Supporting Information

cRGD/TAT dual-ligand reversibly crosslinked micelles loaded with docetaxel penetrate deeply into tumor tissue and show high antitumor efficacy in vivo

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1. Materials. Poly(ethylene glycol) monomethyl ether (MeO-PEG, M_n = 5.0 kg/mol, PDI = 1.03, Fluka, USA) was dried by azeotropic distillation from toluene. ε-Caprolactone (ε-CL, 99%, Alfa Aesar, USA) was dried by refluxing over CaH_2 and distilled before use. Dichloromethane (DCM) and N, N-dimethylformamide (DMF) were purified using the solvent purification system (Innovative Technology, USA). Maleimide activated poly(ethylene glycol) (MAL-PEG-OH, M_n = 2.0 kg/mol, PDI = 1.04, M_n = 5.0 kg/mol, PDI = 1.08, M_n = 6.0 kg/mol, PDI = 1.04, Suzhou Nord Derivatives Pharm-tech Co. Ltd), diphenyl phosphate (DPP, >99%, TCI, Japan), diethylether, N,N-dimethylformamide (DMF), absolute ethanol, paraformaldehyde (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China), methanol, acetonitrile (HPLC grade, Sigma, USA), docetaxel (DTX, >99%, Beijing ZhongShuo Pharmaceutical Technology Development Co., Ltd., Beijing, China), TAT (YGRKKRRQRRRC, >98%) and cRGDfK, Cyclo(-Arg-Gly-Asp-D-Phe-Lys), (cRGD, >98%) (ChinaPeptides Co., Ltd., Shanghai, China), glutathione (GSH, >98%, Amresco, USA), dithiothreitol (DTT, 99%, Merck, Darmstadt, Germany), Cy5-NH_2 (Mycomebio Biomedical Science and Technology Center, Beijing, China), 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl, 98%, J&K, Beijing, China), mercaptopropionic acid (99%, J&K, Beijing, China), 2,2′-Azobisisobutyronitrile (AIBN, 98%, J&K, Beijing, China), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, USA), N-hydroxysuccinimide (NHS, 98%, Alfa Aesar, USA), polysorbate 80 (Tween-80) were purchased from Aladdin (Shanghai, China), lysotracker green (Invitrogen, USA), trypsin (Jinuo Biomedical Technology, Hangzhou, Zhejiang, China), DAPI (Invitrogen, USA), rat monoclonal anti-mouse CD31 (BD Pharmingen, San Jose, California, USA), pentobarbital sodium (Solarbio Science &Technology, Beijing, China), Alexa 488 conjugated donkey anti-rat secondary antibody (Molecular Probes, Eugene, OR, USA), heparin sodium (>140 IU/mg, Solarbio, Shanghai, China), chlorpromazine and amiloride hydrochloride (>98%,

Shanghai Jianglai Biotechnology, Shanghai, China), MβCD (>98%, Solarbio, Shanghai, China).

2. Synthesis of polymers. mPEG$_{5k}$-P(DTC-co-CL) was synthesized by ring-opening polymerization of DTC and ε-CL in DCM at 30 °C using MeO-PEG$_{5k}$-OH as an initiator and DPP as a catalyst. Briefly, in a glove-box under a nitrogen atmosphere, DPP (0.23 g, 1 mmol) was quickly added to a stirred solution of PEG (0.50 g, 100 μmol), DTC (0.10 g, 0.52 mmol) and ε-CL (0.20 g, 1.75 mmol) in DCM (4.0 mL). The reaction vessel was sealed and placed into an oil-bath thermostated at 30 °C. The polymerization was allowed to proceed with magnetic stirring for 72 h. The resulting polymer was isolated by precipitation in cold diethylether, filtration, and drying in vacuo at room temperature. Yield: 93.1%. ^{1}H NMR (400 MHz, CDCl3, Figure S1): PEG: δ 3.38, 3.65; DTC: δ 2.99, δ 4.13, δ 4.17; CL: δ 1.40, δ 1.67, δ 2.32, δ 4.06; M_n (^{1}H NMR) = 7.8 kg/mol, M_n (GPC) = 14.5 kg/mol, PDI (GPC) = 1.1.

