Supporting Information

Dual-amplified ROS-responsive nanosystem with self-accelerating drug release for synergistic chemotherapy

Experimental Section

Materials and Methods

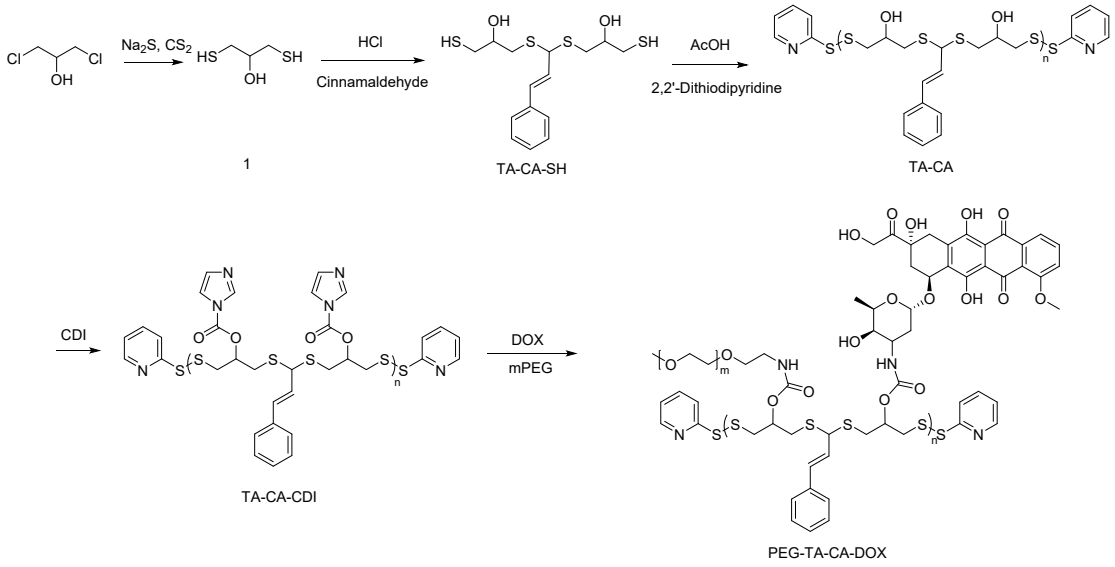
Sodium disulfide (Na_2S) and hydrochloric acid (HCl) were purchased from Aladdin Industrial, Inc. (Shanghai, China). 1,3-Dichloro-2-propanol, carbon disulfide (CS_2), cinnamaldehyde (CA), 2,2'-dithiodipyridine, *N*, *N*'-carbonyldiimidazole (CDI), acetic acid (AcOH), *N*, *N*'-diisopropylethylamine (DIPEA), 4-(hydroxymethyl) phenylboronic acid pinacol ester and triphosgene (BTC) were purchased from Energy Chemical (Shanghai, China). Doxorubicin hydrochloride (DOX·HCl) was purchased from Dalian Meilun Biotechnology Co. LTD. Amine terminated methoxy poly (ethylene glycol) (mPEG-NH_2) was purchased from JenKen Co. LTD. (Beijing, China). Hemicyanine dye $CyNH_2$ was prepared according to the previous literature.[1]

Dulbecco's modifed eagle's medium (DMEM), 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 were obtained from Life Technologies. Intracellular reactive oxygen species (ROS) fluorescent probe 2,7-dichlorodi-hydrofluorescein diacetate (DCFH-DA), mitochondrial membrane potential fluorescent probe (JC-1 dye) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich. Mito-tracker Green were obtained from Beyotime Institute of Biotechnology (China).

NMR spectra were measured on a Bruker ARX 400 NMR spectrometer (Bruker, Billerica, MA). Deuterated dimethyl sulfoxide was used as the solvent for NMR measurements. Nanoparticle hydrodynamic diameters (Dh) measurements were carried out in aqueous solution using a Malvern ZS90 dynamic light scattering instrument (DLS) with a He-Ne laser (633 nm) and 90° collecting optics. The data were analyzed using Malvern Dispersion Technology Software 7.0.2. The absorption spectra were measured on a UV-3802 (UNICO, Shanghai, China) spectrophotometer. Photoluminescence (PL) spectra were measured on a Shimadzu RF-6000 spectrofluorometer (Shimadzu UV-2600, Japan). The polymer dispersity index (PDI) of polymers were determined bygel permeation chromatography (GPC) measurements on a Waters GPC system (Waters, Milford,

MA). The system was equipped with a Waters 2414 refractive index detector and a Waters 1515 HPLC solvent pump. Four Waters styragel high-resolution columns (HR4, HR2, HR1, and HR0.5) were also equipped with molecular weights of 5000−600,000, 500−20,000, 100−5000, and 0−1000 respectively. HPLC grade *N*, *N*'-dimethylformamide (DMF) was used as eluent at 35 °C with a flow rate of 1.0 mL/min. Monodispersed polystyrene standards with a molecular weight range of 1.31×10^3 to 5.51×10^4 were used to generate the calibration curve. Confocal images were acquired by confocal microscope (CLSM, Nikon Ti-E A1, Japan). Absorbance and fluorescence intensity were measured by a multifunctional micropore detection board analysis system (Biotek Cytation5, BioTek, United States). The mice were imaging by In- Vivo Xtreme (Bruker, German).

Synthesis of PEG-TA-CA-DOX



Scheme S1. Synthetic procedures of PEG-TA-CA-DOX. CS_2: carbon disulfide, CDI: *N*, *N*'-carbonyldiimidazole.

Synthesis of 1, 3-dimercapto-2-propanol (1). Compound **1** was prepared by following previously published procedures.[2] To a solution of sodium disulfide nonahydrate (48 g, 200 mmol) in 24 mL water, carbon disulfide (13.2 mL, 220 mmol) was added, the reaction mixture was stirred at 40 °C for 5 h, and then the excess carbon disulfide was removed under reduced pressure. The remaining solution was diluted with 70 mL of water to get a ~33% solution of sodium trithiocarbonate and to this, 1,3-dichloro-2-propanol (6.72 mL, 70 mmol) was added drop-wise at room temperature and the reaction mixture was stirred at 60 °C for 5 h. Afterward, heating was stopped and the solution was cooled to room temperature and then washed with diethyl ether (5 × 200 mL). The aqueous part was collected and acidified by adding concentrated sulphuric acid slowly, then extracted with

diethyl ether (3 × 200 mL). The combined ether extract was washed with water and dried over anhydrous $MgSO_4$. After vacuum distillation at 90 °C, compound **1** was purified as a light-yellow liquid (3.1 g, 35.7%).

Synthesis of TA-CA-SH. Compound **1** (0.51 g, 4.1 mmol), CA (0.27 g, 2 mmol), and hydrochloric acid (8 μL, 9.6 mmol) were added into a glass flask with a magnetic stirrer, and the reaction mixture was stirred at 0 °C for 20 min under argon atmosphere. The reaction mixture was extracted with ethyl acetate, and dried with anhydrous $MgSO_4$ to get the crude product, which was further purified by silica gel chromatography. The desired product came at hexane and ethyl acetate in the volume ratio of 5:1, yielding the product as a colorless liquid (0.25 g, 34.5%). It is worth mentioning that since the terminal sulfhydryl group of the product is easy to be oxidized, the next step will be taken immediately after the product is obtained.

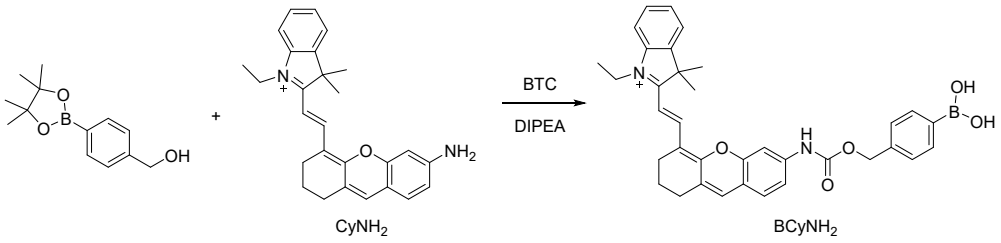
Synthesis of TA-CA. TA-CA-SH (0.5 g, 1.38 mmol) was taken into a glass Schlenk flask with a magnetic stirrer. Then 2,2'-dithiodipyridine (0.55 g, 2.5 mmol) was added, followed by 4 mL dry degassed THF and 2 drops of acetic acid as the catalyst. Afterward, the reaction mixture was stirred at room temperature for 24 h under argon atmosphere. Finally, concentrated the solution and then purified via a gel column using THF as eluents and dried under vacuum to obtain TA-CA as a light-yellow solid (0.4 g, 76.3%).

