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Electronic Supplementary Information

Sequential self-assembly and release of camptothecin prodrug for tumor-targeting therapy

Wujuan Zhu,[a] Minghui Yu,[a] Minghui Wang,[a] Miaomiao Zhang,[b,*] and Zijuan Hai[a,*]

[a]Institutes of Physical Science and Information Technology, Anhui University, Hefei, Anhui 230601, China

[b]College of Chemistry and Center for Advanced Analysis & Gene Sequencing, Zhengzhou University, Zhengzhou 450001, China

Correspondence and requests for materials should be addressed to e-mail:
zmm@zzu.edu.cn (M. Zhang) and zijuan@ahu.edu.cn (Z. Hai)

Contents

1. General Method

Experimental materials and instruments

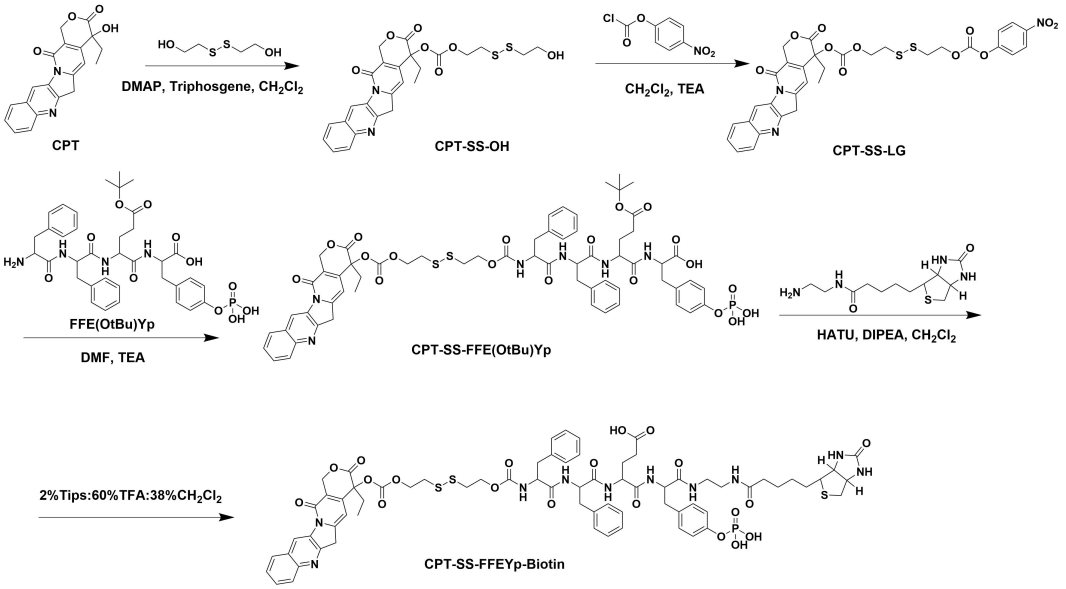
All the starting materials were obtained from Aladdin, Sigma or Sangon Biotech. Commercially available reagents were used without further purification, unless noted otherwise. All other chemicals were reagents grade or better. Calf intestinal alkaline phosphatase (ALP) was obtained from Takara Bio Inc. (Beijing, China). HPLC analyses were performed on an Agilent 1260 HPLC system equipped with a G7111A pump and an in-line diode array UV detector using an Agilent Zorbax 300SB-C18 RP column, with CH_3CN (0.1% of TFA) and ultrapure water (0.1% of TFA) as the eluent. Electrospray ionization (ESI) mass spectra were obtained on a Finnigan LCQ Advantage ion trap mass spectrometer (ThermoFisher Corporation). ^1H NMR spectrum was obtained on a JNM-ECZ400S. ^{13}C NMR spectrum was obtained on a JNM-ECZ600R.

Cell culture

NIH-3T3 and HeLa cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. 4T1 cells were cultured in RPMI 1640 Medium with 10% fetal bovine serum. The culture dishes were maintained at 37°C under a humid atmosphere with 5% CO_2.

2. Syntheses and Characterizations of CPT-SS-FFEYp-Biotin

Scheme S1. The synthetic route for CPT-SS-FFEYp-Biotin.



Synthesis of CPT-SS-OH: CPT (250 mg, 0.718 mmol), triphosgene (78.5 mg, 0.265 mmol) were dissolved in anhydrous CH_2Cl_2 with stirring at 0°C for 30 min. Then, DMAP (280 mg, 2.3 mmol), Bis(2-hydroxyethyl) Disulfide (1.105 g, 7.15 mmol) were added to react overnight. The mixture was washed with 0.1 M HCl (3 × 80 mL), brine (1 × 80 mL) and water (1 × 80 mL). After removing the liquid by rotary evaporator and vacuum drier, the crude product CPT-SS-OH (300 mg, yield 87.2%) was obtained. MS: calc. $[M + H]^+$ = 529.1025, obsvd. ESI-MS: m/z = 529.1091 (Figure S1).

Synthesis of CPT-SS-LG: CPT-SS-OH (30 mg, 0.0568 mmol), 4-Nitrophenyl chloroformate (17 mg, 0.0852 mmol), Triethylamine (10.4 mg, 0.10224 mmol) were dissolved in anhydrous CH_2Cl_2 (1.5 mL) with stirring at room temperature for 3 h. Compound CPT-SS-LG (25 mg, yield 63.6%) was obtained after HPLC purification. MS: calc. $[M + H]^+$ = 694.1087, obsvd. ESI-MS: m/z = 694.1158 (Figure S2).