cRGD-PEG$_{6k}$-P(DTC-co-CL) diblock copolymer was synthesized in two steps (Figure S2A). Firstly, MAL-PEG$_{6k}$-P(DTC-co-CL) was obtained as described above except that MAL-PEG-OH (Mn = 6.0 kg/mol) was used as a macro-initiator. Yield: 87.5%. ^{1}H NMR (400 MHz, CDCl3, Figure S3): PEG: 3.65; DTC: δ 2.99, δ 4.13, δ 4.17; CL: δ 1.40, δ 1.67, δ 2.32, δ 4.06; MAL: δ 6.71. M_n (^{1}H NMR) = 8.9 kg/mol, M_n (GPC) = 16.1 kg/mol, PDI (GPC) = 1.2. Secondly, cRGD was conjugated to MAL-PEG$_{6k}$-P(DTC-co-CL) polymer through the reaction between the thiol and maleimide groups. Briefly, dry DMF (2 mL) was bubbled with nitrogen gas for 10 min, then MAL-PEG$_{6k}$-P(DTC-co-CL) (0.10 g, 11.2 μmol) was added and stirred under nitrogen for 10 min, and cRGD-SH (10 mg, 16.2 μmol) was quickly added to the solution and the reaction proceeded for 24 h at 30 °C. The final product, cRGD-PEG$_{6k}$-P(DTC-co-CL), was isolated through dialysis (MWCO 7000) against DMF and then precipitation in cold diethylether, filtration, and drying in vacuo at room temperature. Yield: 83.4 %. ^{1}H NMR (400 MHz, DMSO-d6, Figure S4): PEG: δ 3.51; DTC: δ 3.06, δ 4.07, δ 4.11; CL: δ 1.30, δ 1.54, δ 2.28, δ 3.98; cRGD: δ 7.17. The degree of cRGD conjugation

was 95.8 % as determined with the Micro BCA protein assay kit (Thermo scientific). TAT-PEG$_{2k}$-P(DTC-co-CL) diblock copolymer was synthesized similar to the method described above. Firstly, MAL-PEG$_{2k}$-P(TMC-co-DTC) was obtained as described above except that MAL-PEG-OH (Mn = 2.0 kg/mol) was used as a macro-initiator. Yield: 84.6%. ^{1}H NMR (400 MHz, CDCl3, Figure S5): PEG: 3.64; DTC: δ 2.99, δ 4.13, δ 4.16; CL: δ 1.40, δ 1.67, δ 2.33, δ 4.06; MAL: δ 6.71. M_n (^{1}H NMR) = 4.8 kg/mol, M_n (GPC) = 10.9 kg/mol, PDI (GPC) = 1.2. TAT peptide was also conjugated to the MAL-PEG$_{2k}$-P(DTC-co-CL) polymer via the thiol-maleimide coupling reaction. Briefly, dry DMF (2 mL) was bubbled with nitrogen gas for 10 min to remove oxygen, then MAL-PEG$_{2k}$-P(DTC-co-CL) (0.10 g, 20.8 μmol) was added and stirred under nitrogen for 10 min, and TAT-SH (50 mg, 30 μmol) was quickly added to the solution and the reaction proceeded for 24 h at 30 °C. Yield: 83.3%. The final product, TAT-PEG$_{2k}$-P(DTC-co-CL), was isolated through dialysis (MWCO 7000) against DMF and then precipitation in cold diethylether, filtration, and drying in vacuo at room temperature. Yield: 81.3 %. ^{1}H NMR (400 MHz, DMSO-d6, Figure S6): PEG: δ 3.50; DTC: δ 2.99, δ 4.05, δ 4.13; CL: δ 1.28, δ 1.54, δ 2.28, δ 3.98; TAT: δ 7.07, δ 7.89. The degree of TAT conjugation was determined to be 96.4% by determining the amino groups using the trinitrobenzenesulfonic acid (TNBS) assay.

Cy5-labeled PEG$_{5k}$-P(DTC-co-CL) polymer was synthesized in three steps (Figure S2B). Firstly, MAL-PEG$_{5k}$-P(TMC-co-DTC) was obtained as described above except that MAL-PEG-OH (Mn = 5.0 kg/mol) was used as a macro-initiator. Yield: 85.3%. ^{1}H NMR (400 MHz, $CDCl_3$): PEG: 3.64; DTC: δ 2.99, δ 4.13, δ 4.16; CL: δ 1.40, δ 1.67, δ 2.33, δ 4.06; MAL: δ 6.71. M_n (^{1}H NMR) = 7.8 kg/mol, M_n (GPC) = 15.1 kg/mol, PDI (GPC) = 1.2. Secondly, MAL-PEG$_{5k}$-P(DTC-co-CL) polymer (100 mg, 12.8 μmol) was reacted with mercaptopropionic acid (5.6 μL, 64 μmol) in the presence of AIBN (10.5 mg, 64 μmol) in DMF (1 mL). The mixture was stirred at 70 °C for 24 h. The HOOC-PEG$_{5k}$-P(DTC-co-CL), was isolated by precipitation in cold diethyl ether and dried in vacuo at room temperature.

Yield: 82.4%. Finally, HOOC-PEG_{5k}-P(DTC-co-CL) (50 mg, 6.4 μmol) was activated with EDC (3.7 mg, 19.2 μmol) and NHS (1.1 mg, 9.6 μmol) in DMF (1 mL) for 15 min and then Cy5-NH_2 (4.7 mg 7.7 μmol) was added. The reaction was carried out for 24 h at 30 ℃. The final product, Cy5-labeled PEG_{5k}-P(DTC-co-CL), was isolated through extensive dialysis against DMF followed by precipitation in cold diethyl ether and drying in vacuo at room temperature. Yield: 81.3%. The degree of Cy5 conjugation was determined to be 92.6 % using fluorescence spectrophotometry (Thermo Scientific, USA).