Synthesis of TA-CA-CDI. TA-CA (0.38 g, 0.1 mmol) and *N*, *N*'-carbonyldiimidazole (CDI, 0.32 g, 2 mmol) were dissolved in *N*, *N*'-dimethylformamide (DMF, 5 mL) and stirred at room temperature for 24 h under argon atmosphere. Subsequently, the solution was evaporated at reduced pressure, purified through a gel column using DMF as eluents, and dried under vacuum to obtain TA-CA-CDI as a light-yellow solid (0.34 g, 59.6%).

Synthesis of PEG-TA-CA-DOX. DOX·HCl (0.09 g, 0.15 mmol) and triethylamine (TEA, 0.03 g, 0.3 mmol) were dissolved in DMF (10 mL), and the solution was stirred at room temperature for 6 h under argon atmosphere. Then, TA-CA-CDI (0.16 g, 0.028 mmol) and mPEG-NH_2 (0.06 g, 0.03 mmol) were dissolved in DMF (2 mL) and added to the reaction mixture at room temperature for 24 h under argon atmosphere. After that, the solution was evaporated at reduced pressure and precipitated with cold diethyl ether, then purified through a gel column using DMF as eluents, dried under vacuum to obtain PEG-TA-CA-DOX as a red solid (0.14 g, 47.4%).

Synthesis of PEG-TA-CA. PEG-TA-CA was synthesized by a similar procedure just without addition of DOX·HCl and obtained as a light-yellow solid (0.09 g, 43.1%).

Synthesis of $BCyNH_2$



Scheme S2. Synthetic procedures of $BCyNH_2$. BTC: triphosgene, DIPEA: *N*, *N*'-diisopropylethylamine.

Synthesis of $BCyNH_2$. To a mixture of triphosgene (0.16 g, 0.31 mmol) and anhydrous acetonitrile (5 mL) was added the mixture of $CyNH_2$ (0.1 g, 0.25 mmol) and DIPEA (0.063 g, 0.48 mmol) in anhydrous acetonitrile (10 mL) dropwise in an ice bath. The resulting solution was refluxed for 4 h and the solvent was removed thoroughly under vacuum. Afterward, 4-(hydroxymethyl) phenylboronic acid pinacol ester (0.13 g, 0.55 mmol) dissolved in anhydrous acetonitrile, was added and the reaction stirred at room temperature for 24 h. After the reaction, the solvent was removed under reduced pressure and the crude product was purified by silica gel chromatography with dichloromethane and methanol in a volume ratio of 20:1 to obtain $BCyNH_2$ as a dark blue solid (0.07 g, 48.7%).

Preparation and characterization of nanoparticles

The $BCyNH_2$-loaded nanoparticles (PTCD@B) were prepared by the nanoprecipitation method. Briefly, a mixture containing PEG-TA-CA-DOX (10 mg) and $BCyNH_2$ (1 mg) were dissolved in 1.0 mL of DMSO, and then added dropwise into 9 mL of Milli-Q ultrapure water (Millipore, 18.2 MU, Bedford) under vigorous stirring. After additional stirring for 2 h, the solution was transferred into a dialysis bag (MWCO 3500) to remove DMSO against ultrapure water for 24 h, and the solution was filtered through a 0.45 μm filter to obtain PTCD@B. The loading efficiency of DOX and $BCyNH_2$ were determined by using UV spectrophotometer based on the standard curve of DOX and $BCyNH_2$. The control nanoparticle, PTC@B, was prepared in a similar way except that the PEG-TA-CA-DOX was replaced with PEG-TA-CA. The self-amplifying degradable polyprodrug nanoparticles, PTCD were prepared by self-assembly of PEG-TA-CA-DOX as a similar method to PTCD@B. The size distribution of PTCD, PTC@B and PTCD@B were performed in triplicate using a zeta sizer (Nano ZS, Malvern Instruments) at 25 °C. After incubation in PBS for different time periods, the diameters of nanoparticles were monitored. The size changes of PTCD@B after treatment with H_2O_2 were determined by DLS.

Fluorescence changes of $CyNH_2$ and DOX

The fluorescence change of $BCyNH_2$ in response to H_2O_2 was investigated by fluorescence spectrophotometer. Briefly, $BCyNH_2$ was treated with 100 μM H_2O_2 at different times, and the fluorescence change of $CyNH_2$ was studied with excitation wavelength at 670 nm.

The solutions of PTCD@B (2 μg/mL DOX, 0.5 μg/mL $CyNH_2$) co-incubation with H_2O_2 (10 mM) for 24 h, the fluorescence changes in DOX channel (Ex: 480 nm) and $CyNH_2$ channel (Ex: 670 nm) were studied by fluorescence spectrophotometer.

ROS-mediated release of DOX and $CyNH_2$

To study the release profile of DOX and $CyNH_2$ from PTCD@B, the solutions of PTCD@B were transferred into the dialysis bags (MWCO 3500 Da) and then were immersed in the PBS (pH 7.4, 30 mL) containing 2% tween 80 with gentle shaking (100 rpm) at 37 °C. After incubation for different time periods, 1 mL of external PBS was collected and replaced with fresh PBS. The collected solutions were lyophilized and analyzed by UV spectrophotometer to determine the DOX and $CyNH_2$ concentrations. The same drug release procedure was carried out in triplicate in the presence of 0.1 mM or 10 mM H_2O_2, respectively.

Cell culture

4T1 mouse breast cancer cells and L929 mouse fibroblasts were cultured in DMEM (37 °C, 5% CO_2), supplemented with 10% FBS (Gibco) and (1%, v/v) penicillin-streptomycin.

Intracellular ROS-generating capacity of CA and $CyNH_2$

The level of intracellular ROS was evaluated using the cell-permeable ROS sensor 2′,7′-dichlorofluorescin diacetate (DCFH-DA), which can be oxidized by ROS to form green fluorescent dichlorofluorescein (DCF). The fluorescence intensity of DCF indicates the level of intracellular ROS. 4T1 cells were seeded into 35 mm glass-bottom dishes at a density of 5×10^4 cells and incubated for 24 h. Afterward, the medium was discarded, and cells were incubated with fresh medium containing $CyNH_2$ (5 μg/mL) or CA (10 μg/mL) for 12 h. Cells were then washed with PBS followed by DCFH-DA staining at 37°C for 30 min. DCF fluorescence images were acquired by CLSM and quantitatively measured by flow cytometry.

Mitochondrial membrane potential study

Mitochondrial membrane potential (MMP) was measured by MitoProbe™ JC-1 Assay Kit. JC-1 could assemble into J-aggregates, with red fluorescence in high MMP mitochondrial matrix, but dispersed into the cytoplasm in a monomeric form with green fluorescence in low MMP mitochondrial matrix. 4T1 cells were seeded into a 35 mm glass-bottom dishes at a density of 5×10^4 cells and incubated overnight. Then the cells were treated with $CyNH_2$ (5 μg/mL) or CA (10 μg/mL) for 6 h at 37 °C, the cells were washed three times with PBS and incubated with 50 nM JC-1 for 30 min. After staining with Hoechst 33342, the cells were washed, incubated with fresh medium, and imaged immediately by CLSM.

Detection of intracellular ROS levels in 4T1 tumor cells after different treatments

The levels of intracellular ROS level after different treatments were evaluated using DCFH-DA. 4T1 cells were seeded into 35 mm glass-bottom dishes at a density of 5 × 10^4 cells and incubated for 24 h. Afterward, the medium was discarded, and cells were incubated with fresh medium containing PTCD, $BCyNH_2$, PTC@B, PTCD@B or PTCD@B+NAC (N-Acetyl-L-cysteine, ROS scavenger) for 4h. Cells were then washed with PBS followed by DCFH-DA staining at 37°C for 30 min. DCF fluorescence images were acquired by CLSM and quantitatively measured by flow cytometry.

Intracellular activation of $CyNH_2$

4T1 cells were seeded into 35 mm glass-bottom dishes at a density of 5 × 10^4 cells and incubated overnight. Cells were incubated with $BCyNH_2$, PTC@B or PTCD@B (in equivalent doses of 2 μg/mL $CyNH_2$) for 2, 4 or 8 h, then the medium was discarded. After staining with Hoechst 33342, the medium was removed, and cells were rinsed with PBS. $CyNH_2$ fluorescence recovery images were acquired by CLSM and quantitatively measured by flow cytometry.

