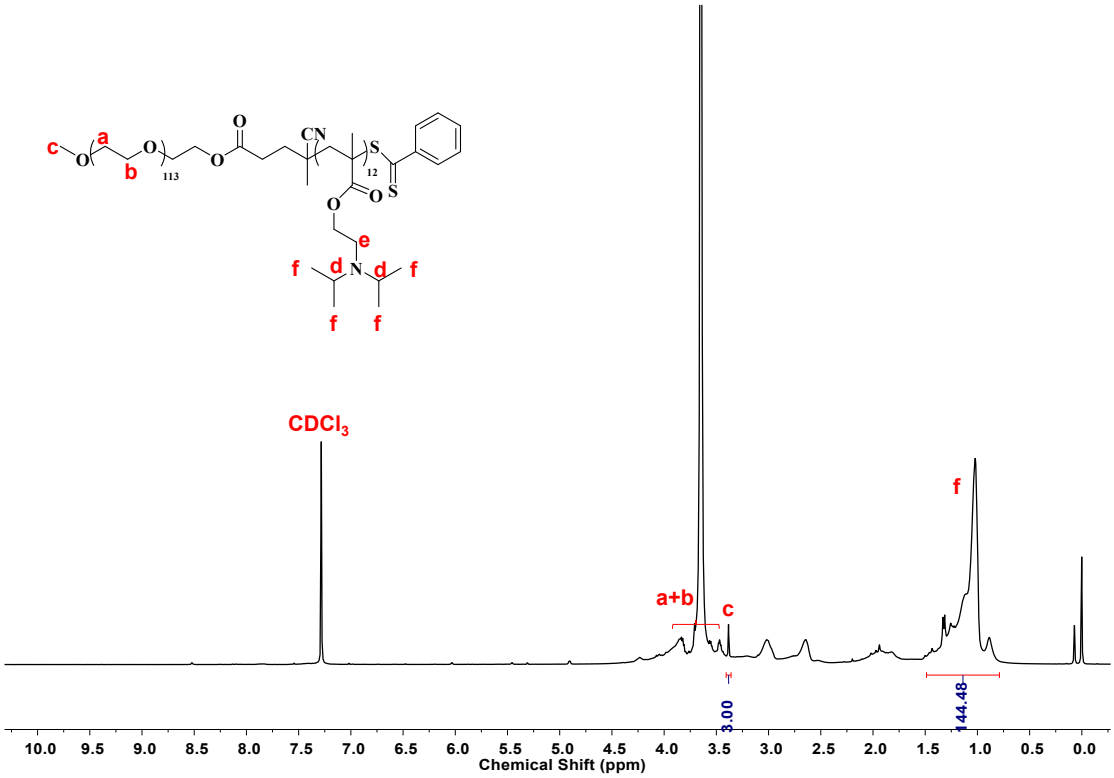


Figure S12. ^{1}H NMR spectrum of PEG-PDPA in $CDCl_3$.

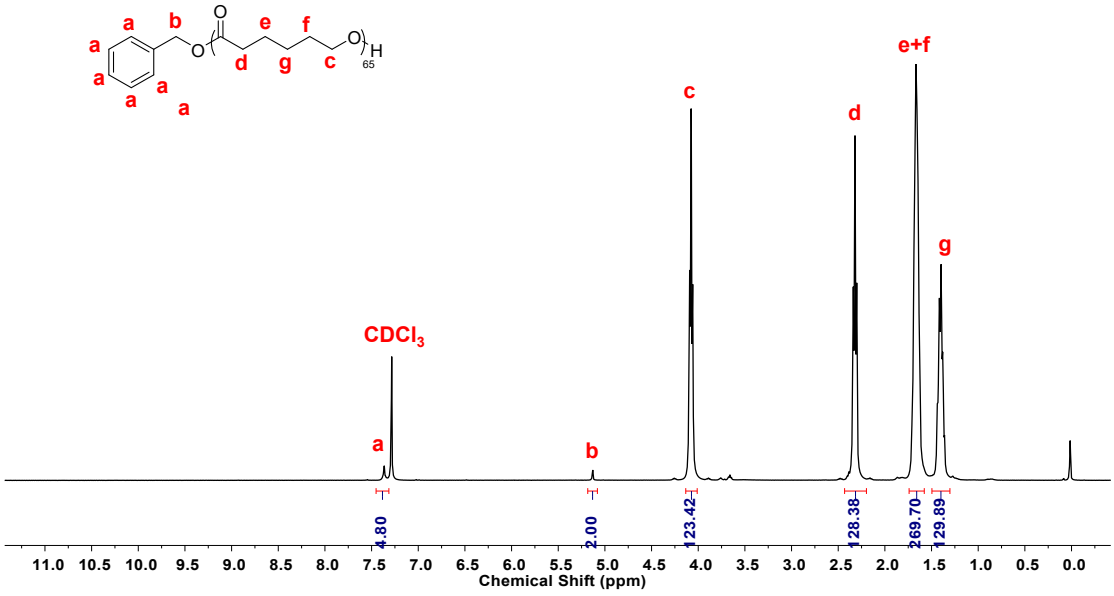


Figure S13. ^{1}H NMR spectrum of PCL-OH in $CDCl_3$.

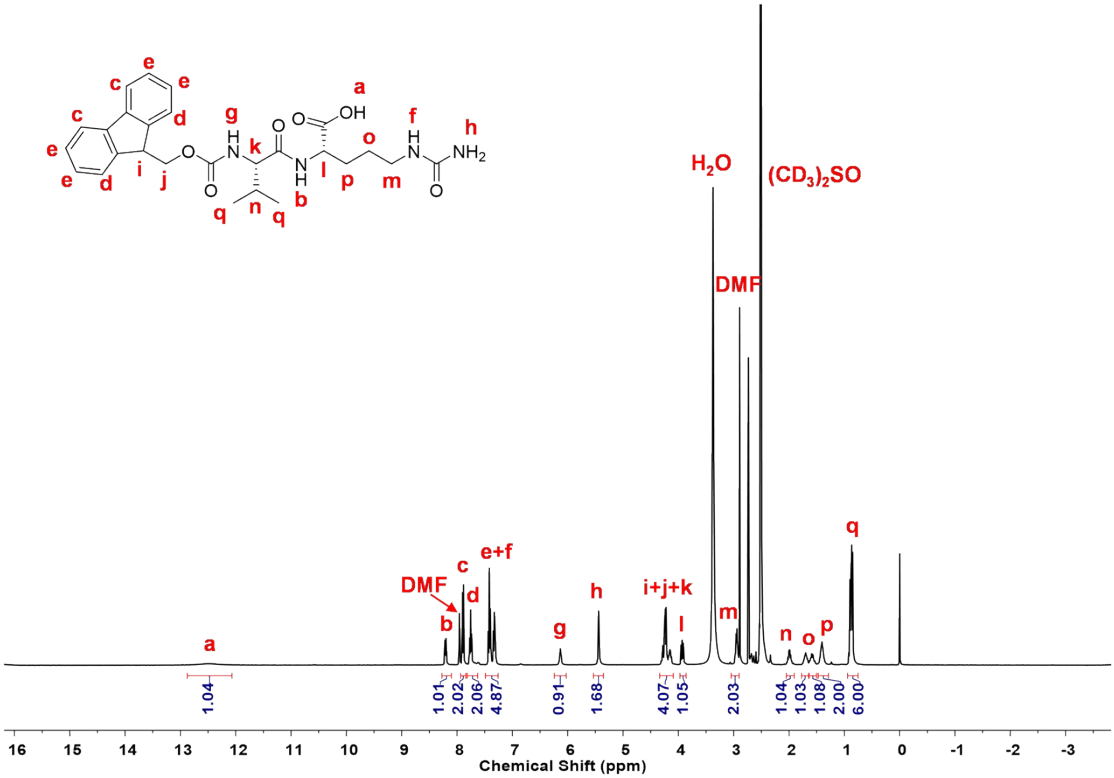


Figure S14. ^{1}H NMR spectrum of Fmoc-Val-Cit in DMSO-d_6.