Synthesis of FFE(OtBu)Yp: Peptide FFE(OtBu)Yp was synthesized by solid phase peptide synthesis (SPPS) using 2-chlorotrityl chloride resin and the corresponding Fmoc-protected amino acids with side chains properly protected. The solution of 20% piperidine in N, N -Dimethylformamide (DMF) was used to remove the Fmoc group. MS: calc. $[M + H]^+$ = 741.2822, obsvd. ESI-MS: m/z = 741.2878 (Figure S3).

Synthesis of CPT-SS-FFE(OtBu)Yp: CPT-SS-LG (45 mg, 0.0649 mmol), FFE(OtBu)Yp (62 mg, 0.08437 mmol), Triethylamine (19.7 mg, 0.1947 mmol) were dissolved in anhydrous DMF (1 mL) with stirring at room temperature for 5 h. Compound CPT-SS-FFE(OtBu)Yp (35 mg, yield 41.6%) was obtained after HPLC purification. MS: calc. $[M + H]^+$ = 1295.3640, obsvd. ESI-MS: m/z = 1295.3705 (Figure S4).

Synthesis of **CPT-SS-FFEYp-Biotin**: CPT-SS-FFE(OtBu)Yp (20 mg, 0.01544 mmol), HATU (17.6 mg, 0.046 mmol), DIPEA (15 μL, 0.085 mmol) were dissolved in anhydrous CH_2Cl_2 (2 mL) with stirring at 0°C for 30 min. Then, the compound Biotin (8.8 mg, 0.03 mmol) was dissolved with 1 mL anhydrous CH_2Cl_2 and added to the above mixture and stirred at room temperature for 6 h. After removing the reaction liquid by rotary evaporator and vacuum drier, the yellow solid powder was stirred in

CH_2Cl_2 (570 μL) containing triisopropylsilane (30 μL) and trifluoroacetic acid (900 μL) at room temperature for 2 h. Compound CPT-SS-FFEYp-Biotin (10 mg, yield 43.1%) was obtained after HPLC purification. ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 8.64 (s, 1 H), 8.30 (s, 1 H), 8.16 – 7.95 (m, 6 H), 7.80 (t, J = 7.7 Hz, 1 H), 7.76 – 7.59 (m, 2 H), 7.15 (ddt, J = 27.1, 20.4, 5.9 Hz, 16 H), 6.97 (d, J = 8.2 Hz, 2 H), 5.48 (s, 2 H), 5.26 (s, 3 H), 4.53 (s, 2 H), 4.35 (s, 1 H), 4.29 – 4.22 (m, 3 H), 4.09 (dd, J = 10.9, 6.5 Hz, 3 H), 3.95 (dq, J = 14.0, 6.2 Hz, 2 H), 3.15 – 2.99 (m, 5 H), 2.88 (dt, J = 11.4, 5.1 Hz, 3 H), 2.78 – 2.52 (m, 4 H), 2.21 – 2.05 (m, 5 H), 2.04 – 1.76 (m, 2 H), 1.74 – 1.40 (m, 3 H), 1.22 (dd, J = 21.6, 6.0 Hz, 6 H), 0.93 – 0.77 (m, 5 H) (Figure S5). ^{13}C NMR (150 MHz, DMSO-d_6) δ (ppm): 173.55 (2 C), 171.81 (1 C), 167.60 (1 C), 163.30 (1 C), 157.05 (1 C), 155.951 (1 C), 153.34 (1 C), 152.72 (1 C), 151.64 (3 C), 149.76 (1 C), 148.44 (1 C), 146.81 (1 C), 145.29 (1 C), 143.86 (1 C), 140.17 (2 C), 138.57 (1 C), 138.10 (1 C), 135.16 (2 C), 132.15 (1 C), 130.99 (1 C), 130.28 (1 C), 129.67 (1 C), 129.37 (3 C), 128.48 (2 C), 126.66 (1 C), 125.12 (1 C), 124.31 (1 C), 121.25 (3 C), 119.94 (1 C), 119.72 (2 C), 94.93 (1 C), 78.44 (1 C), 67.00 (1 C), 66.73 (1 C), 62.42 (1 C), 61.60 (1 C), 59.77 (1 C), 55.92 (1 C), 54.06 (3 C), 50.86 (1 C), 42.32 (1 C), 42.00 (1 C), 38.77 (1 C), 36.94 (1 C), 35.74 (1 C), 30.86 (1 C), 28.77 (1 C), 25.52 (1 C), 19.27 (1 C),18.56 (3 C), 16.99 (3 C), 12.92 (1 C), 8.07 (1 C) (Figure S6). MS: calc. $[M + H]^+$ = 1507.4372, obsvd. ESI-MS: m/z = 1507.4481 (Figure S7).