Intracellular localization of $CyNH_2$

Mouse breast cancer cells (4T1) were seeded into 35 mm glass-bottom dishes at a density of 5 × 10^4 cells and incubated overnight. The cells were incubated with $BCyNH_2$ (5 μg/mL) for 4 h and 8 h, then 200 nM Mito-Tracker Green (Thermo Fisher, USA) was added and incubated at 37 °C for 45 min. After staining with Hoechst 33342, the medium was removed, and cells were rinsed with PBS and imaged immediately by CLSM.

Intracellular activation of DOX

4T1 cells were seeded into 35 mm glass-bottom dishes at a density of 5 × 10^4 cells and incubated overnight. Cells were incubated with PTCD or PTCD@B (in equivalent doses of 2 μg/mL DOX) for 4 or 12 h, then the medium was discarded. After staining with Hoechst 33342, the medium was removed, and cells were rinsed with PBS and imaged immediately by CLSM.

To confirm the enhanced release of DOX from PTCD@B in tumor cells. PTCD and PTCD@B co-incubated with 4T1 tumor cells for 1, 2, 4, 8, 12 or 24 h. Then, the cells were collected and lysed, the protein concentration was detected by an enhanced BCA protein assay kit. After that, the cell lysates were extracted with chloroform/acetonitrile component solvent (1.0 mL, 4: 1, v/v) on a vortex mixed for 5 min. Following centrifugation at a speed of 10000 g for 10 min, the organic phase was gathered and dried under vacuum condition and added 100 μL acetonitrile to dissolve. The concentration of DOX was measured by HPLC (Waters e2695, Germany).

***In vitro* cytotoxicity assay**

In vitro cell cytotoxicity was evaluated in 4T1 breast cancer cells and L929 mouse fibroblasts using a standard MTT assay. Briefly, cells at a density of 1×10^4 per well were seeded into 96-well plates. After overnight incubation, PTCD, $BCyNH_2$, PTC@B and PTCD@B (DOX: $CyNH_2$ = 4: 1) were added at different concentrations to the wells for 24 h incubation. Afterward, MTT solution (10 μL, 5 mg/mL) was added to each well for an additional 4 h. The medium containing MTT solution was discarded, and dimethyl sulfoxide (DMSO, 150 μL per well) was added to dissolve formazan crystals for 30 min. Finally, a microplate reader was used to measure absorbance at 490 nm.

To investigate the synergistic effects of DOX and $CyNH_2$, *In vitro* cell cytotoxicity was evaluated in 4T1 tumor cells using a standard MTT assay. DOX, $CyNH_2$ and DOX + $CyNH_2$ (4: 1) were added at different concentrations to the wells for 24 h incubation. Combination index (CI) values were calculated by CompuSyn software (Version 1.4, Combo-Syn Inc., U.S.) for drug combinations with a fractional effect between 0.1 and 0.9 (10–90% of cell growth inhibition relative to control).

Animals and tumor model

Female BALB/c mice (5-6 weeks old) and BALB/c nude mice 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 a subcutaneous breast tumor model. At the end of experiments, all mice were killed by CO_2 inhalation. All animal studies were approved by the Ethics Committee of the South China University of Technology and followed the guidelines of “Guangdong Province Experimental Animal Policies and Regulations” and “National Experimental Animal Management Regulations”.

Tumor accumulation

4T1 tumor-bearing nude mice were randomly divided into five groups and intravenously injected with $BCyNH_2$, PTC@B and PTCD@B (in equivalent doses of 2.5 mg/kg $CyNH_2$). The *in vivo* fluorescence distribution in tumor-bearing mice was detected at predetermined time points by a Xenogen IVIS® Lumina system. After 36 h post-injection, the tumors were harvested and detected for *ex vivo* imaging.

ROS generation *in vivo*

4T1 tumor-bearing mice were randomly divided into five groups and intravenously injected with PBS, PTCD, $BCyNH_2$, PTC@B and PTCD@B. After 24 h post-injection, DCFH-DA was intratumorally injected. 4 hours later, the tumors were harvested, frozen in liquid nitrogen, embedded in optimum-cutting temperature (OCT) tissue compound (Sakura, Tokyo, Japan), and sectioned into 8 μm slices, stained with Hoechst 33342 and observed by CLSM.

***In vivo* antitumor activity assessment**

BALB/c mice were inoculated with 4T1 cells (5×10^5 cells per mouse) in the right mammary pad. When the tumor volume reached to approximately 100 mm^3, the mice were randomly divided into five groups and intravenously injected with PBS, PTCD (5mg/kg DOX), $BCyNH_2$ (5mg/kg $CyNH_2$), PTC@B (5mg/kg $CyNH_2$) and PTCD@B (4 mg/kg DOX and 1 mg/kg $CyNH_2$) for four times, respectively. The tumor volumes and body weights were measured every other day. The tumor volume was calculated by using the formula: V = L×W×W/2 (L, the longest dimension; W, the shortest dimension). The mice were sacrificed 14 days and the histological analysis was carried out after treatments. The tumor tissues of the mice in five groups were excised, fixed in 4% paraformaldehyde, embedded in paraffin, sliced and stained with H&E. Tumor sections were also stained with TUNEL apoptosis detection kit assay and then observed by CLSM.

Statistical Analysis

All the data were presented as mean ± standard deviation. One-way analysis of variance (ANOVA) was used to assess the significance of the difference. ns = not significant, $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

References

1. K. Wang, X. Xiao, M. Jiang, J. Li, J. Zhou and Y. Yuan, Small, 2021, **17**, e2102610.
2. (*a*) H. Ma, J. Zhang, Z. Zhang, Y. Liu and J. Fang, Chem Commun (Camb), 2016, **52**, 12060-12063; (*b*) L. Zhang, D. Duan, Y. Liu, C. Ge, X. Cui, J. Sun and J. Fang, J Am Chem Soc, 2014, **136**, 226-233.

Supporting Figures

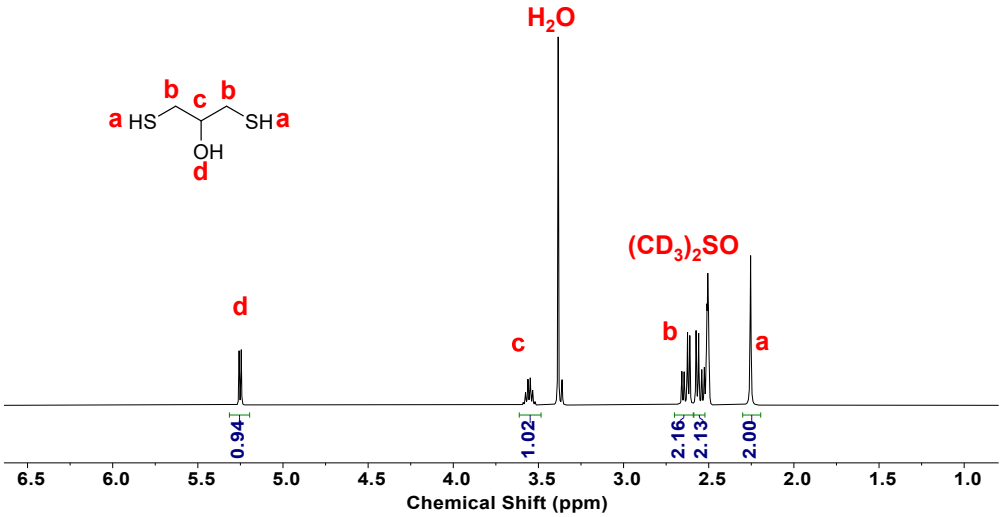


Figure S1. ^{1}H NMR spectrum of 1, 3-dimercapto-2-propanol (compound **1**) in DMSO-d_6.