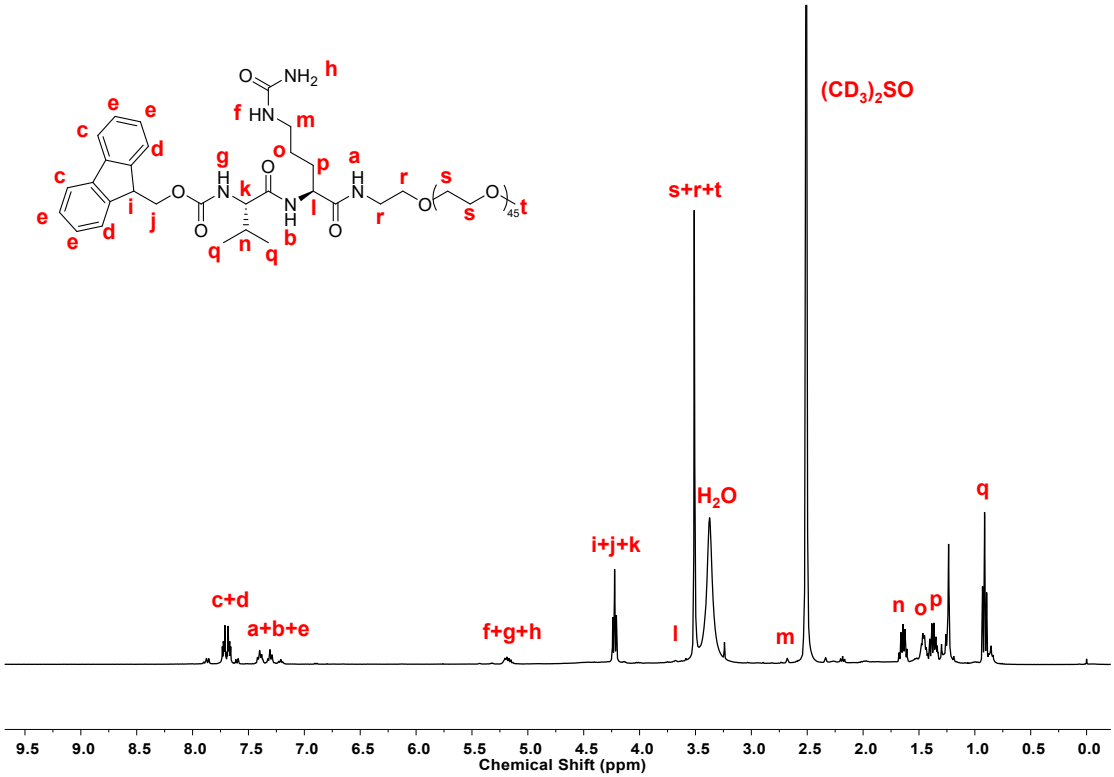


Figure S15. ^{1}H NMR spectrum of Fmoc-VC-PEG in DMSO-d_6.

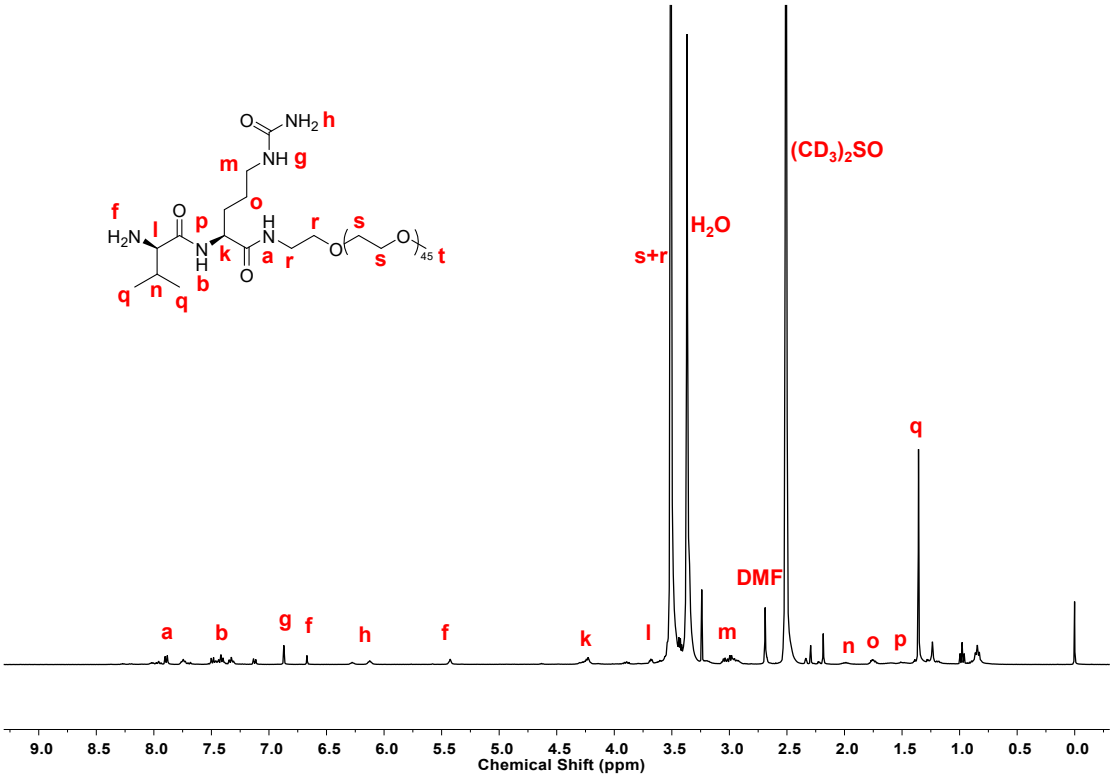


Figure S16. ^{1}H NMR spectrum of VC-PEG in DMSO-d_6.

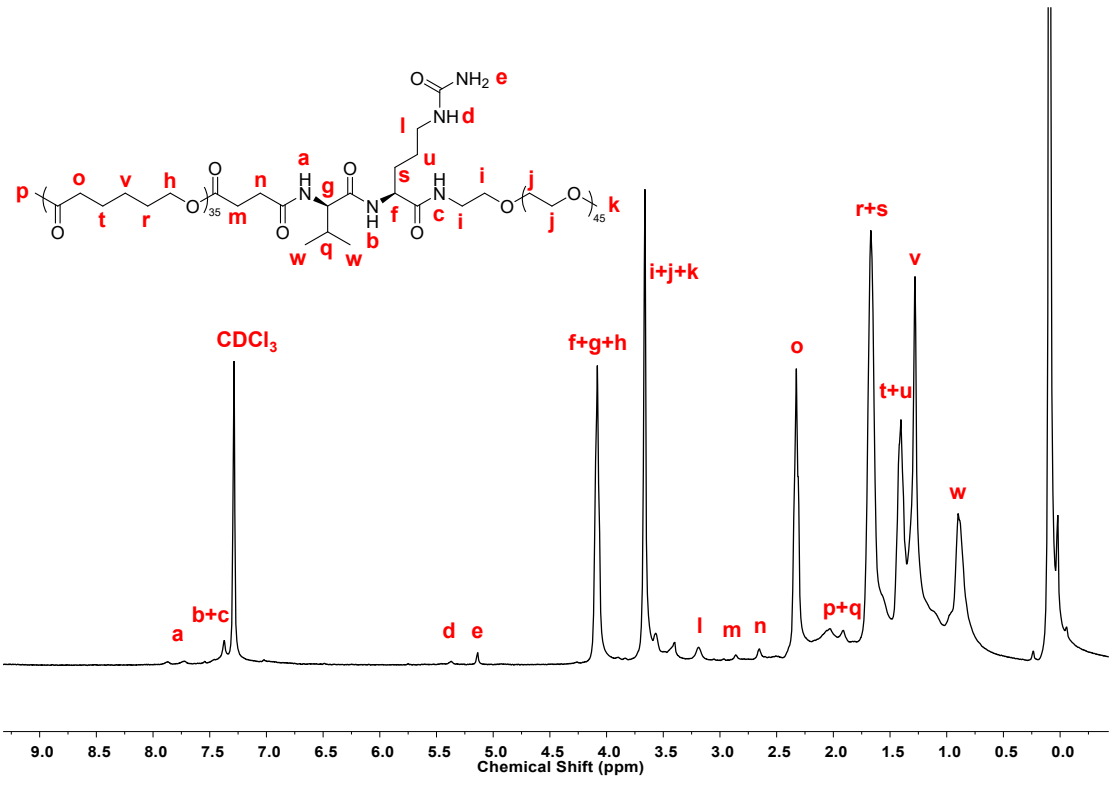


Figure S17. ^{1}H NMR spectrum of PEG-VC-PCL in DMSO-d_6.