3. Supporting figures and table

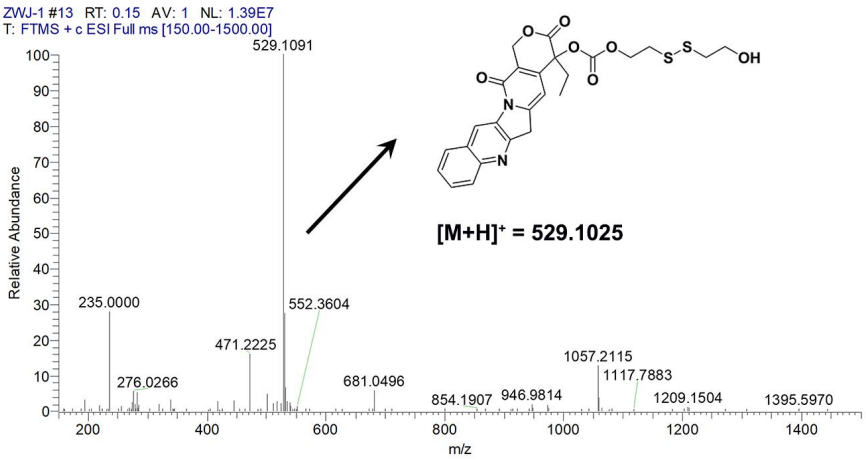


Figure S1. ESI-MS spectrum of CPT-SS-OH.

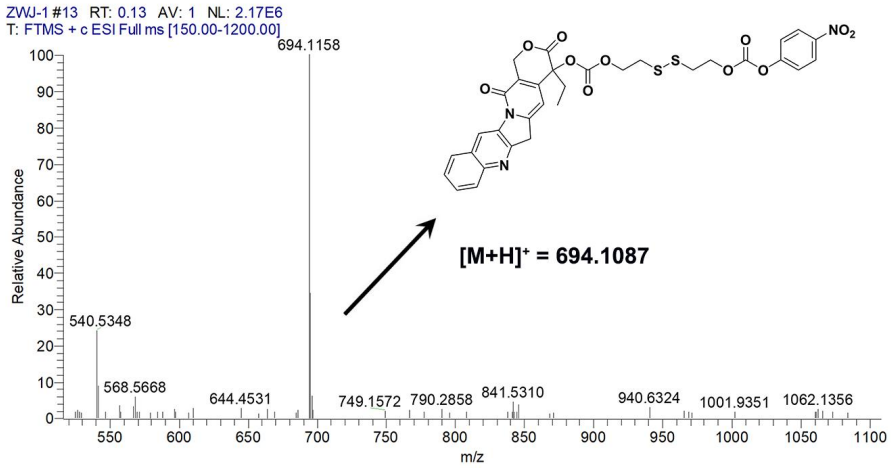


Figure S2. ESI-MS spectrum of CPT-SS-LG.

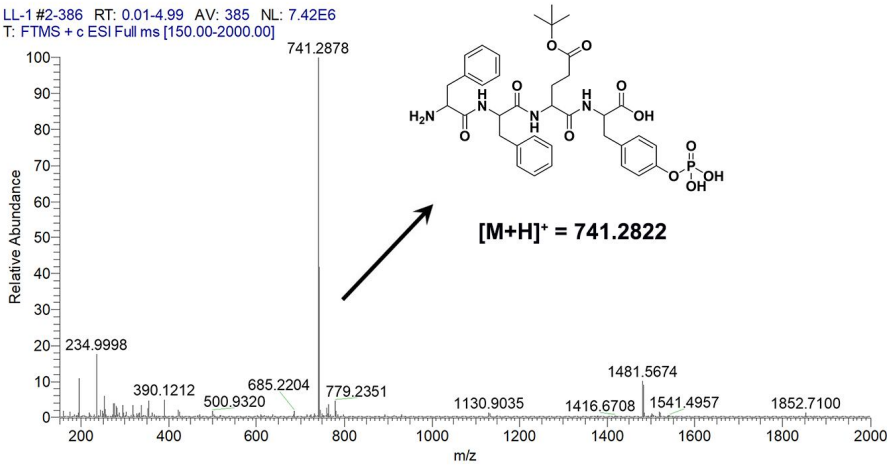


Figure S3. ESI-MS spectrum of FFE(OtBu)Yp.

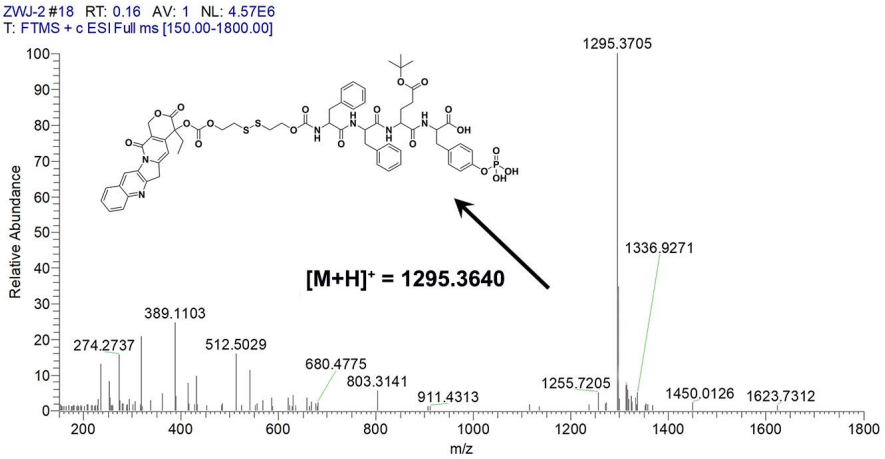


Figure S4. ESI-MS spectrum of CPT-SS-FFE(OtBu)Yp.