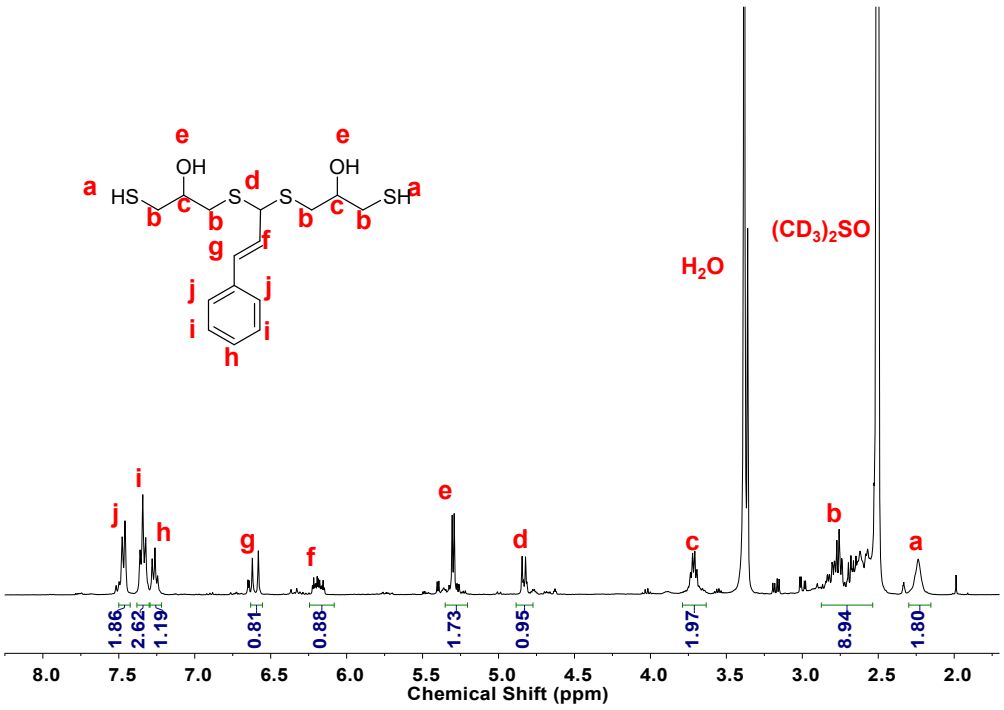


Figure S2. ^{1}H NMR spectrum of TA-CA-SH in DMSO-d_6.

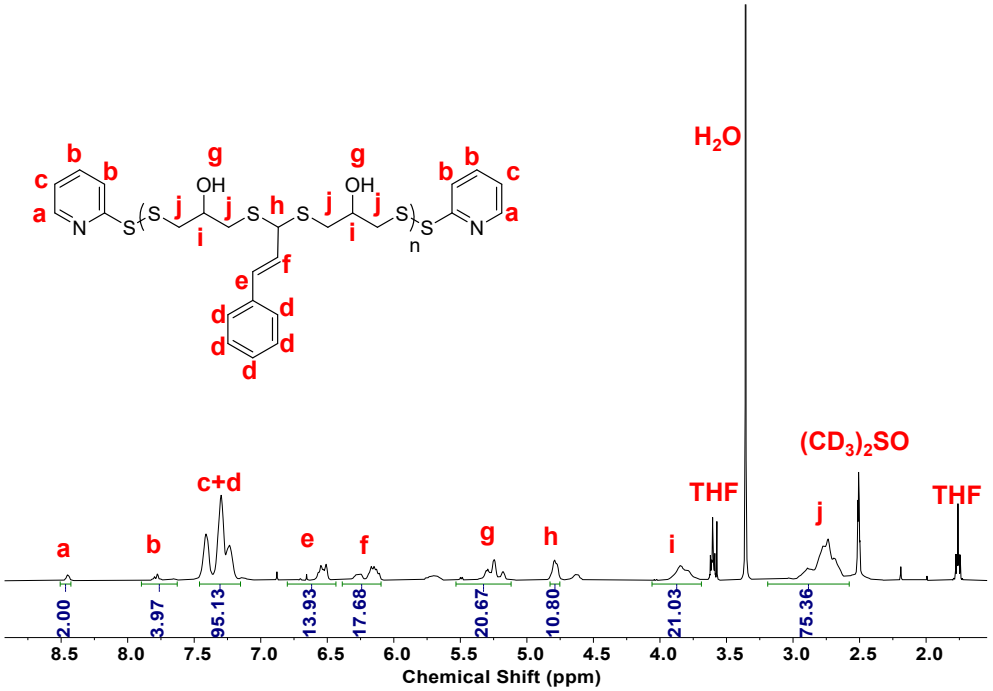


Figure S3. ^{1}H NMR spectrum of TA-CA in DMSO-d_6.

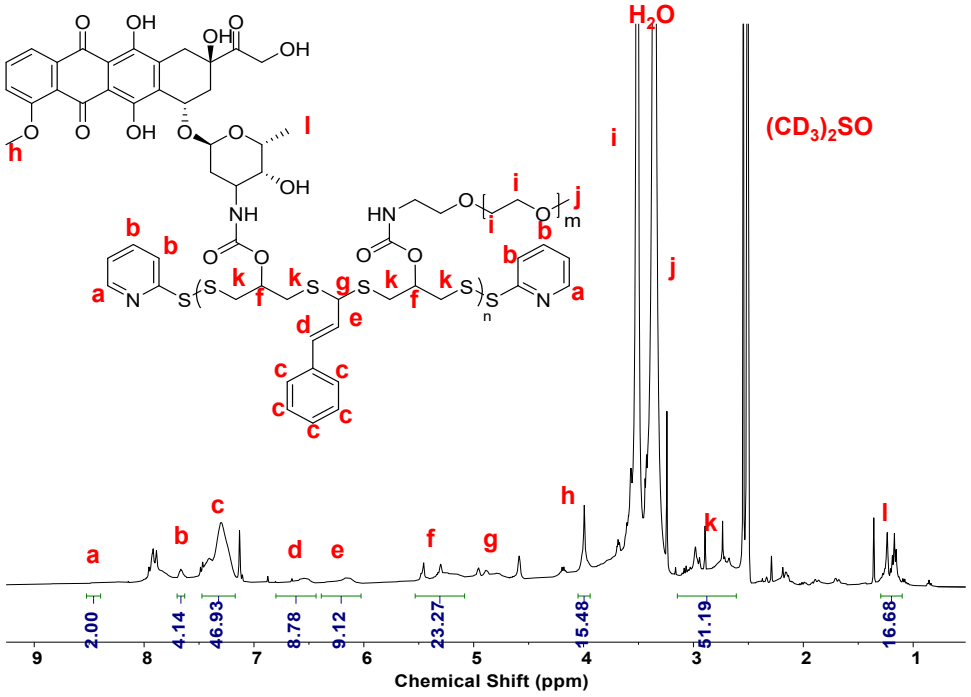


Figure S4. ^{1}H NMR spectrum of PEG-TA-CA-DOX in DMSO-d_6.

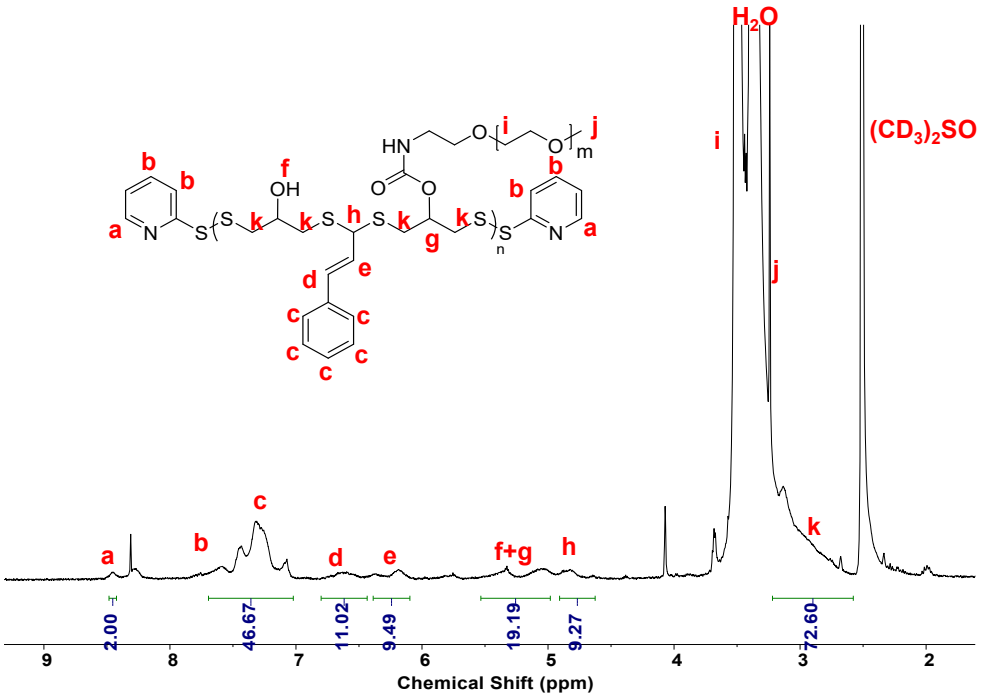


Figure S5. ^{1}H NMR spectrum of PEG-TA-CA in DMSO-d_6.