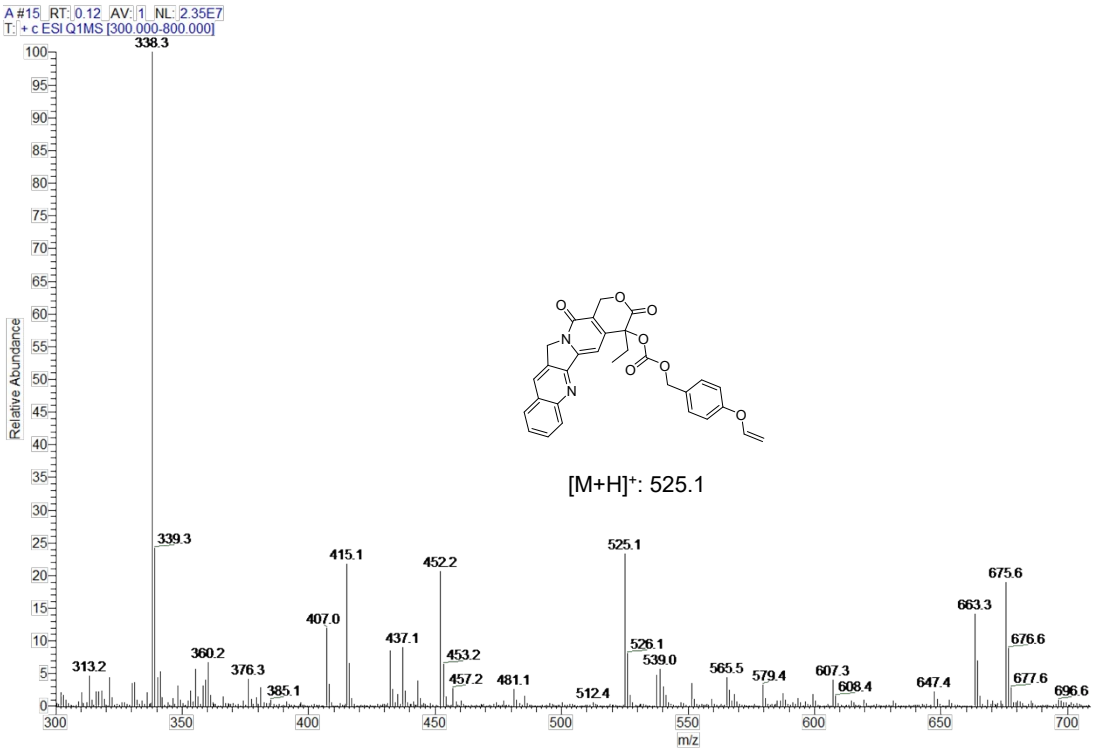


Figure S18. Mass spectra of VE-CPT.

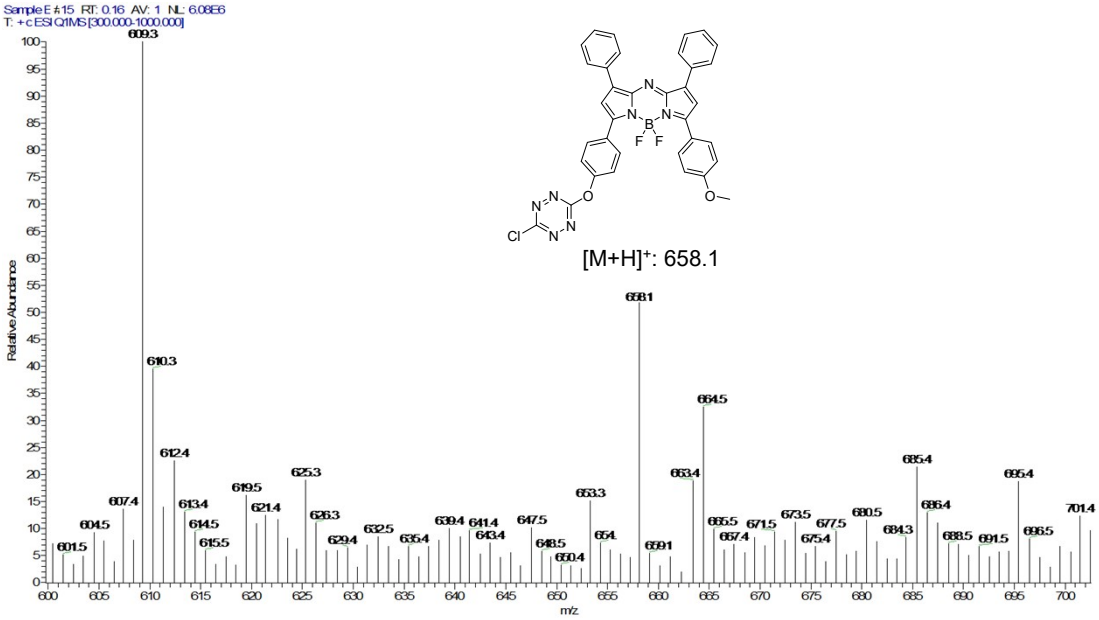


Figure S19. Mass spectra of TZ-BOD.

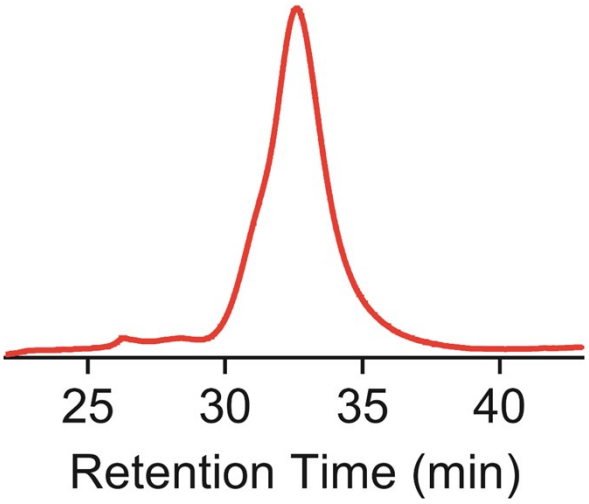


Figure S20. The GPC profiles of PEG-PDPA.

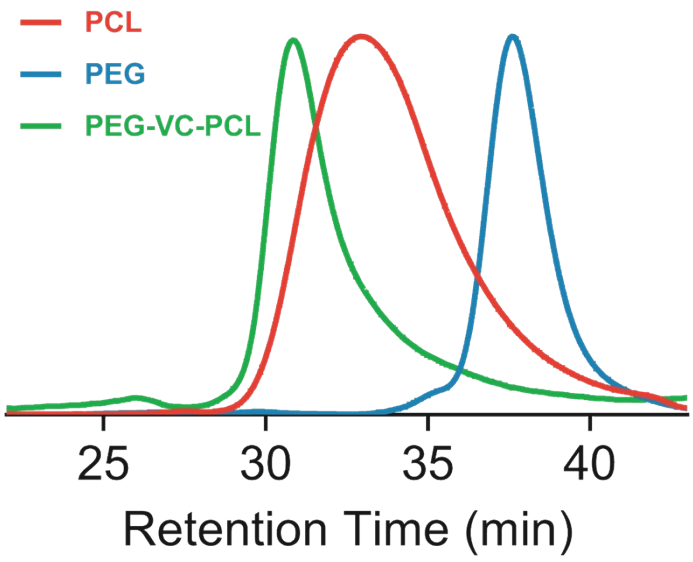


Figure S21. The GPC profiles of PCL-VC-PEG, PCL and PEG.

Table S1. Characterization of PEG-PDPA and PEG-VC-PCL.

Samples	M_n (g/mol) [a] (NMR)	M_w (g/mol) [b] (GPC)	M_n (g/mol) [c] (GPC)	PDI[d]
PEG_{113}-$PDPA_{12}$	7900	10100	9500	1.06
PEG_{45}-VC-PCL_{65}	9100	11000	12000	1.11

[a]Average degrees of polymerization were determined by ^{1}H NMR. [b]Number-average molecular weights of polymer determined by ^{1}H NMR. [c]Weight-average molecular weight was evaluated by GPC with polystyrene standards. [d]Polymer dispersity index (PDI) = M_w/M_n.

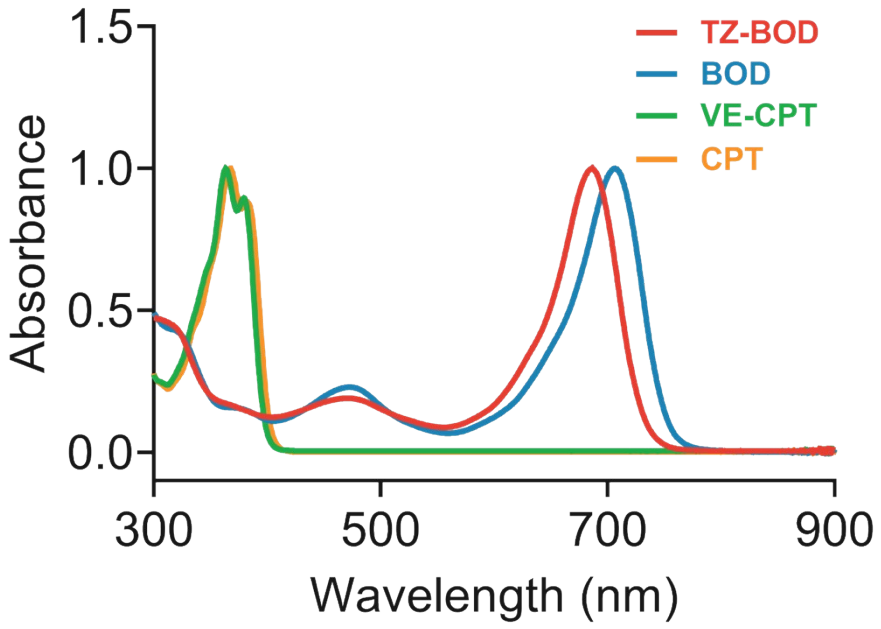


Figure S22. UV-Vis absorption spectra in pH 7.4 PBS containing 20% (v/v) THF.