3. Characterization. ^{1}H NMR spectra were recorded on a Unity Inova 400 spectrometer (Agilent, USA) operating at 400 MHz using deuterium oxide (D_2O, CIL, Andover, MA, USA) or deuterated dimethylsulfoxide (DMSO-d_6, CIL, Andover, MA, USA) as a solvent. The molecular weights and polydispersities of the copolymers were determined using a gel permeation chromatograph (GPC) instrument (Waters 1515, USA) equipped with two linear PL gel columns (500 Å and Mixed-C) following a guard column and a differential refractive-index detector (RI 2414). The measurements were performed using DMF as the eluent at a flow rate of 1.0 mL/min at 30 ℃ and a series of narrow poly (methyl methacrylate) (PMMA) standards for the calibration of the columns. The size of the micelles was determined using dynamic light scattering (DLS). Measurements were carried out at 25 ℃ using a Zetasizer Nano-ZS from Malvern Instruments equipped with a 633 nm He-Ne laser using back-scattering detection. Transmission electron microscopy (TEM) was performed using a Tecnai G220 TEM operated at an accelerating voltage of 200 kV.

4. Serum stability and GSH-triggered de-crosslinking of micelles. The stability of cRGD20/TAT10 CMs in the presence of 10% FBS was investigated by DLS, TAT100 CMs were used as control group. Samples were maintained at 37 ℃ in a shaking bath at 200 rpm for 24 h. At desired time intervals, the sizes were determined by DLS.

The destabilization of cRGD20/TAT10 CMs micelles in response to 10 mM GSH in PB buffer (5 mM, pH 7.4) was investigated by DLS measurements. Briefly, cRGD20/TAT10

CMs dispersion and GSH solution (the pH was adjusted to 7.4 with 1 M NaOH) were gently bubbled with nitrogen gas for 10 min, respectively. Then the GSH solution was quickly added into the micelle dispersion (final GSH concentration: 10 mM) and the mixture was immediately placed in a shaking bath (200 rpm) at 37 °C. At different time intervals, the micelle size was measured using DLS. Samples without GSH were used as control groups.

5. Reduction-triggered drug release. The *in vitro* release of DTX from cRGD20/TAT10 CMs was studied using a dialysis tube (Spectra/Pore, MWCO 12000) at 37 °C in PB (5 mM, pH 7.4) containing 0.1% (v/v) Tween 80 either in the presence or absence of 10 mM GSH. To acquire sink conditions, drug release studies were performed at a micelle concentration of 1.0 mg/mL (DTX concentration ~ 63 μg/mL) with 0.5 mL of micelle dispersion dialyzed against 25 mL of the same media. At desired time intervals, 5 mL of release medium was taken out and replenished with an equal volume of fresh medium. The amount of DTX released was determined by the HPLC method as described above.

6. MTT assay. The antitumor activity of DTX-loaded micelles and free DTX were determined by the MTT assay. Briefly, U87MG cells were seeded at a density of 8×10^3 cells/well in 96-well plates and cultured for 24 h. DTX-loaded micelles or free DTX (final DTX concentration: 0.0001, 0.001, 0.01, 0.1, 0.5, 1, 2.5 and 5 μg/mL) in 10 μL of PBS were added. The cells were incubated for 4 h, the medium was removed and replaced with fresh medium, and the cells were incubated for another 44 h. Subsequently, 20 μL of MTT stock solution (5 mg/mL) was added to each well, and the plates were further incubated for 4 h at 37 ºC in the dark. The medium was discarded and 150 μL of DMSO was added to dissolve the blue formazan crystals. Cell viability was assessed by the absorbance at 492 nm of the DMSO solution measured on a microplate reader (Multiskan FC, Thermo). The data were expressed as the percentages of viable cells compared to the survival of a control group (untreated cells). The morphology of cells treated with the different groups of DTX loaded micelles and free DTX was observed by microscopy (Leica QWin, Germany).

7. Cellular uptake of micelles. To establish the optimal density of TAT peptide, the cellular uptake of Cy5-labeled cRGD/TAT CMs with a molar ratio of 20% cRGD and different molar ratios of TAT peptide (5%, 7.5%, 10%, 15%, 20%) were studied by flow cytometry. Briefly, U87MG cells were seeded at a density of 5×10^5 cells/well in 6-well plates and cultured for 24 h. Different Cy5-labeled micelles were added and incubated for 4 h at a final micelle concentration of 100 μg/mL. The cells were washed three times with PBS, detached with trypsin, centrifuged at 156.5 × g for 5 min and suspended in 0.5 mL of PBS. The cells were analyzed using flow cytometry (FACS Calibur, BD Biosciences, USA). For each sample, 10,000 events were collected and U87MG cells cultured under normal conditions were used as the control. The cellular uptake of different micelles was also studied with confocal laser scanning microscopy (CLSM, Leica TCS SP5, Wetzlar, Germany). Briefly, U87MG cells were seeded on round glass coverslips at a density of 2×10^4 cells/well in 24-well plates and cultured at 37 °C for 24 h. Cy5-labeled micelles were added and incubated for 4 h at a micelle concentration of 100 μg/mL. Cells were then washed with PBS and subsequently fixed using 4% paraformaldehyde, treated with DAPI for 8 min for nuclei staining and washed again with PBS, and finally examined with CLSM using a 60 × oil immersion objective.