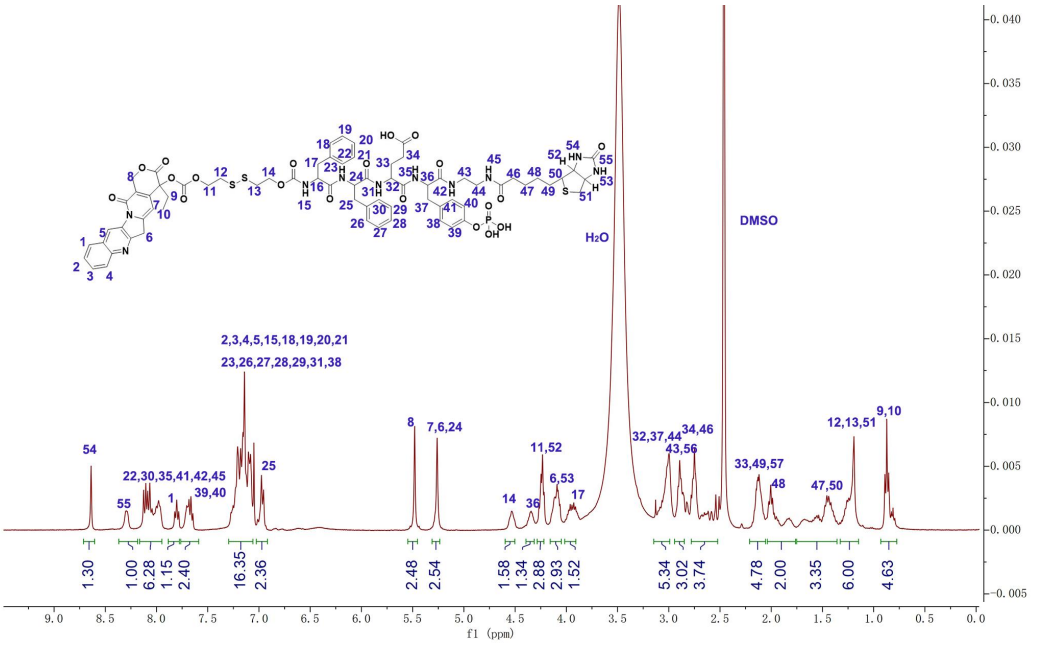


Figure S5. ^{1}H NMR spectrum of CPT-SS-FFEYp-Biotin.

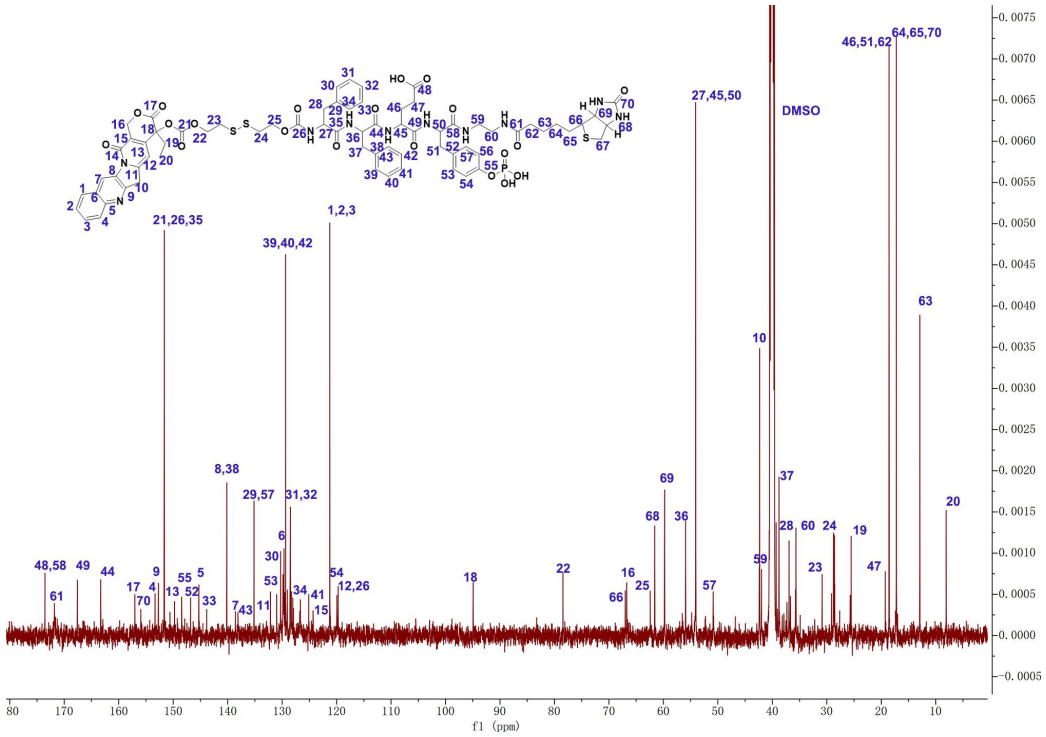


Figure S6. ^{13}C NMR spectrum of CPT-SS-FFEYp-Biotin.