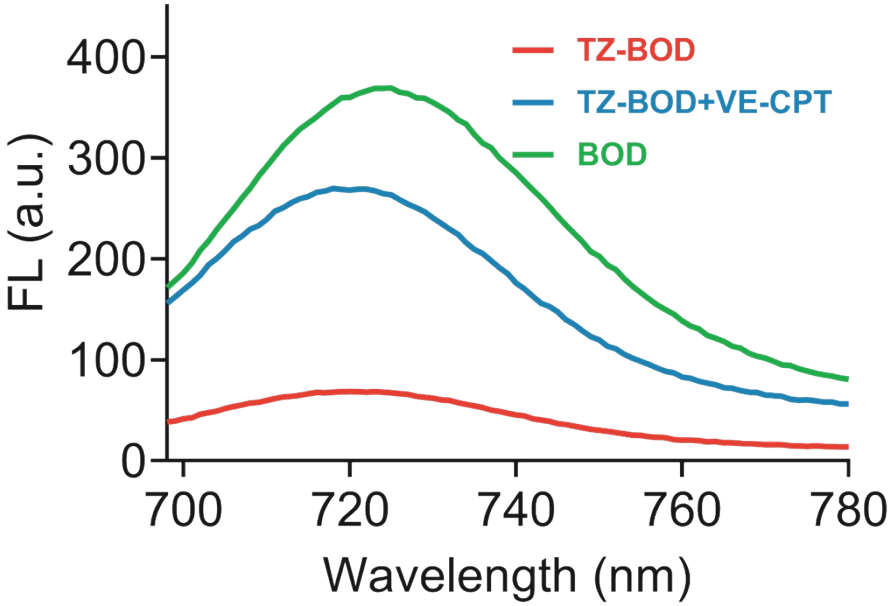


Figure S23. Fluorescence spectra for BOD, TZ-BOD and TZ-BOD treated with VE-CPT in pH 7.4 PBS containing 20% (v/v) THF.

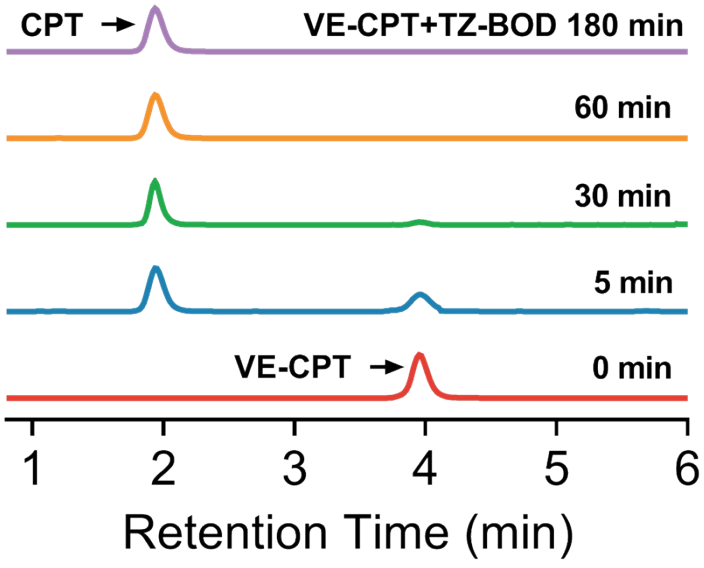


Figure S24. HPLC profiles at different times for VE-CPT treated with TZ-BOD in ultrapure water containing 40% (v/v) acetonitrile.

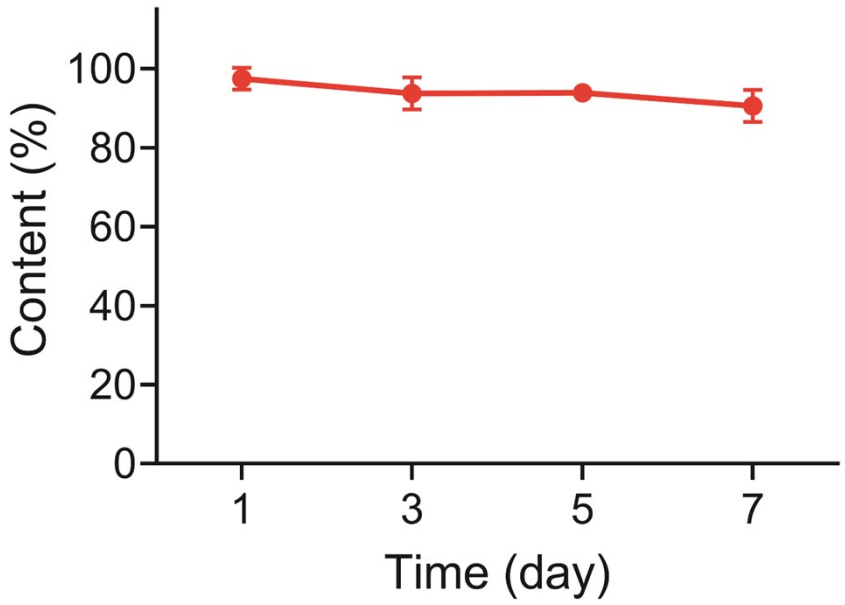


Figure S25. The content of VE-CPT after incubation with RPMI 1640 culture medium for 7 days.

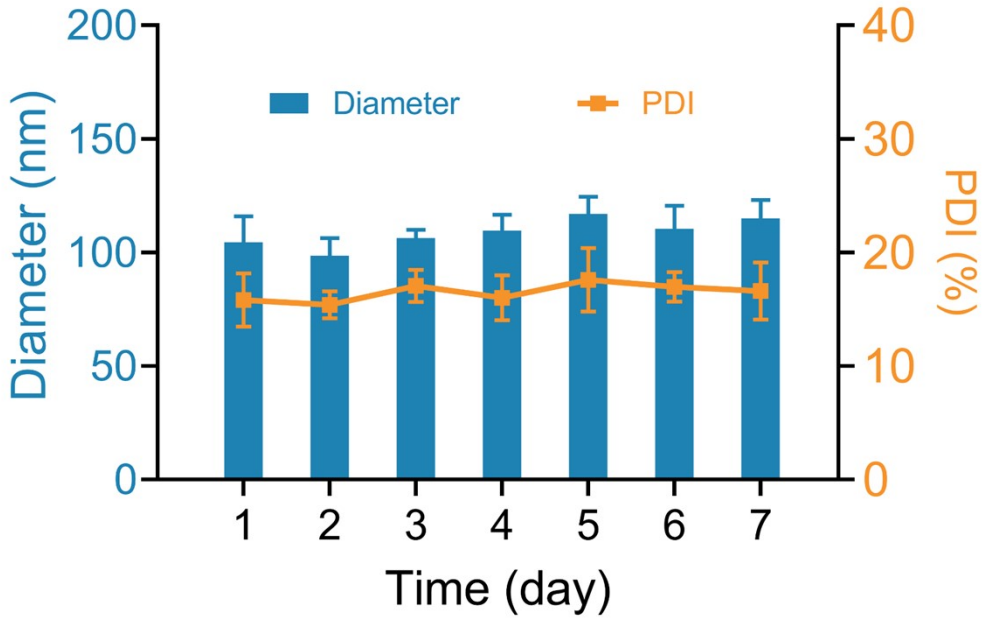


Figure S26. The Size and polydispersity index (PDI) changes of pH@VE-CPT after incubation with RPMI 1640 culture medium for 7 days.