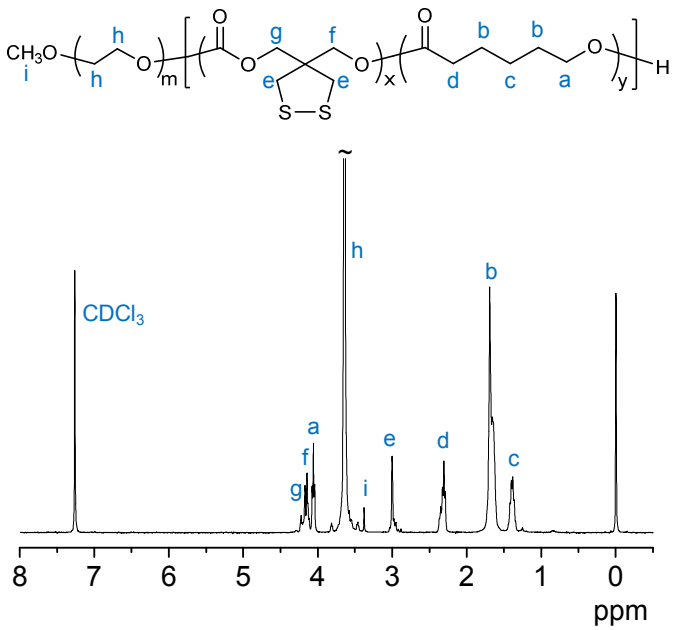

Figure S1. ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of $mPEG_{5k}$-P(DTC-*co*-CL).