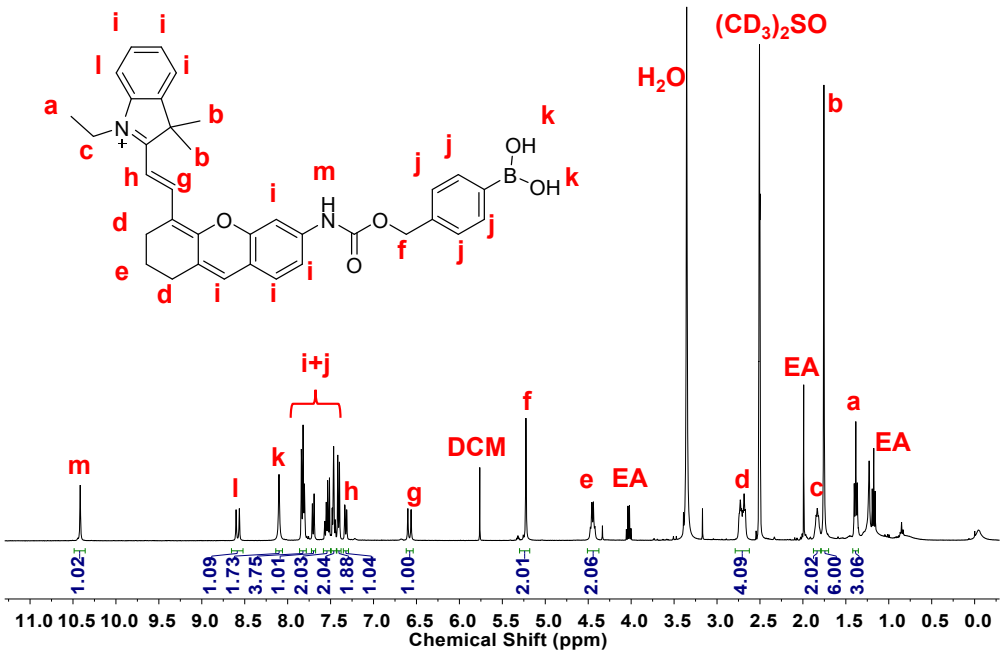


Figure S6. ^{1}H NMR spectrum of BCyNH$_2$ in DMSO-d_6.

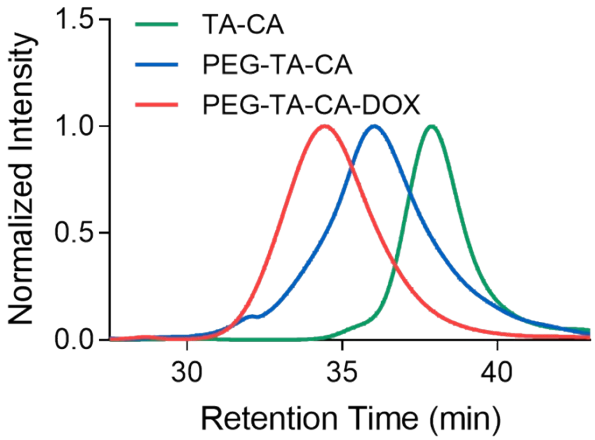


Figure S7. GPC profiles of TA-CA, PEG-TA-CA and PEG-TA-CA-DOX.

Table S1. Characterization of PEG-TA-CA-DOX and PEG-TA-CA.

□	M_n (g/mol) [a]	M_w (g/mol) [b]	M_n (g/mol) [b]	□
Samples	(NMR)	(GPC)	(GPC)	PDI[b]
PEG-TA-CA-DOX	10540	11200	8900	1.25
PEG-TA-CA	7450	7600	5450	1.40

[a]Number averaged molecular weights of polymer determined by ^{1}H NMR. [b]Molecular weights and molecular weight distributions, Mw/Mn, were evaluated by GPC with polystyrene standards.

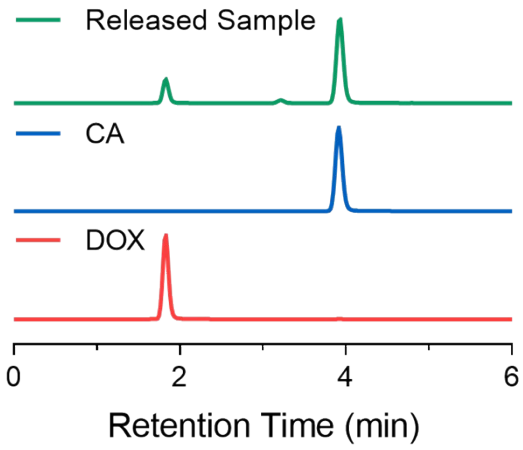
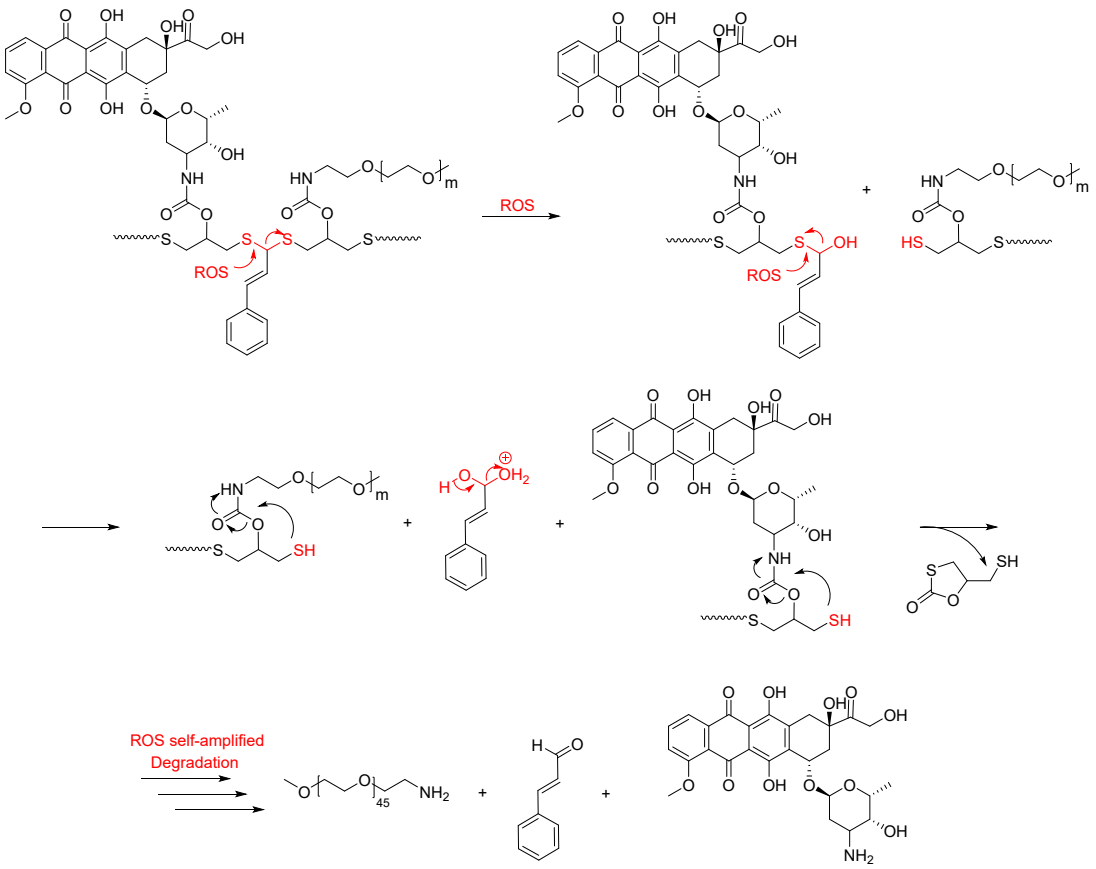


Figure S8. HPLC elution spectra of DOX, CA, and released DOX and CA from PEG-TA-CA-DOX after co-incubation with H_2O_2 (10 mM). The red line is the standard sample of DOX. The blue line is the standard sample of CA. The green line is the testing sample. The retention time (t_R) of DOX and CA is 1.8 min and 3.9 min respectively. Chromatographic conditions: Samples were analyzed at 290 nm by HPLC on Waters C-18 reverse phase column (4.6 × 250 mm, 5 µm) with a mobile phase of acetonitrile/5% acetic acid (v/v, 75/25, 1.0 ml/min).



Scheme S3. Proposed degradation mechanism of PEG-TA-CA-DOX triggered by ROS.

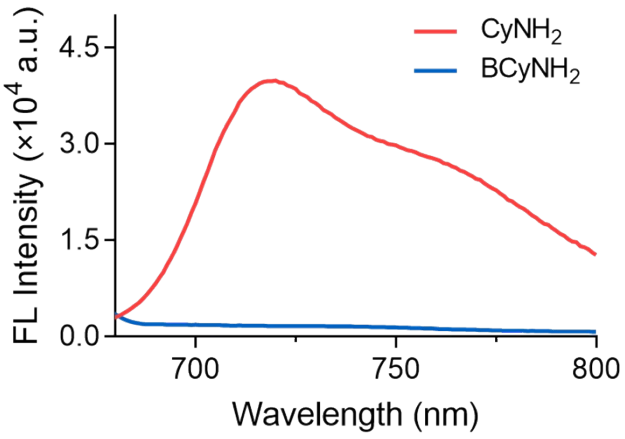


Figure S9. Fluorescence spectra of $BCyNH_2$ and $CyNH_2$.