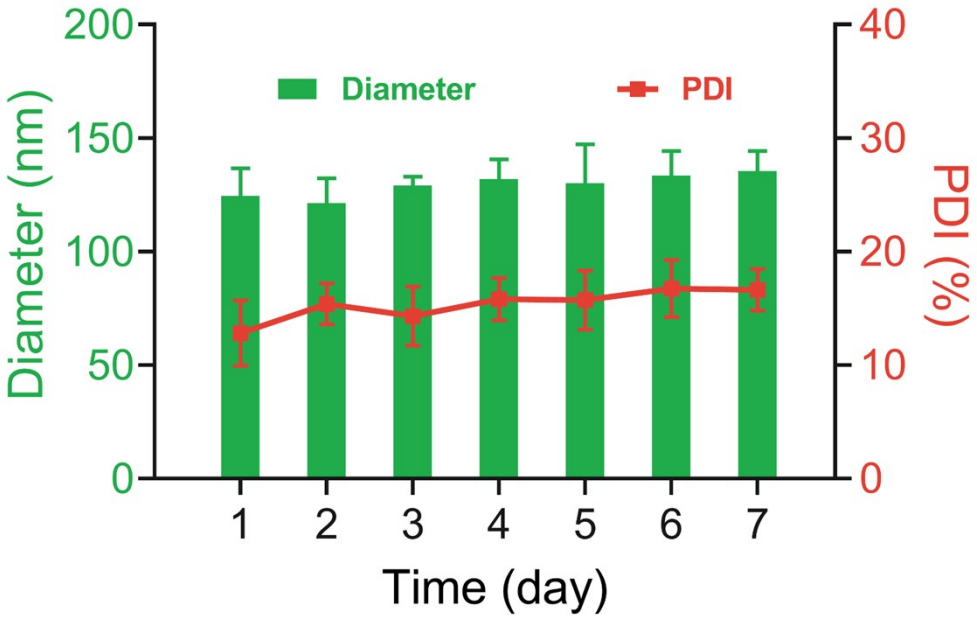


Figure S27. The Size and polydispersity index (PDI) changes of CTB@TZ-BOD after incubation with RPMI 1640 culture medium for 7 days.

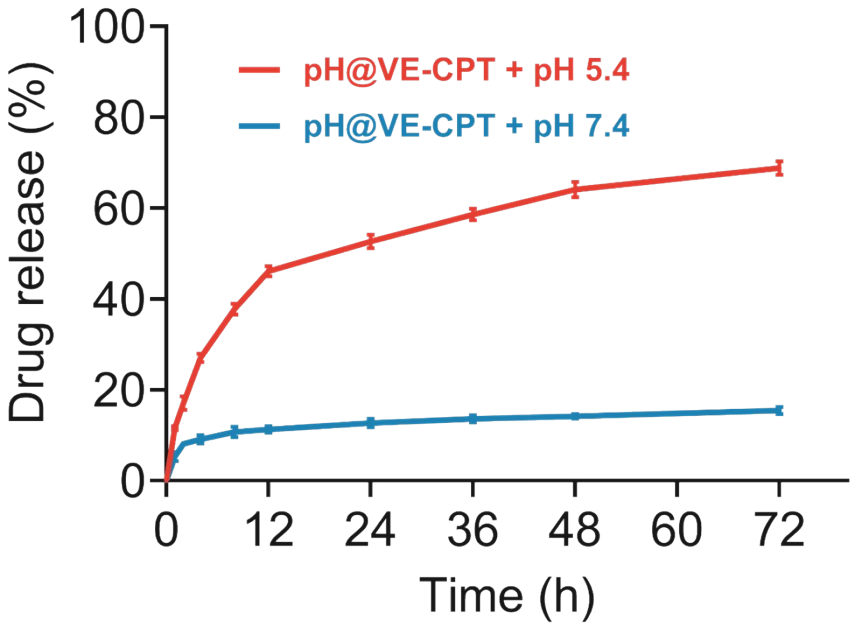


Figure S28. Release profiles of VE-CPT from pH@VE-CPT in PBS at pH 7.4 or pH 5.4.

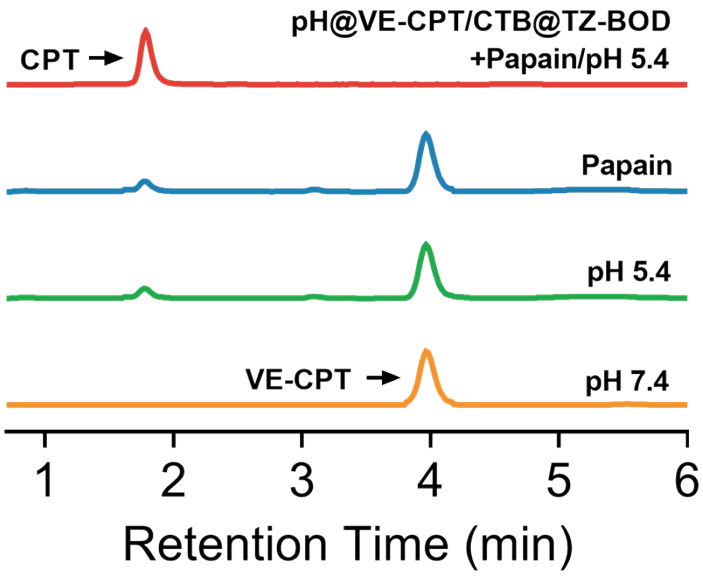


Figure S29. HPLC profiles ($\lambda_{ex}/\lambda_{em}$ = 365/430 nm) at different medium for pH@VE-CPT treated with CTB@TZ-BOD in ultrapure water containing 40% (v/v) acetonitrile for 8 h.

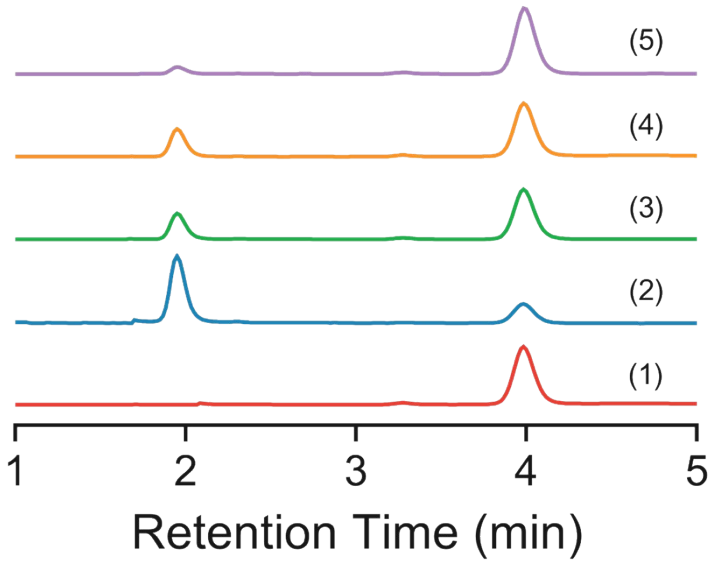


Figure S30. HPLC profiles ($\lambda_{ex}/\lambda_{em}$ = 365/430 nm) for pH@VE-CPT and CTB@TZ-BOD incubated with 4T1cells for 8 h. (1) pH@VE-CPT, (2) pH@VE-CPT+CTB@TZ-BOD, (3) pH@VE-CPT/CTB@TZ-BOD + CA-074-Me, (4) pH@VE-CPT/CTB@TZ-BOD + Baf-A1, (5) pH@VE-CPT/CTB@TZ-BOD + CA-074-Me/Baf-A1. CA-074-Me: a CTB inhibitor. Baf-A1: a low pH inhibitor.

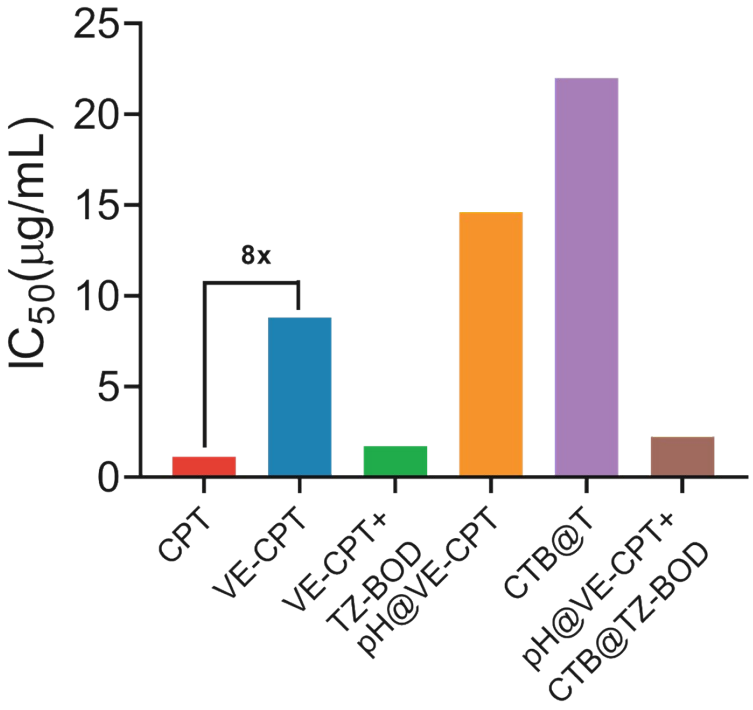


Figure S31. The IC_{50} of different formulations statistics from Fig. 2A and B.