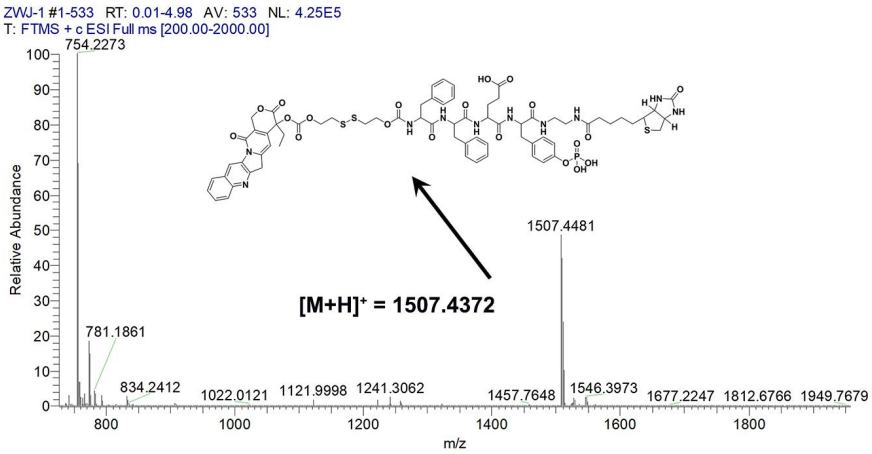


Figure S7. ESI-MS spectrum of CPT-SS-FFEYp-Biotin.

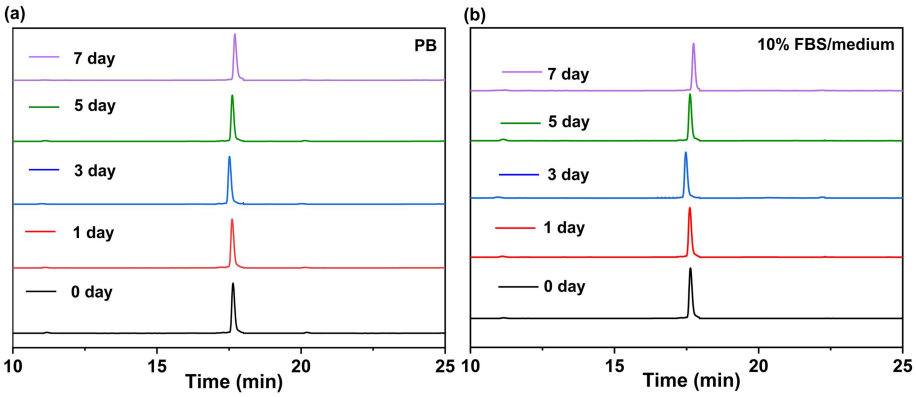


Figure S8. HPLC traces of 25 μM CPT-SS-FFEYp-Biotin in (a) PB or (b) 10% FBS/medium at 37 °C within 7 d.

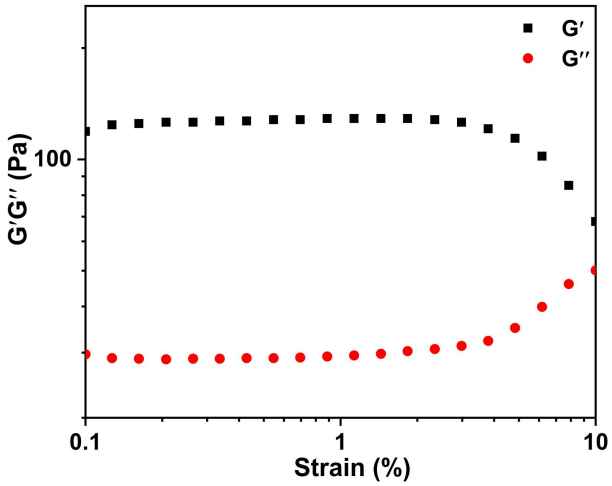


Figure S9. Dynamic strains of storage modulus (G') and the loss modulus (G") of CPT hydrogel at the frequency of 1 Hz, 25 °C.

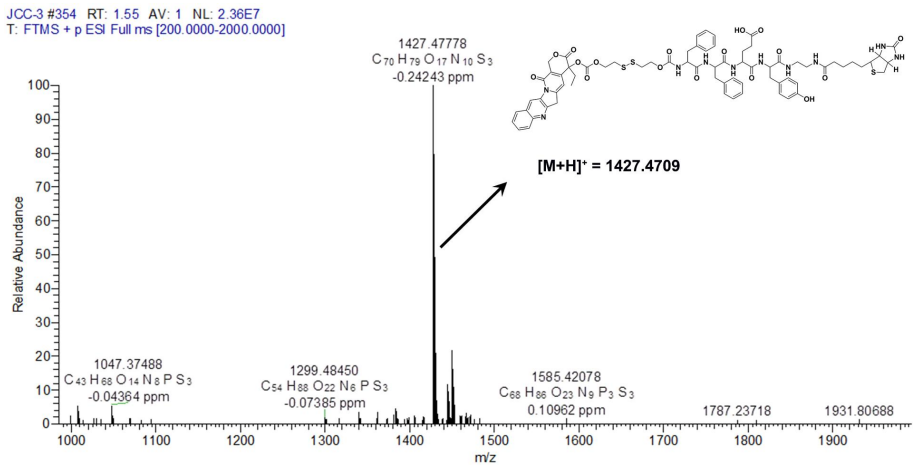


Figure S10. ESI-MS spectrum of **CPT-SS-FFEY-Biotin.**

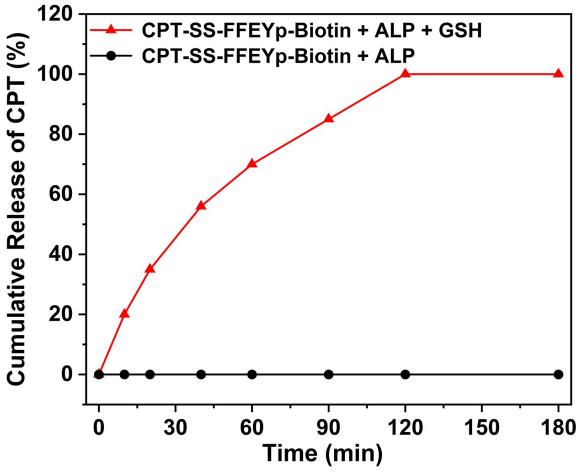


Figure S11. CPT release rate of 25 μM CPT-SS-FFEYp-Biotin with ALP (0.04 U μL^{-1}) or with ALP (0.04 U μL^{-1}) and 5 mM GSH within 180 min.