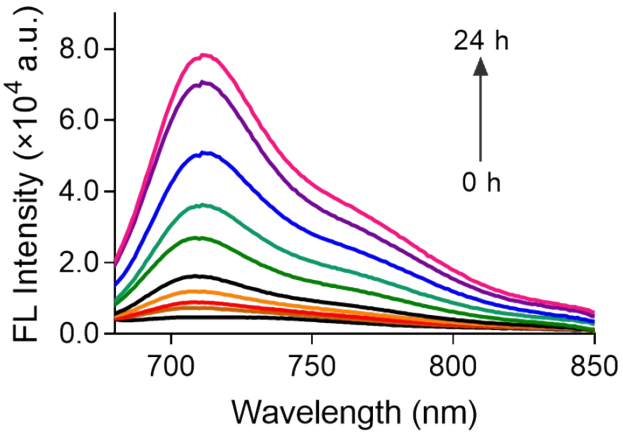
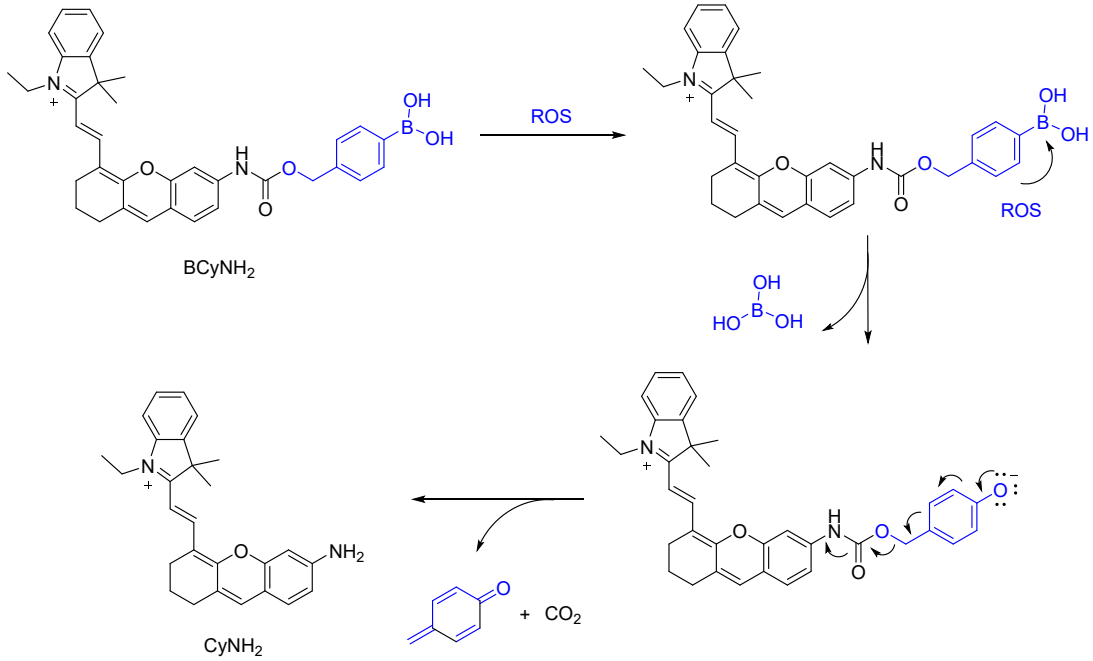


Figure S10. Fluorescence spectra of $BCyNH_2$ (10 μM) after being treated with H_2O_2 (100 μM) for different time.



Scheme S4. Proposed degradation mechanism of $BCyNH_2$ triggered by ROS.

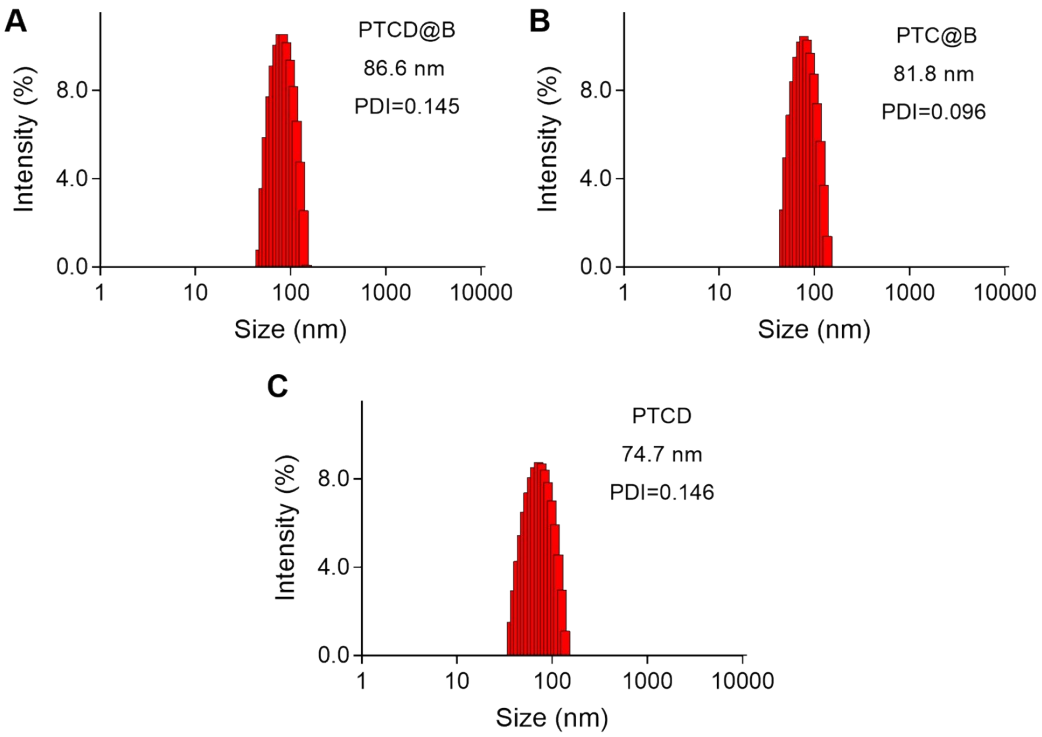


Figure S11. Dynamic light scattering (DLS) characterization of PTCD@B (A), PTC@B (B) and PTCD (C).

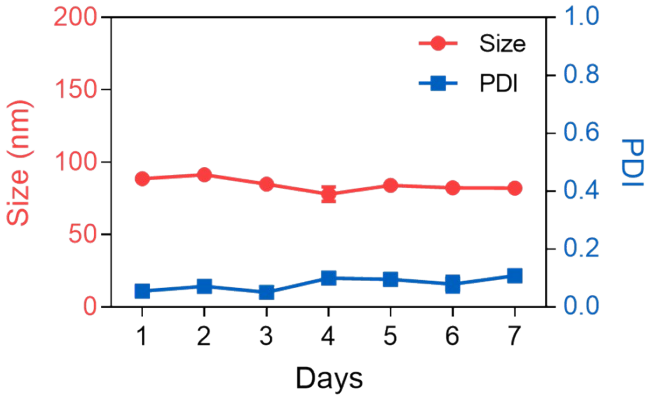


Figure S12. Size and polymer dispersity index (PDI) changes of PTCD@B after incubation in PBS for 7 days.

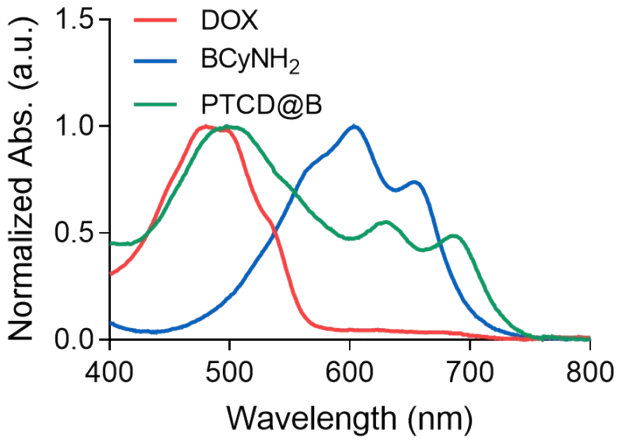


Figure S13. UV-vis absorption spectra of DOX, $BCyNH_2$ and PTCD@B.