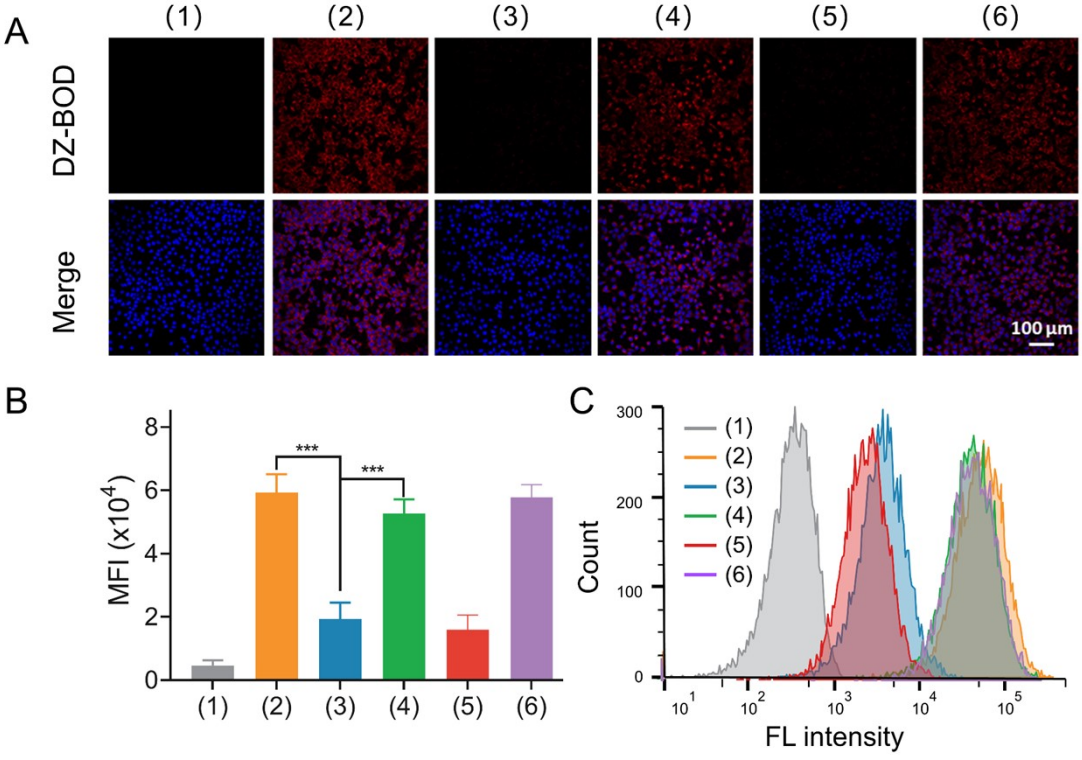


Figure S32. (A) Confocal images (DZ-BOD: red, nuclei: blue), (B) mean fluorescent intensity (MFI) and (C) flow cytometry analysis of 4T1 cells incubated with different formulations. (1) PBS, (2) BOD, (3) TZ-BOD, (4) TZ-BOD + VE-CPT, (5) CTB@TZ-BOD, (6) pH@VE-CPT + CTB@TZ-BOD. ***P < 0.001.

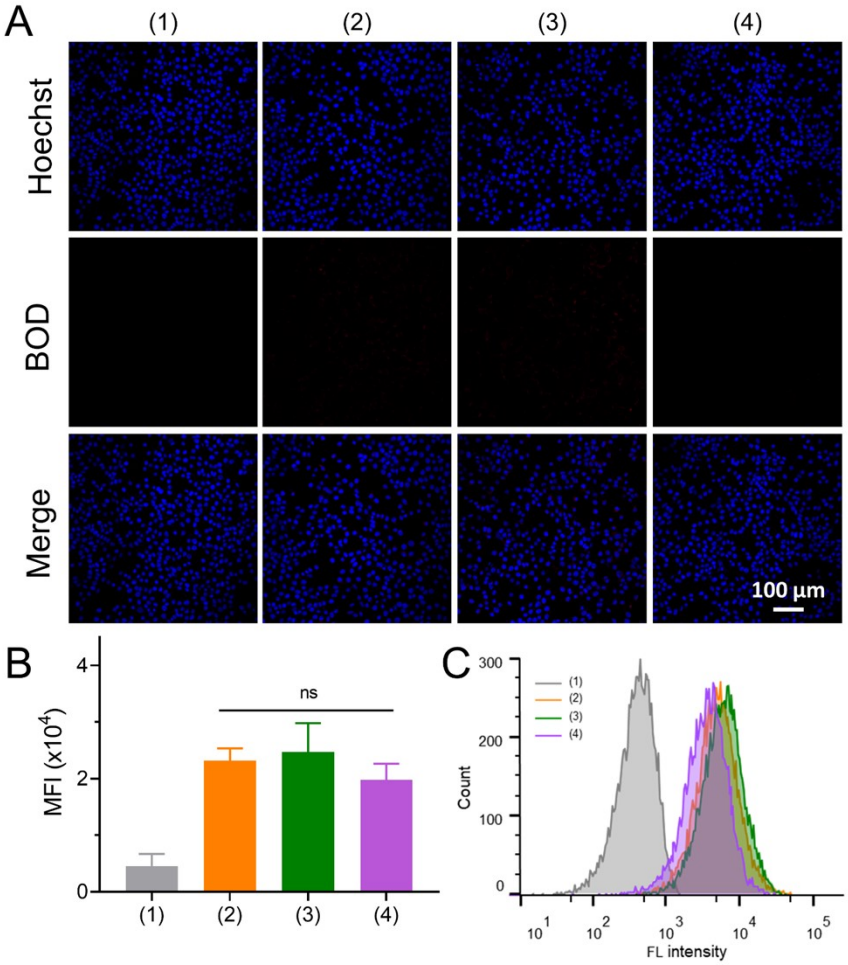


Figure S33. (A) Confocal images, (B) mean fluorescent intensity (MFI) and (C) flow cytometry analysis of 4T1 cells incubated with different formulations for 4 h. ns, non-significant. (1) PBS, (2) pH@V/CTB@TZ-BOD + CA-074-Me, (3) pH@VE-CPT/CTB@TZ-BOD + Baf-A1, (4) pH@VE-CPT/CTB@TZ-BOD + CA-074-Me/Baf-A1.