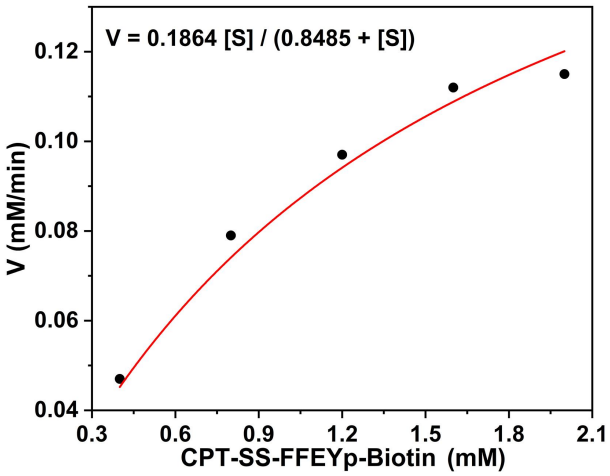


Figure S12. Initial velocities of ALP-catalyzed reaction plotted against the concentrations of CPT-SS-FFEYp-Biotin and fitted to the Michaelis-Menten model.

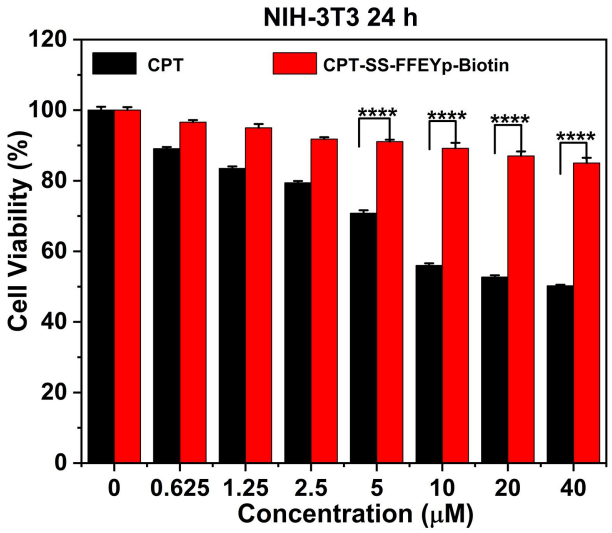


Figure S13. Cell viability of NIH-3T3 cells in CPT and CPT-SS-FFEYp-Biotin groups for 24 h. The error bars represent the standard deviation from three separate measurements. ****$p < 0.0001$, analyzed by Student's t-test.

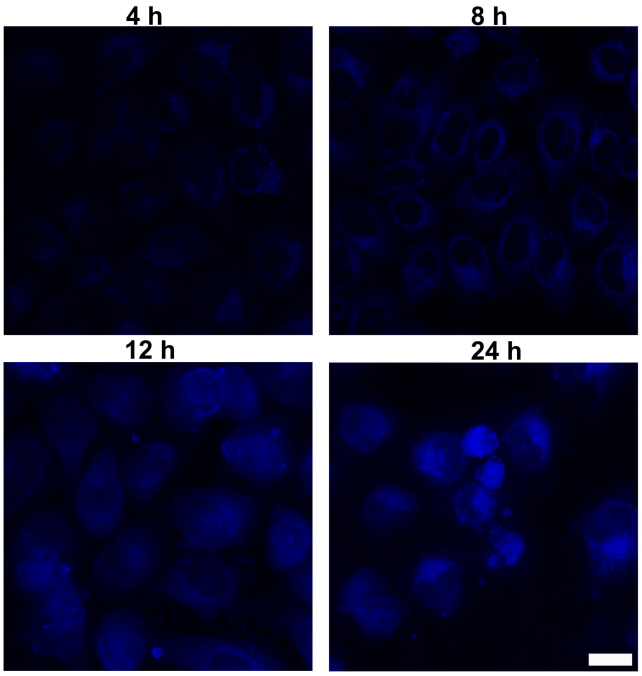


Figure S14. Confocal FL images of HeLa cells after incubation with 25 μM CPT-SS-FFEYp-Biotin at 4, 8, 12 and 24 h. Scale bar: 20 μm. (CPT, pseudo blue, λ_{ex} = 405 nm, λ_{em} = 420-450 nm)

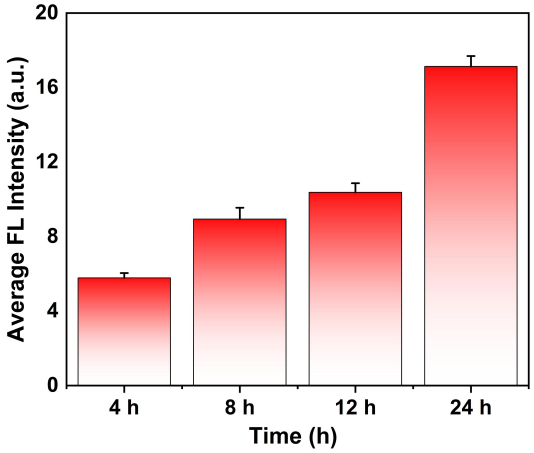


Figure S15. Average FL intensity of HeLa cells in Figure S14.