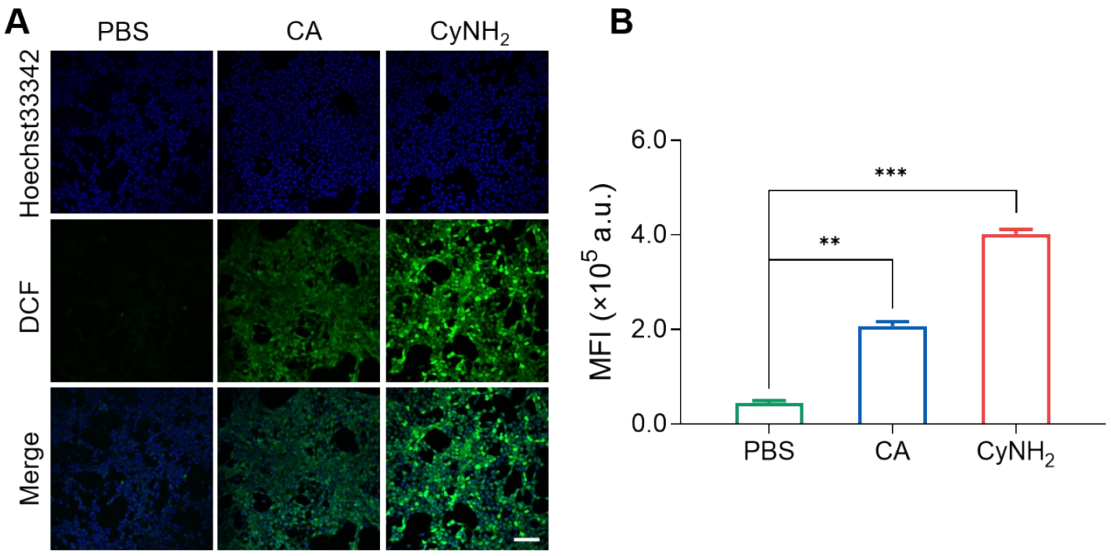


Figure S14. (A) CLSM images of 4T1 cells treated with PBS, CA and $CyNH_2$ by DCFH-DA staining. Scale bar: 100 μm. (B) Flow cytometry of 4T1 cells treated with PBS, CA and $CyNH_2$ by DCFH-DA staining.

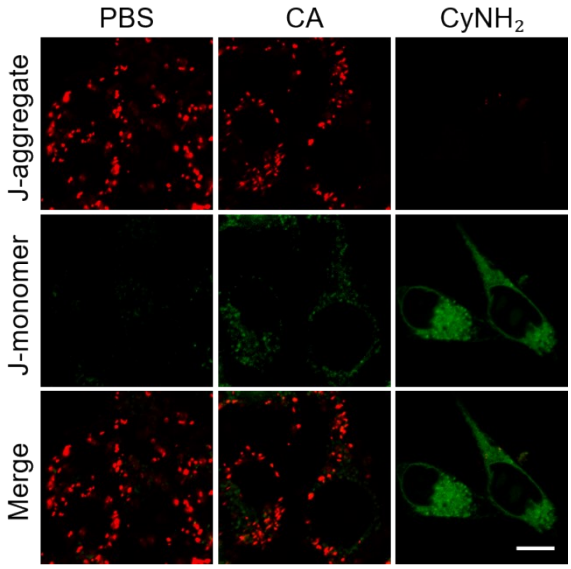


Figure S15. CLSM images of the mitochondrial membrane potential change of 4T1 cells treated with PBS, CA, $CyNH_2$ using JC-1 as the probe. Scale bar: 10 μm.

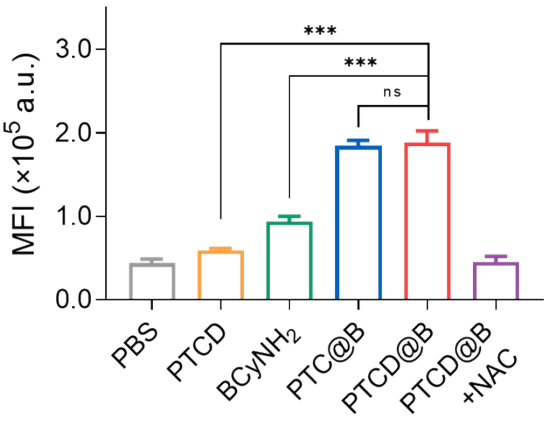


Figure S16. Flow cytometry of 4T1 cells treated with PBS, PTCD, $BCyNH_2$, PTC@B, PTCD@B and PTCD@B + NAC by DCFH-DA staining.

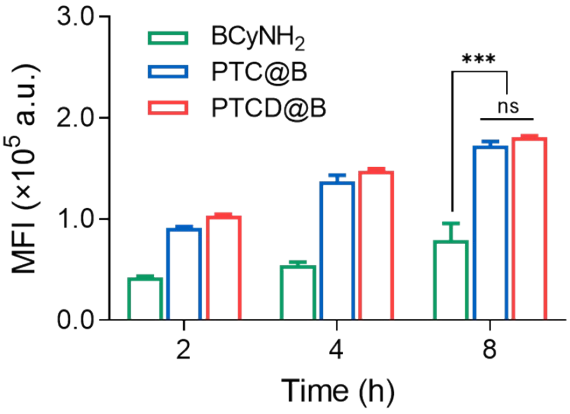


Figure S17. Flow cytometry of $CyNH_2$ activation in 4T1 cells treated with $BCyNH_2$, PTC@B and PTCD@B for different incubation times.

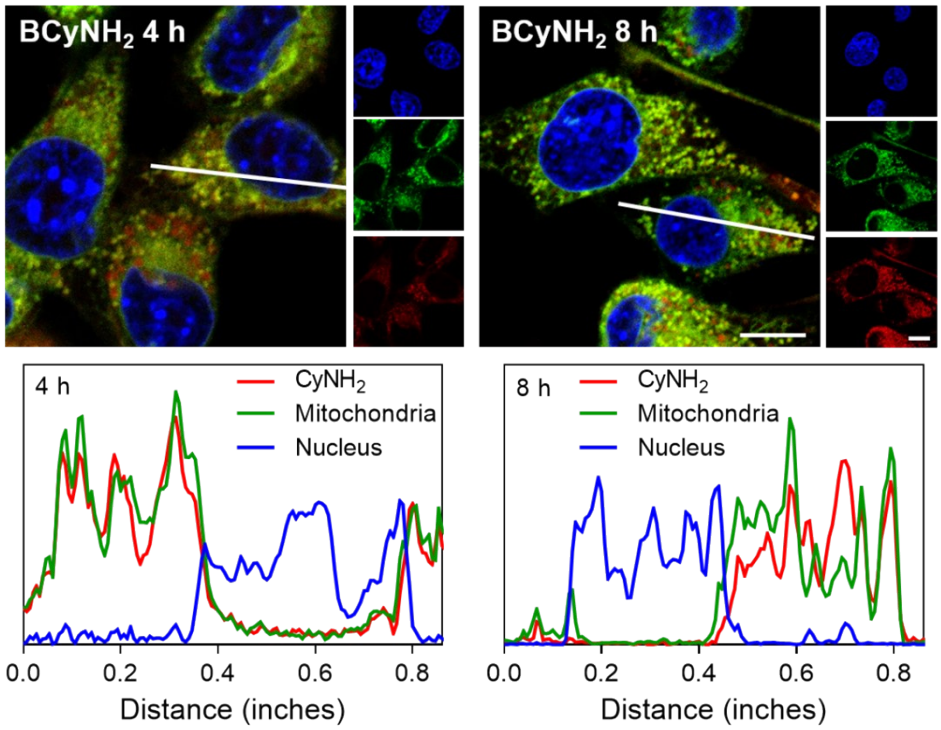


Figure S18. CLSM images and Image J analysis of the colocalization of $CyNH_2$ with mitochondria in 4T1 cells treated with $BCyNH_2$ for 4 h and 8 h. The blue fluorescence is from Hoechst 33342, the green fluorescence is from mitochondria tracker green, and the red fluorescence is from $CyNH_2$. Scale bar: 10 μm.