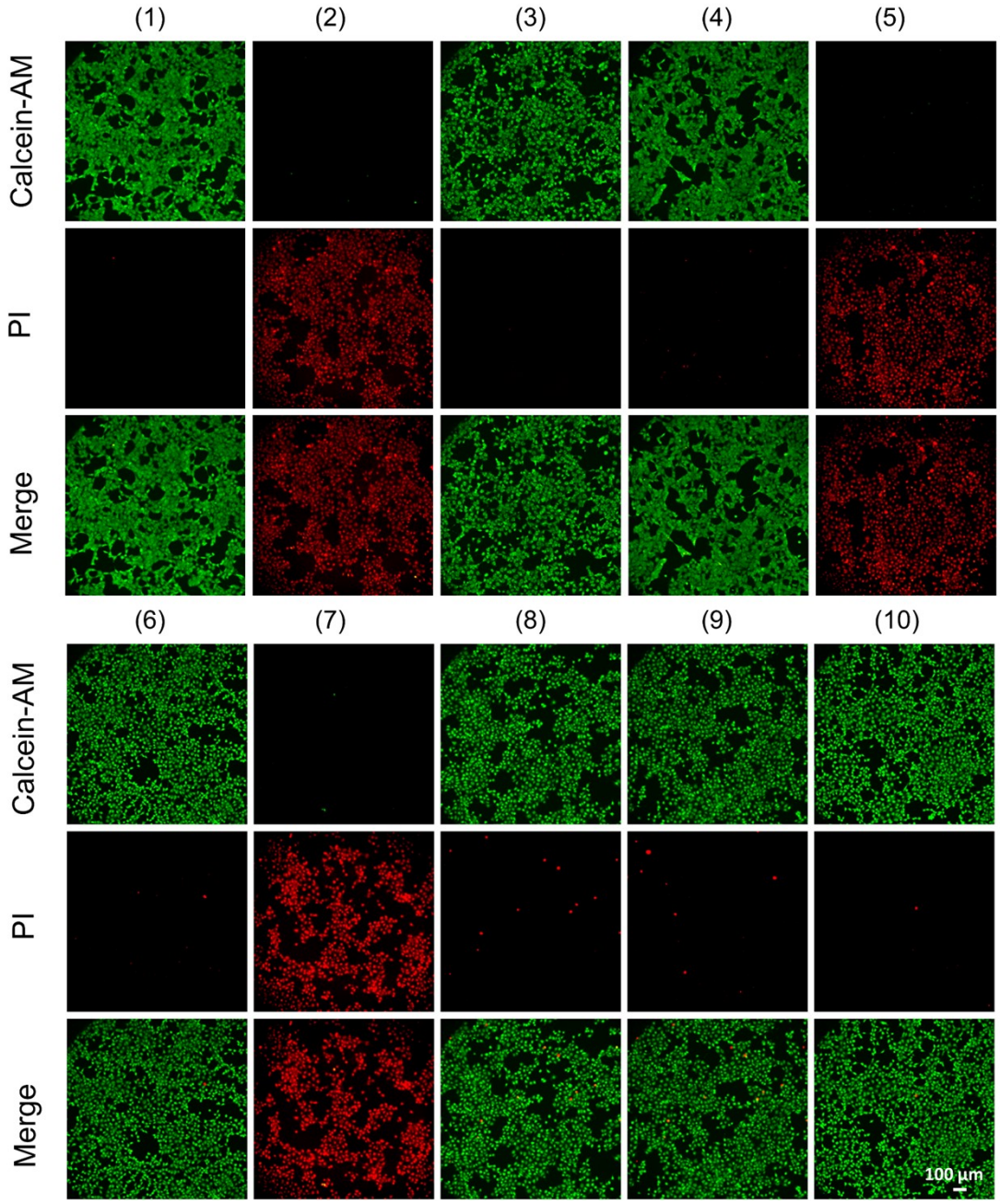


Figure S34. Live /dead staining of 4T1cells treated with different conditions. (1) PBS, (2) CPT, (3) VE-CPT, (4) TZ-BOD, (5) VE-CPT + TZ-BOD, (6) pH@VE-CPT, (7) pH@VE-CPT + CTB@TZ-BOD, (8) pH@VE-CPT/CTB@TZ-BOD + CA-074-Me, (9) pH@VE-CPT/CTB@TZ-BOD + Baf-A1, (10) pH@VE-CPT/CTB@TZ-BOD + CA-074-Me/Baf-A1.

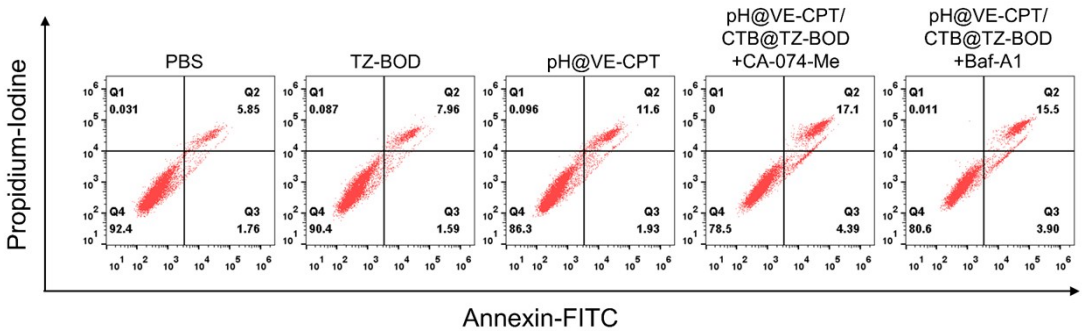


Figure S35. Apoptosis analysis of 4T1 cells incubated with different system compounds.

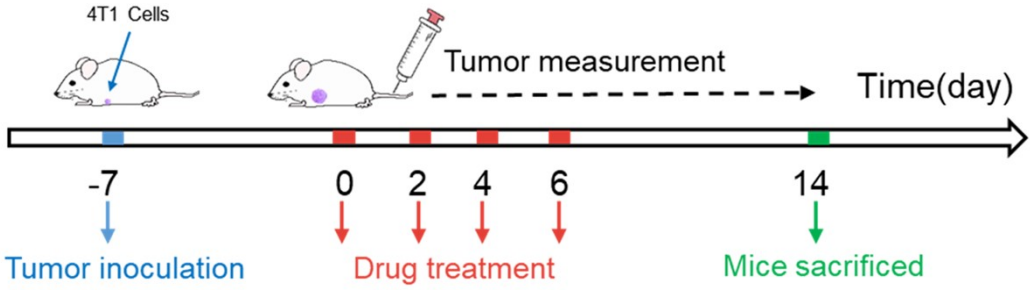


Figure S36. Schematic illustrations of the drug treatment schedule in 4T1-bearing mice.

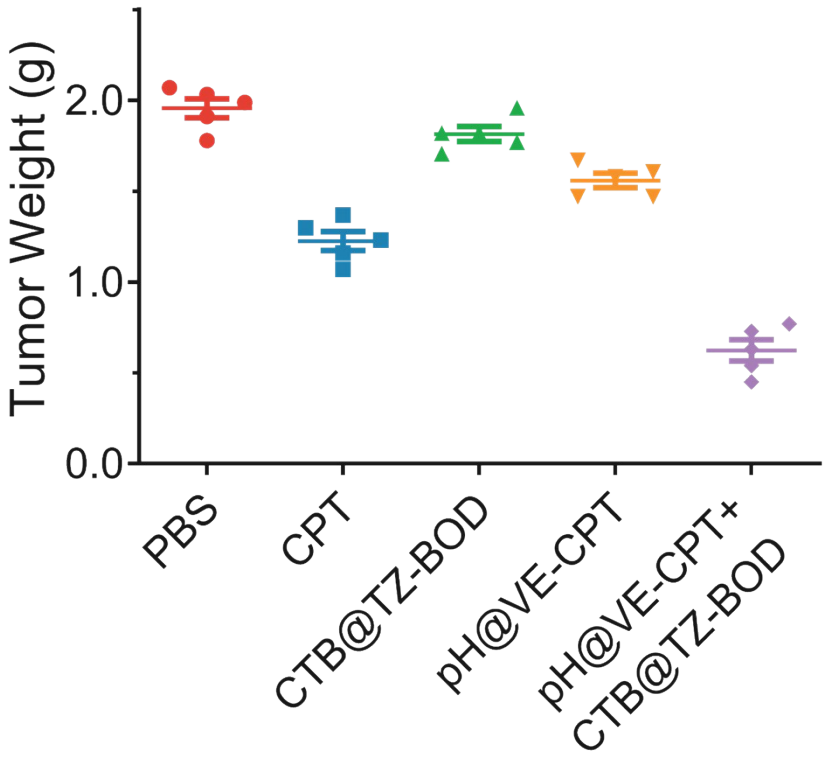


Figure S37. Tumor weight of mice after different treatments.

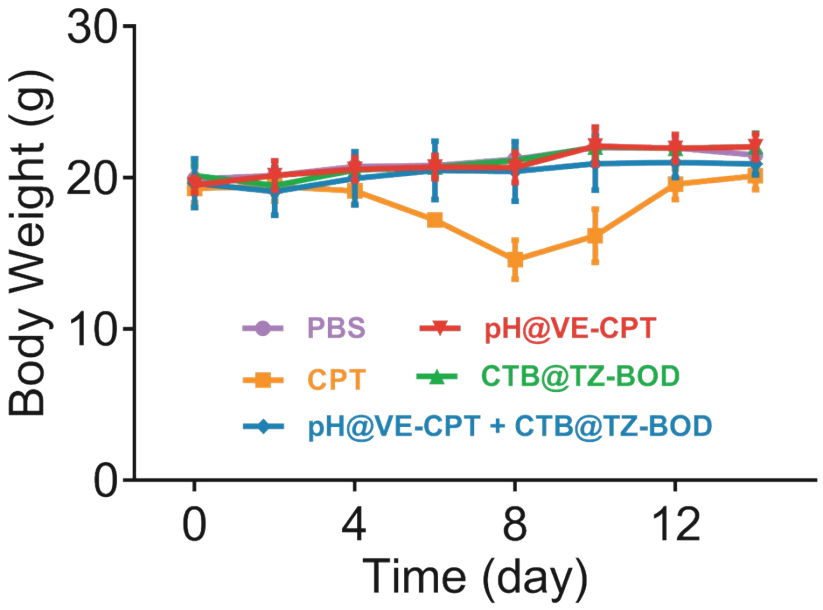


Figure S38. Body weight of mice after different treatments.