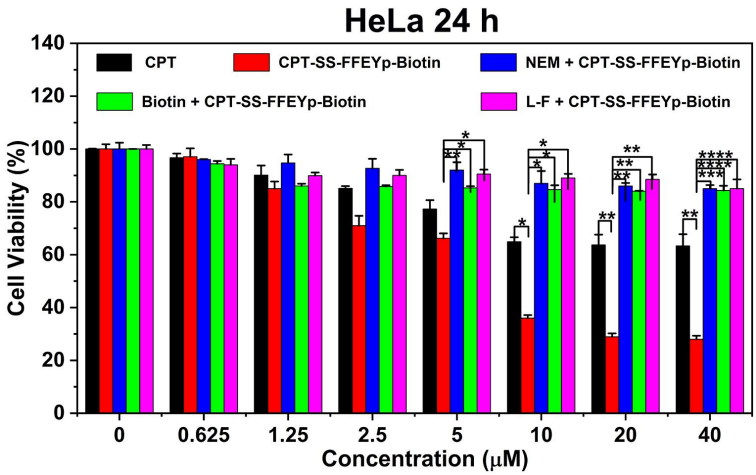


Figure S16. Cell viability of HeLa cells in CPT, CPT-SS-FFEYp-Biotin, NEM + CPT-SS-FFEYp-Biotin, Biotin + CPT-SS-FFEYp-Biotin and L-F + CPT-SS-FFEYp-Biotin groups for 24 h. The error bars represent the standard deviation from three separate measurements. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, analyzed by Student's t-test.

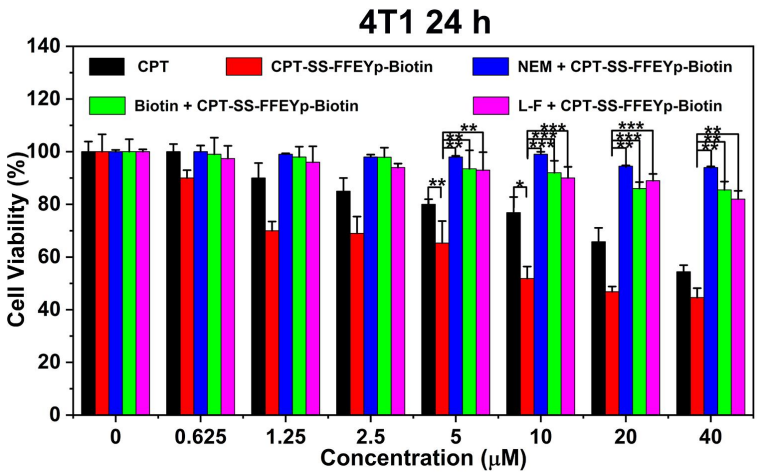


Figure S17. Cell viability of 4T1 cells in CPT, CPT-SS-FFEYp-Biotin, NEM + CPT-SS-FFEYp-Biotin, Biotin + CPT-SS-FFEYp-Biotin and L-F + CPT-SS-FFEYp-Biotin groups for 24 h. The error bars represent the standard deviation from three separate measurements. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, analyzed by Student's t-test.

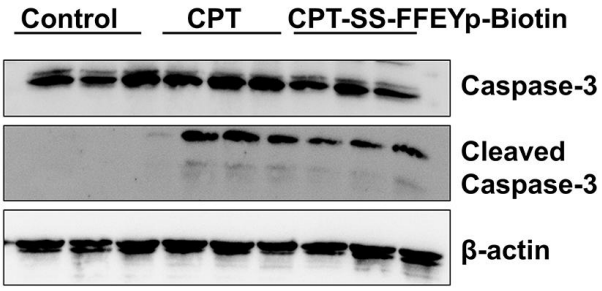


Figure S18. Western blots of Caspase-3, cleaved Caspase-3 and β-actin in HeLa cells without or with 25 μM CPT or CPT-SS-FFEYp-Biotin for 12 h.

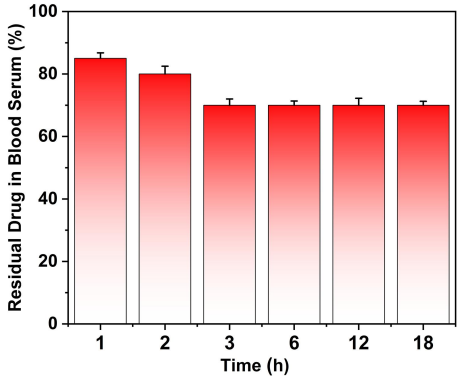


Figure S19. The stability of CPT-SS-FFEYp-Biotin in blood serum.