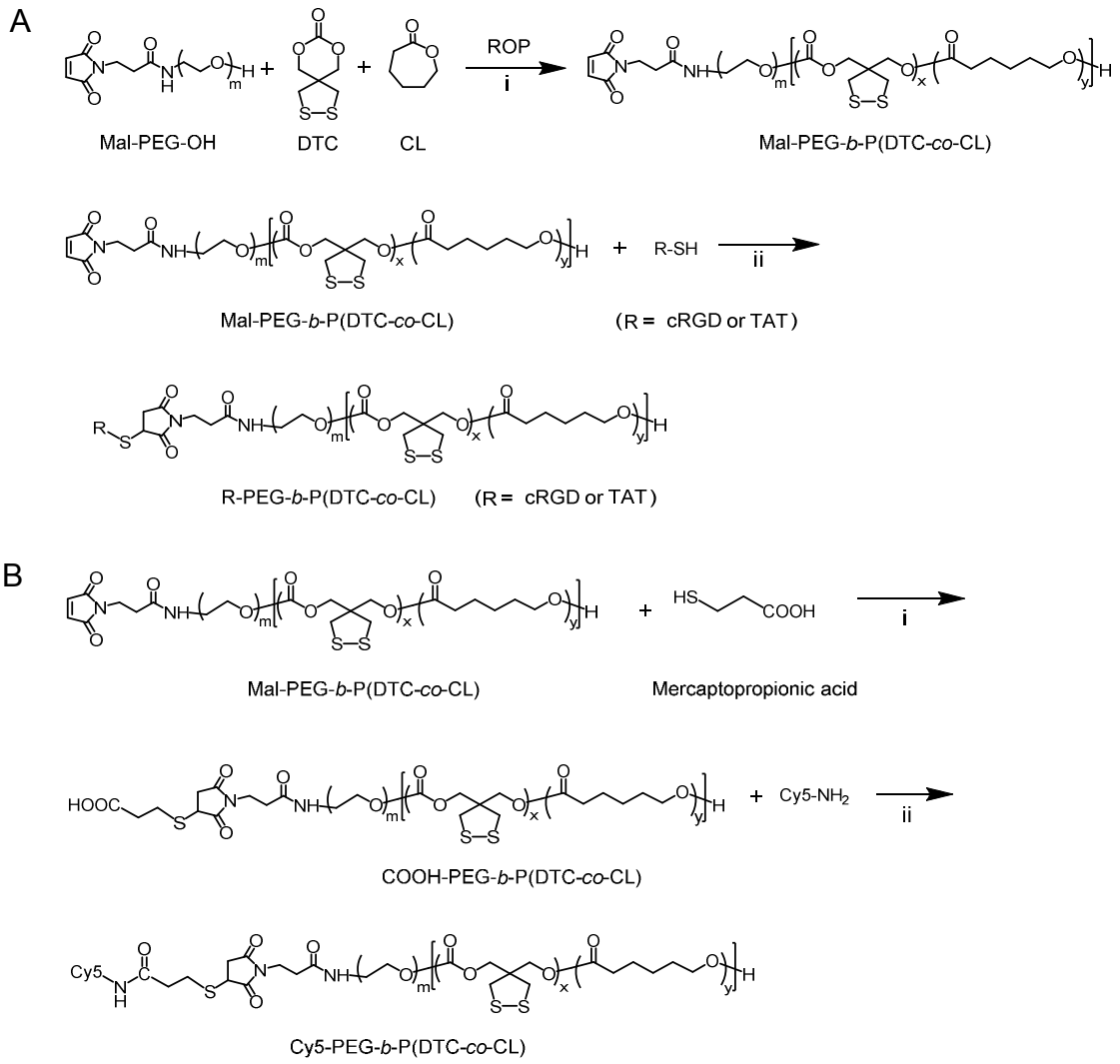

Figure S2. (A) Synthetic routes for cRGD-PEG_{6k}-P(DTC-CL) and TAT-PEG_{2k}-P(DTC-CL) polymers. Conditions: (i) DPP as catalyst, DCM, 30 °C, 72 h. (ii) DMF, 30 °C, 24 h. (B) Synthetic routes for Cy5-PEG_{5k}-P(DTC-CL) polymer. Conditions: (i) DMF, 70 °C, 24 h. (ii) DMF, EDC, NHS, 30 °C, 24 h.

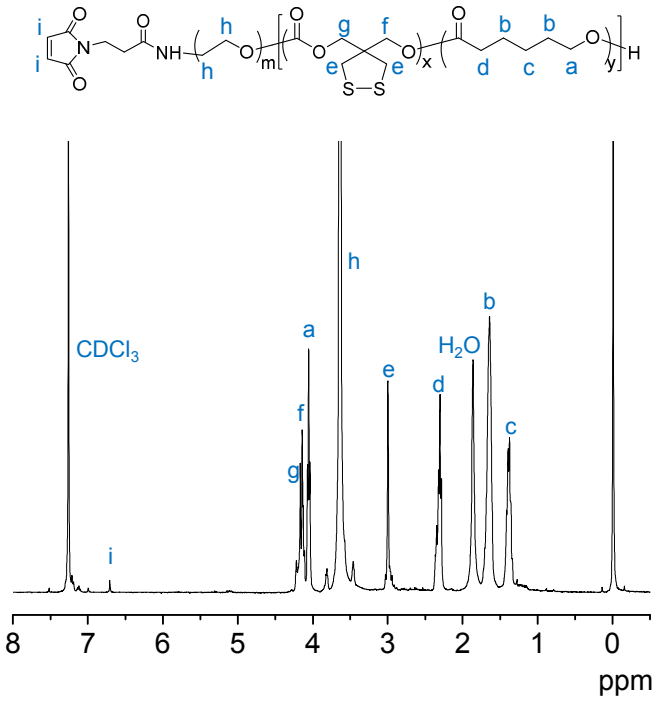

Figure S3. ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of MAL-PEG$_{6k}$-P(DTC-*co*-CL).

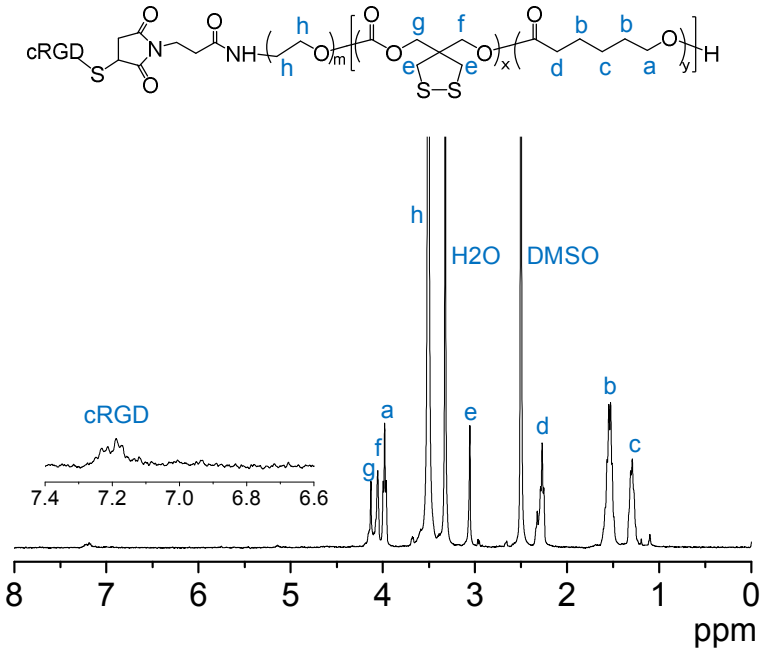

Figure S4. ^{1}H NMR spectrum (400 MHz, DMSO-d_6) of cRGD-PEG$_{6k}$-P(DTC-*co*-CL).