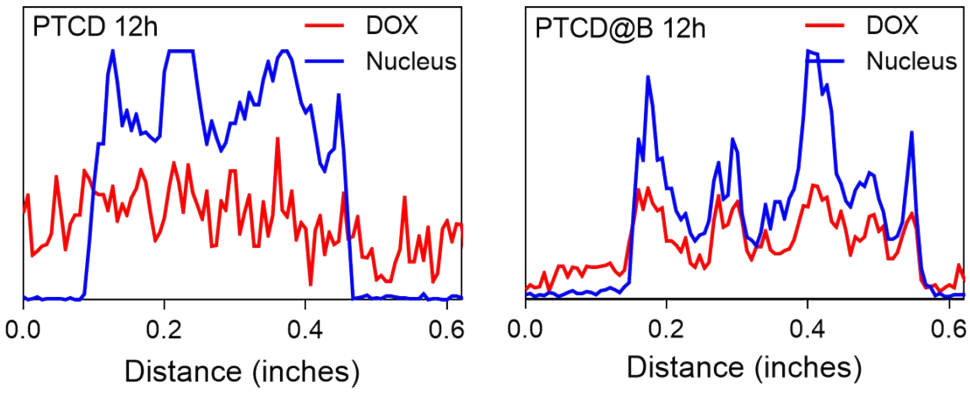


Figure S19. Image J analysis of the colocalization of DOX and Hoechst 33342 stained nucleus in 4T1 cells treated with PTCD and PTCD@B for 12 h. Corresponding Figure 2D.

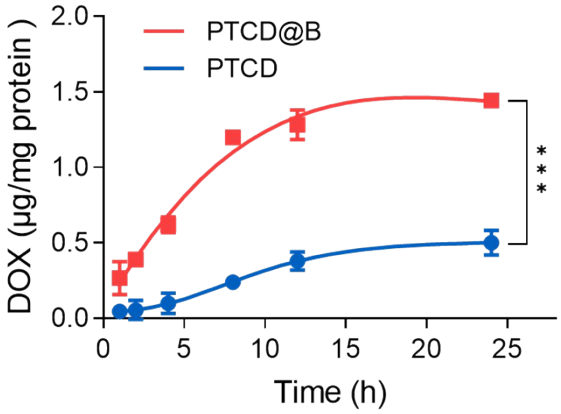


Figure S20. HPLC analysis of DOX activation in 4T1 cells treated with PTCD and PTCD@B for different incubation times.

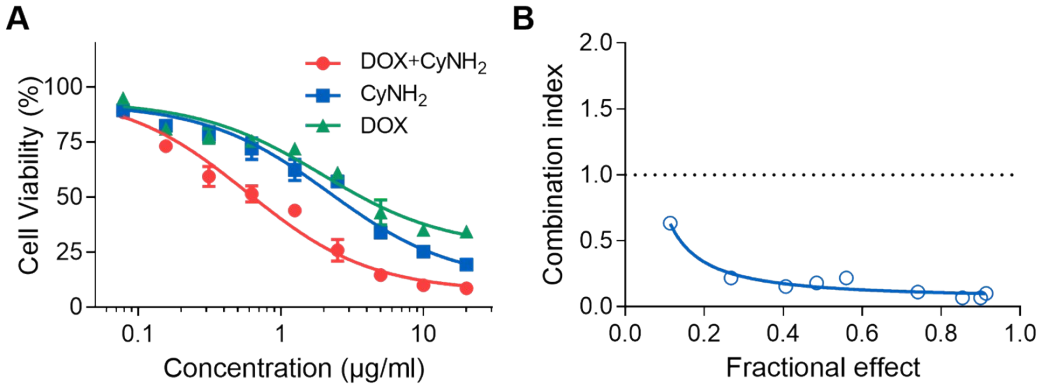


Figure S21. (A) Cytotoxicity of 4T1 cells treated with different concentrations of DOX, $CyNH_2$ and DOX + $CyNH_2$ (the ratio of DOX to $CyNH_2$ drug combination at 4: 1) for 24 h. (B) Combination index (CI) values were determined by CompuSyn software (Version 1.4, Combo-Syn Inc., U.S.) for drug combinations with a fractional effect between 0.1 and 0.9 (10–90% of cell growth inhibition relative to control). CI < 1, CI = 1 (dashed line) and

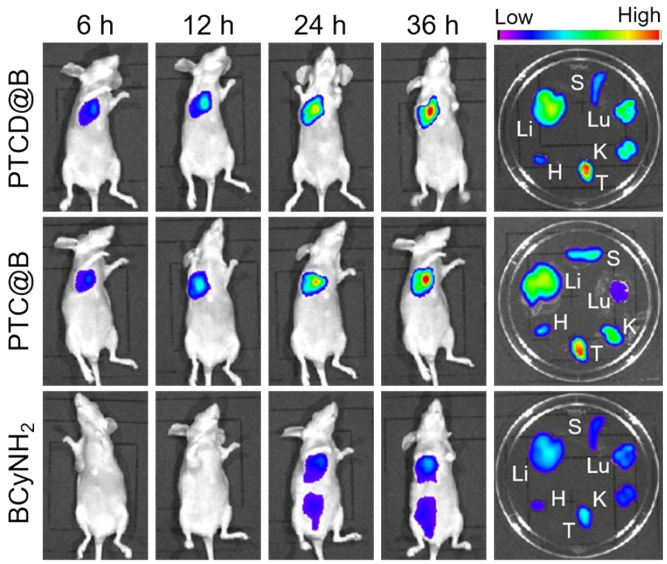


Figure S22. *In vivo* fluorescence imaging of 4T1 tumor-bearing nude mice after intravenous injection of various formulations and *ex vivo* fluorescence images of the tumor and major organs.

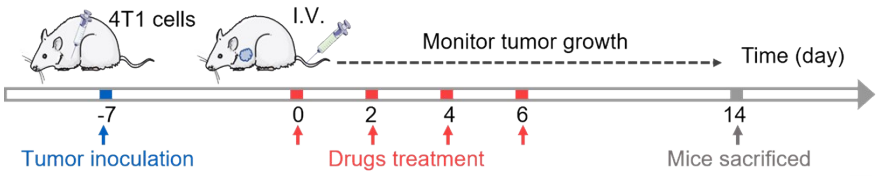


Figure S23. Schematic illustrations of the drug treatment schedule in 4T1-bearing mice.

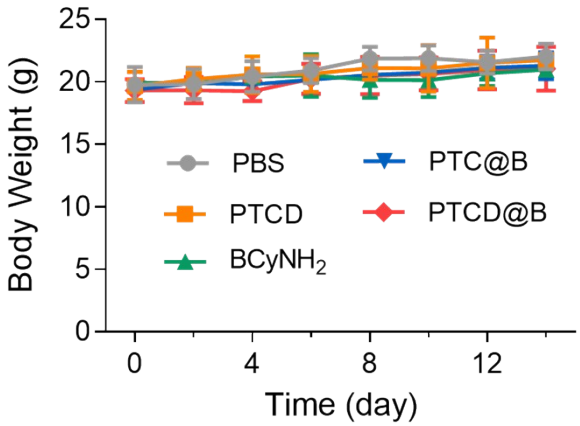


Figure S24. Body weight changes of mice after various treatments.

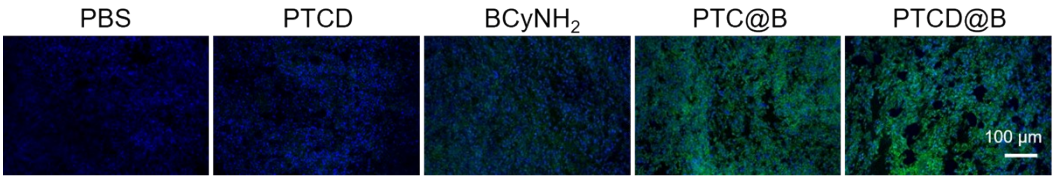


Figure S25. CLSM images of ROS level investigation in tumors after treatment with various formulations.

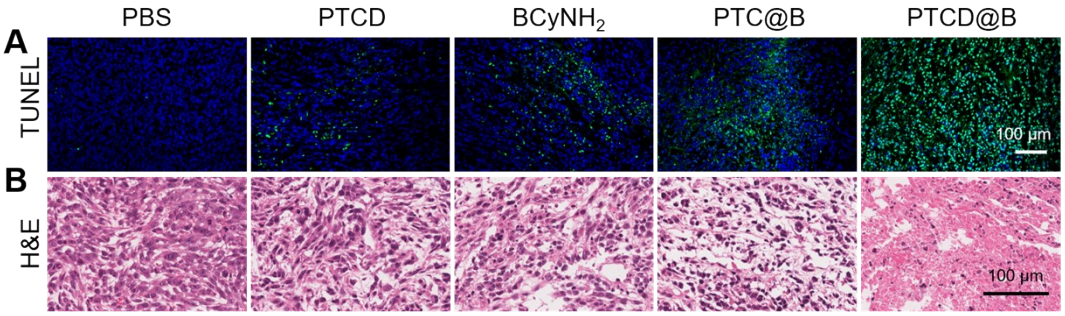


Figure S26. (A) TUNEL staining and (B) H&E staining of the tumor slices upon various treatments.