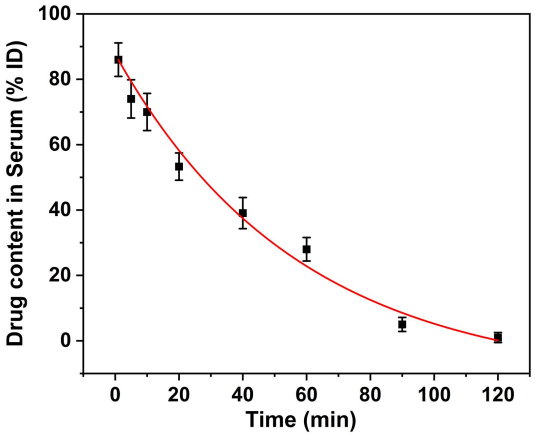


Figure S20. Blood concentration decay of CPT-SS-FFEYp-Biotin upon intravenous injection on normal BALB/c mice. The percentage of decayed CPT-SS-FFEYp-Biotin relative to the injected dose (% ID) was quantified based on its absorption at 350 nm.

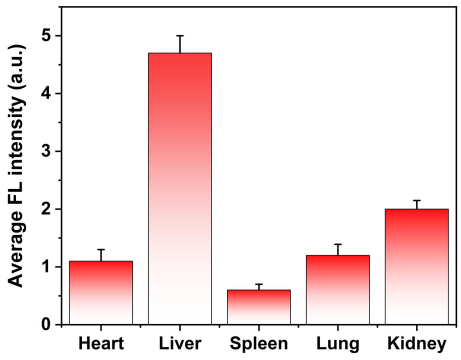


Figure S21. The mean fluorescence intensity of isolated organs in normal BALB/c mice after intravenous injection of CPT-SS-FFEYp-Biotin at 1 h.

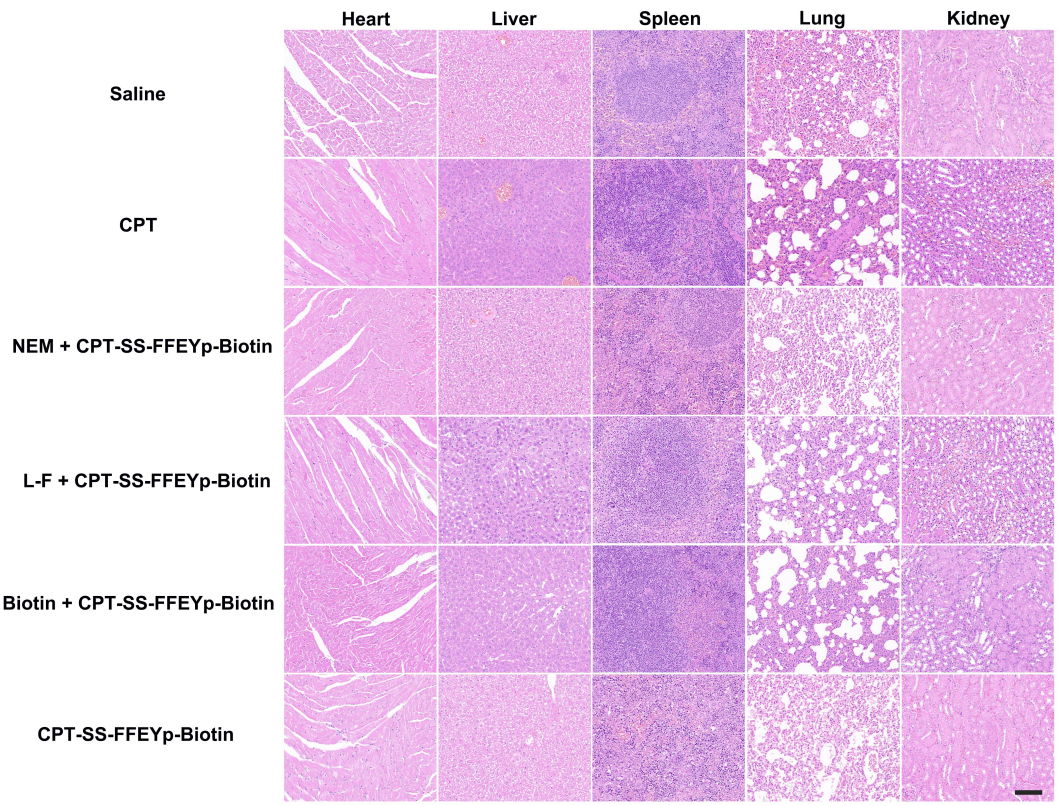


Figure S22. H&E staining of main organs of mice in six groups at day 14. Scale bar: 100 μm.

Table S1. Kinetic parameters for ALP-catalyzed reaction of CPT-SS- FFEYp-Biotin.

	K_m (M)	K_{cat} (s^{-1})	K_{cat}/K_m (M^{-1} s^{-1})
ALP	$8.5 * 10^{-4}$	18.75	$2.2 * 10^4$

Table S2. IC_{50} Values of prodrug CPT-SS-FFEYp-Biotin and CPT on HeLa cells and 4T1 cells for 24 h and 48 h.

IC_{50}	24 h		48 h	
	HeLa	4T1	HeLa	4T1
CPT-SS-FFEYp-Biotin	9.7 μM	17 μM	5.5 μM	4.1 μM
CPT	50.7 μM	41.3 μM	15.1 μM	15.8 μM

Table S3. HPLC condition for Fig. 2f and Fig. S8.

Time(min)	Flow (mL/min)	H_2O % (0.1%TFA)	CH_3CN % (0.1%TFA)
0	1	80	20
3	1	80	20
35	1	0	100
37	1	0	100
39	1	80	20
40	1	80	20