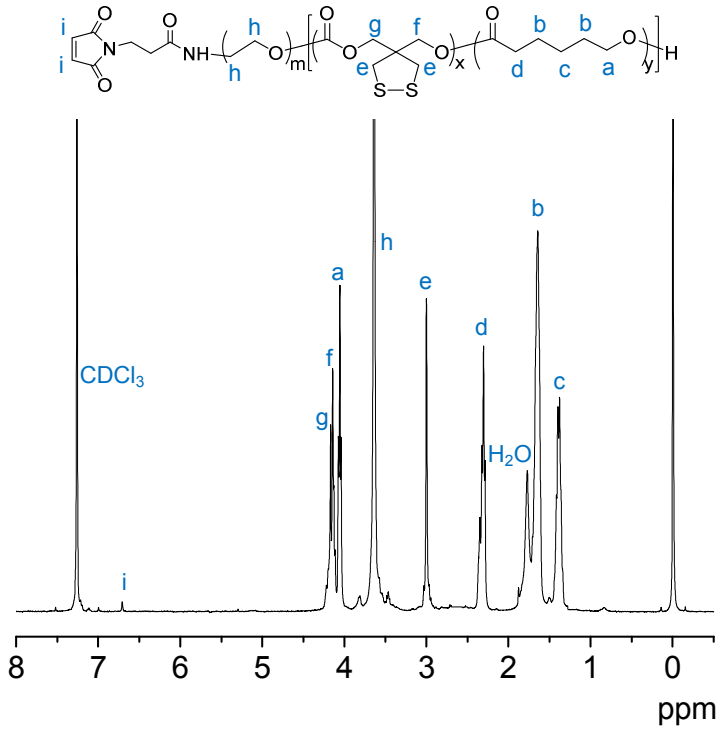

Figure S5. ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of MAL-PEG$_{2k}$-P(DTC-*co*-CL).

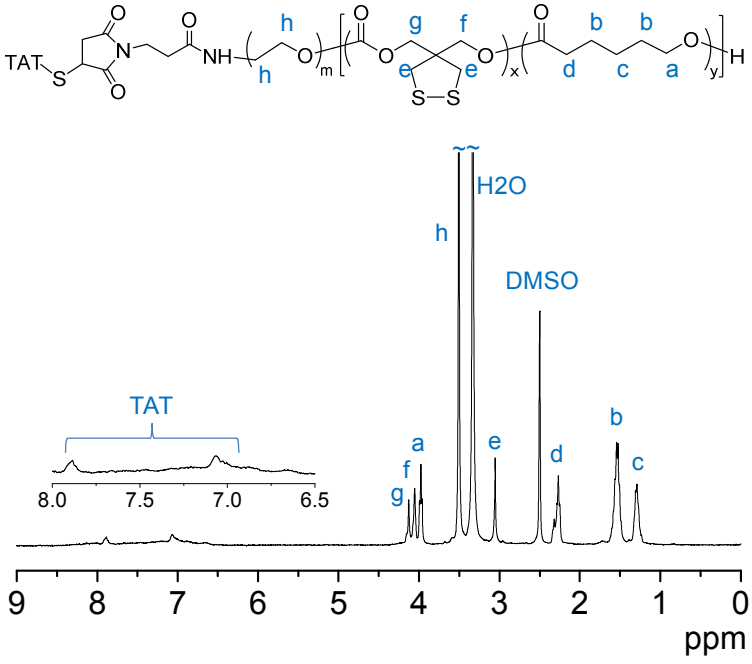

Figure S6. ^{1}H NMR spectrum (400 MHz, DMSO-d_6) of TAT-PEG$_{2k}$-P(DTC-co-CL).