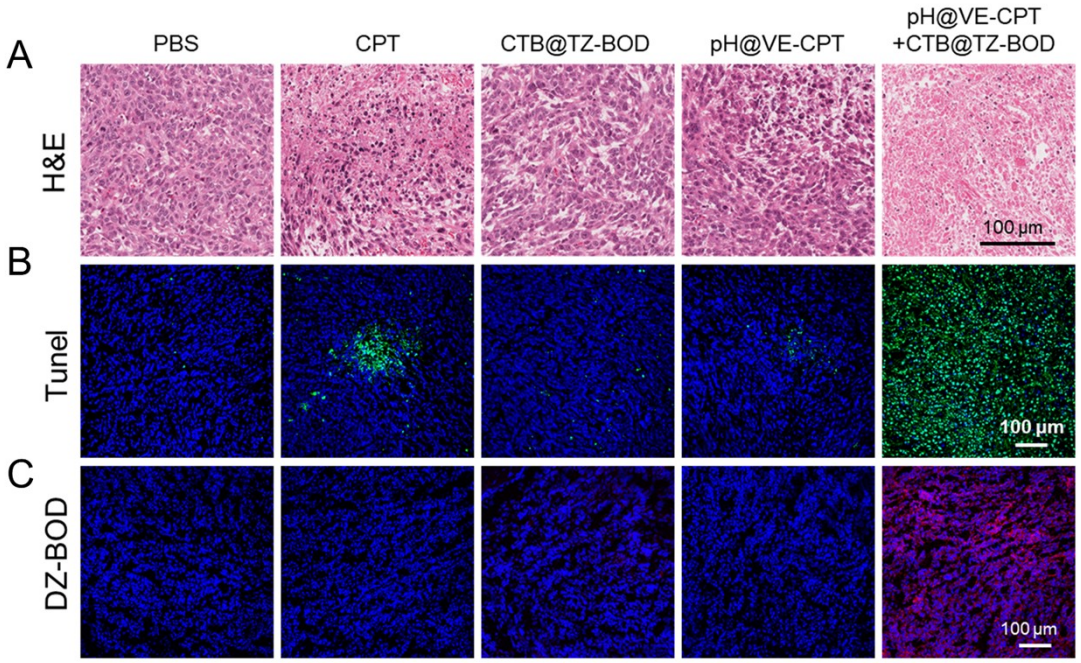


Figure S39. (A) H&E, (B) TUNEL staining (Tunel+ cells: green, nuclei: blue) and (C) the fluorescence at 720 nm of the tumor slices upon treatment of various formulations (DZ-BOD: red).

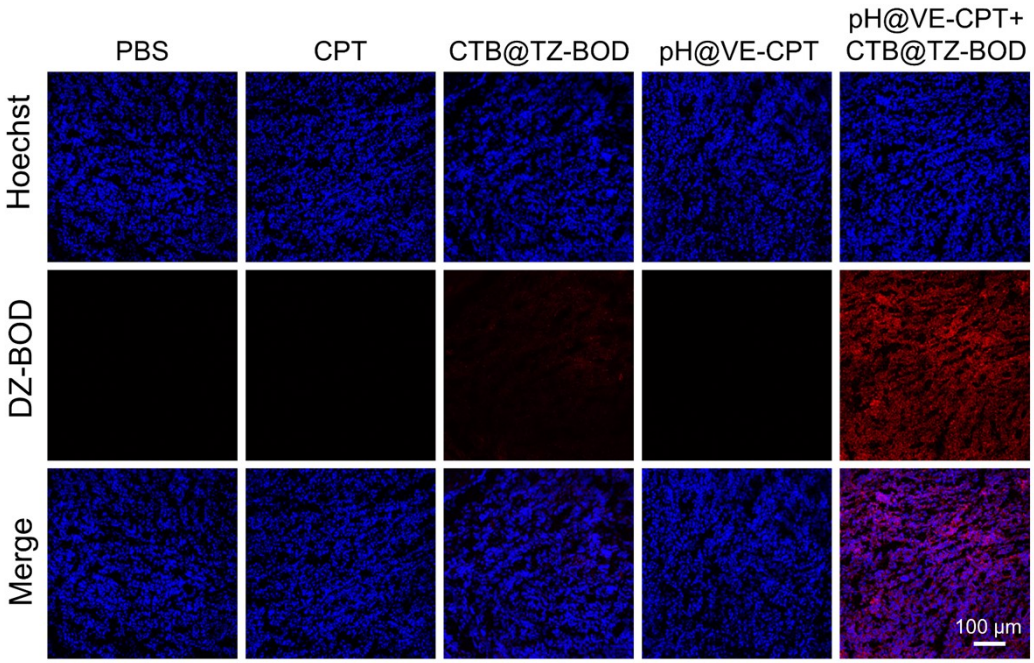


Figure S40. Confocal images of tumor slice after an administration in 4T1 tumor-bearing mice for 24 h.

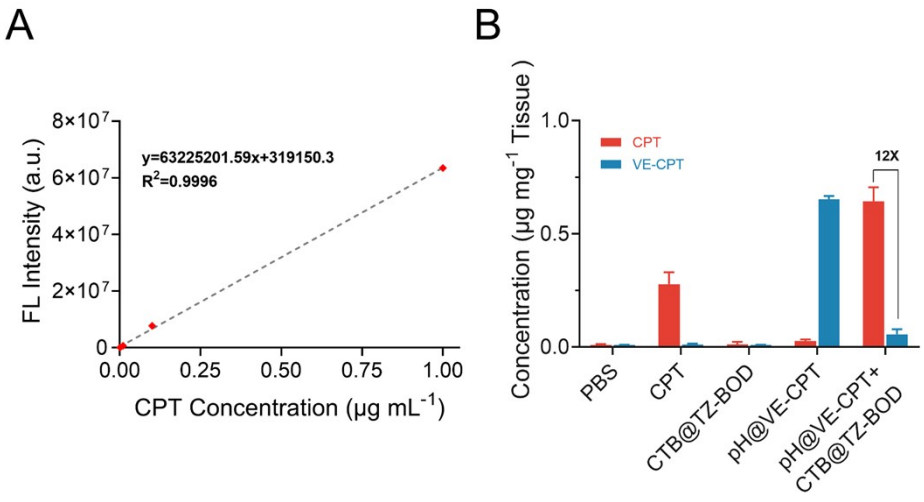


Figure S41. (A) Standard curve of CPT. (B) The concentration of CPT and VE-CPT in the tumor tissue after different treatments.

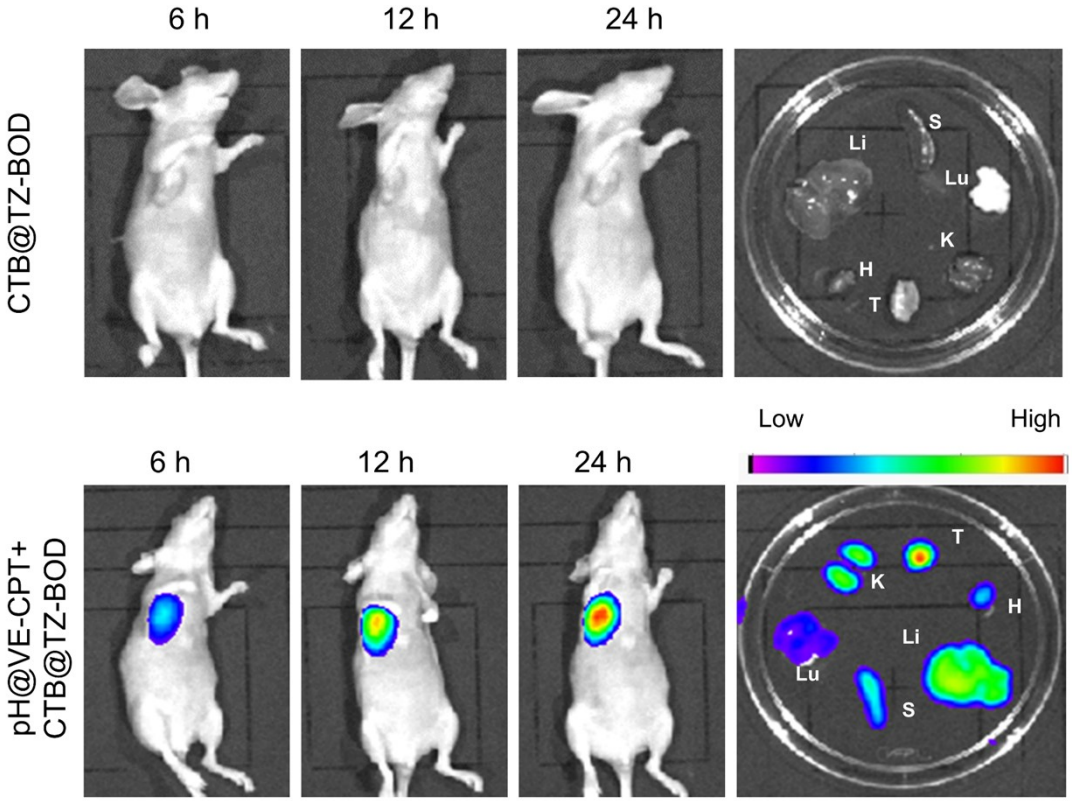


Figure S42. *In vivo* fluorescence images of 4 T1 tumor-bearing nude mice and *ex vivo* fluorescence images of the tumor and major organs. T: tumor, H: heart, Li: liver, S: spleen, Lu: lung, K: kidney.