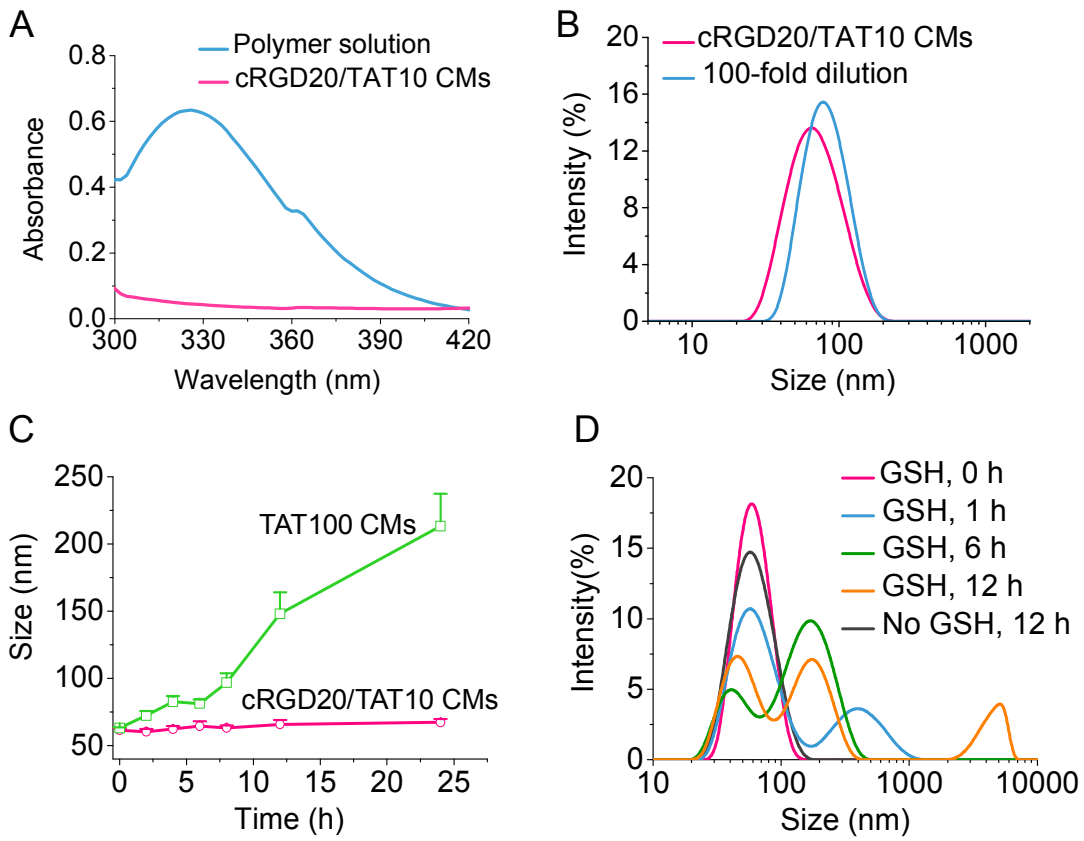

Figure S7. (A) UV spectra of cRGD20/TAT10 CMs in PB (pH 7.4, 5 mM) and the corresponding polymer solution in DMF (micellar concentration: 10 mg/mL). (B) 100-fold dilution of cRGD20/TAT10 CMs (1 mg/mL) in PB (pH 7.4, 5 mM). (C) Serum stability (against 10% FBS, n = 3) of blank cRGD20/TAT10 CMs, blank TAT100 CMs were used as control. (D) GSH-triggered blank cRGD20/TAT10 CMs destabilization.

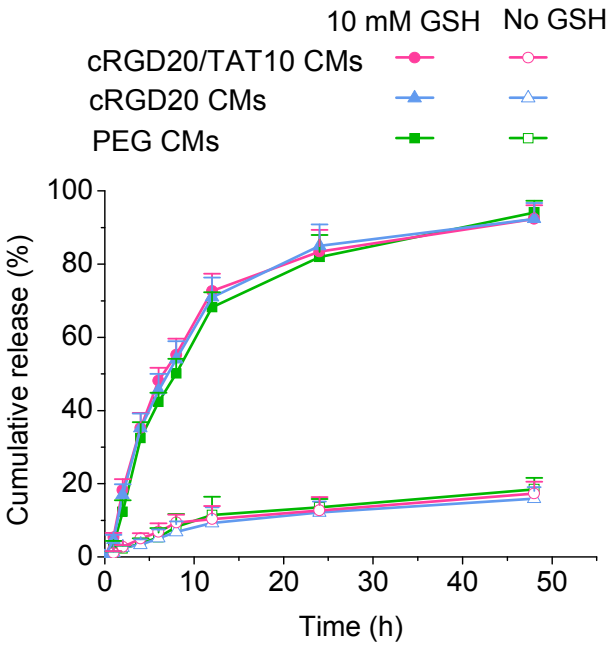

Figure S8. GSH-triggered DTX release from different DTX-loaded micelles. Data are presented as the average ± standard deviation (n = 3).

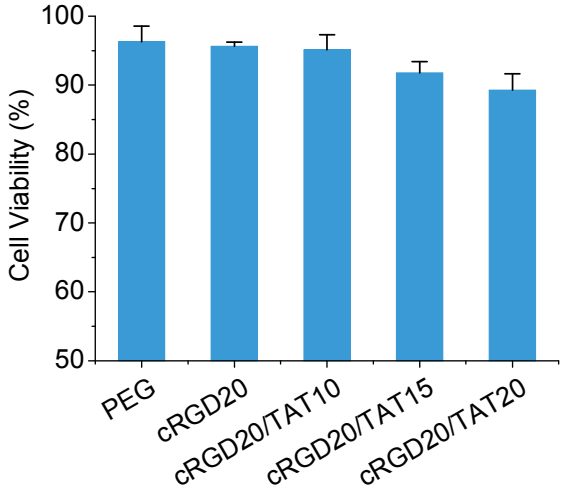

Figure S9. Cytotoxicity of blank micelles against U87MG cells at a micelle concentration of 1 mg/mL after 48 h incubation. Data are presented as the average ± standard deviation (n = 4).

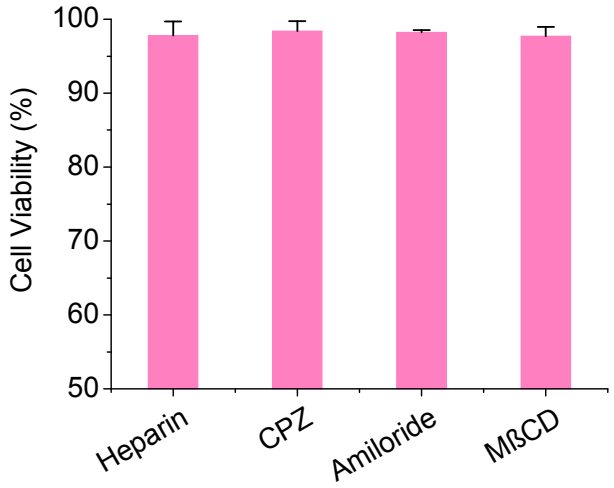

Figure S10. Cytotoxicity of different endocytic inhibitors, (Heparin: 1 mg/mL, CPZ: 10 μg/mL, Amiloride: 1 mg/mL, MβCD: 1 mg/mL). Data are presented as the average ± standard deviation (n = 4).

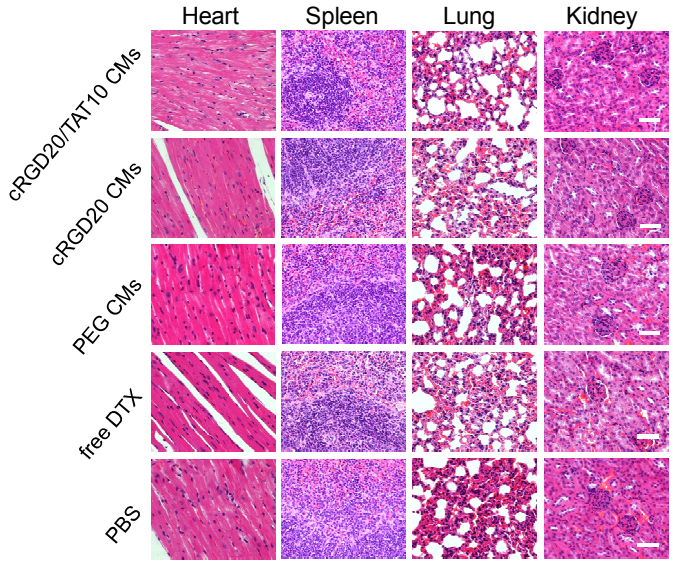

Figure S11. H&E stained heart, spleen, lung and kidney excised from subcutaneous U87MG glioma bearing nude mice following 21 d treatment with DTX-loaded micelles, free PTX or PBS. The images were obtained by a Leica microscope at 400× magnification. Bar: 50 μm.

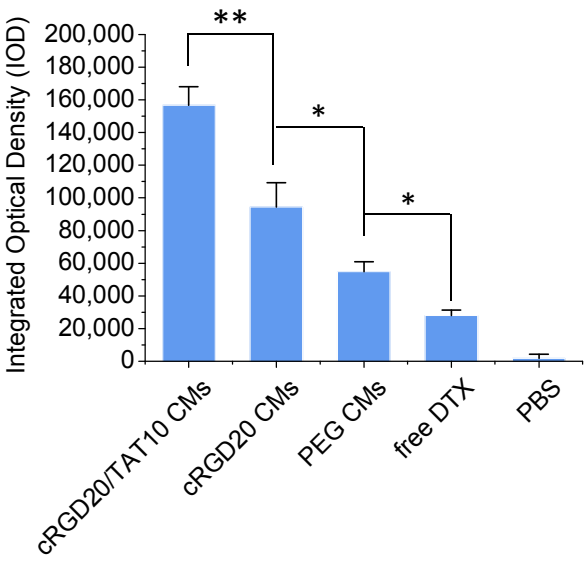

Figure S12. Quantitative analysis for the TUNEL staining presented as average integrated optical density (IOD) level. Data are presented as means ± SD (n = 4).* P<0.05, ** P<0.01.

Table S1. Size distribution and zeta potential of blank micelles

Entry	Micelles	Size (nm)[a]	PDI[a]	Zeta (mV)[a]
1	PEG	56 ± 2	0.11	−4.6 ± 0.2
2	cRGD20	58 ± 2	0.12	−3.8 ± 0.7
3	cRGD20/TAT5	60 ± 1	0.14	−2.3 ± 0.3
4	cRGD20/TAT7.5	60 ± 1	0.15	−1.8 ± 0.3
5	cRGD20/TAT10	61 ± 1	0.14	−1.1 ± 0.2
6	cRGD20/TAT15	62 ± 2	0.15	−0.75 ± 0.1
7	cRGD20/TAT20	65 ± 1	0.16	−0.58 ± 0.1
8	TAT100	63 ± 2	0.14	3.6 ± 0.8
9	PEG/TAT10	60 ± 2	0.12	−1.4 ± 0.3

[a]Determined by dynamic light scattering (DLS) in 5 mM PB (pH 7.4)

Table S2. Characterization of different micelles before and after drug loading (theoretical DTX loading content = 15 wt.%)

Micelles	Before drug loading		After drug loading		DLC[b]	DLE[b]
	Size (nm)[a]	PDI[a]	Size (nm)[a]	PDI[a]	(wt.%)	(%)
PEG	56 ± 2	0.11	58 ± 2	0.13	6.9	42
cRGD20	58 ± 2	0.12	61 ± 1	0.14	7.0	43
cRGD20/TAT10	61 ± 1	0.14	63 ± 1	0.16	6.9	42

[a]Determined by dynamic light scattering (DLS) in 5 mM PB (pH 7.4)

[b]Drug loading content (DLC) and drug loading efficiency (DLE) were determined by HPLC