Supporting Information

Smart Polymer Prodrugs via Responsive Prodrug-Initiated Ring-Opening Polymerization of Lactide for Improved Drug Delivery

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1. EXPERIMENTAL SECTION

1.1 Materials and regents

All the chemicals were purchased and used as received unless otherwise noted. Camptothecin (CPT) (>95%, reagent grade) was purchased from Matrix Scientific (Columbia, SC). Bis(2-hydroxyethyl) disulfide (90%, technical grade) was purchased from Alfa Aesar (Tewksbury, MA). Triphosgene (99.5%, reagent grade) was purchased from Chem-Impex (Wood Dale, IL). 4-Nitrophenyl chloroformate (97%, reagent grade) was purchased from AmBeed (Arlington Hts, IL). Polyethylene glycol monomethyl ether 2000 (average Mn, ~2,000 Da) was purchased from TCI (Portland, OR). 4-Dimethylaminopyridine (DMAP, ≥ 99%, reagent grade), 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU) (98%, reagent grade), (3S,6S)-3,6-dimethyl-1,4-dioxane-2,5-dione (98%, reagent grade), (3R,6R)-3,6-dimethyl-1,4-dioxane-2,5-dione (95%, reagent grade), 3,6-Dimethyl-1,4-dioxane-2,5-dione (99%, reagent grade) and all the anhydrous solvents were purchased from Sigma-Aldrich (St. Louis, MO). All NMR solvents were used as received from Cambridge Isotope Laboratories, Inc. RPMI 1640 medium with L-glutamine and 25 mM HEPES, 0.25% trypsin with 2.21 mM EDTA, and Dulbecco's Phosphate Buffered Silane (DPBS) were purchased from Corning. Penicillin-Streptomycin solution was purchased from Cytiva. Thiazolyl blue tetrazolium bromide (MTT) was purchased from Acros Organics. Dimethyl Sulfoxide (DMSO) was purchased from Fisher Chemical.

1.2 Instrumentation

NMR spectra were recorded on an Avance Bruker 500 MHz spectrometer. UV-vis-NIR spectra were recorded on a Cary5000 equipped with a Peltier-controlled heating stage (Versa 20 from Quantum Northwest). Mass spectra were obtained on a MicroQ-TOF ESI mass

spectrometer. Dynamic light scattering (DLS) measurements and the particle zeta potential values were determined using a Malvern ZEN1600 (MALVERN, Ltd., Worcestershire, UK). Transmission electron microscope (TEM) images were acquired with a JEOL 1400-X TEM located at the Miller School of Medicine TEM Core at the University of Miami. HPLC was performed on a Shimadzu HPLC system (LC-20AT) connected with PDA detector (SPD-M20A) and fluorescence detector (RF-20A). The DMF gel permeation chromatography (GPC) was conducted on a Shimadzu (Columbia, MD) system equipped with an isocratic pump model LC-40D, a differential refractometer model RID-20A. The system was equilibrated at 55 °C in pre-filtered DMF, which served as polymer solvent and eluent. Polymer solutions were prepared at a concentration of ca. 10 mg/mL and an injection volume of 100 μL was used. Flow rate was set to 1.00 mL/min. The system was calibrated with polyethylene glycol standards ranging from 1000 to 40,000 Da.

1.3 Synthesis procedure

1.3.1 Synthesis of CPT-ss-OH

CPT (1 g, 2.87 mmol) was dissolved in 200 mL of dichloromethane (DCM), and then triphosgene (0.536g) was added to the mixture. 4-dimethylaminopyridine (DMAP) (1.05 g, 8.6 mmol) was put into a 10 mL DCM solution, and this preparation was added dropwise to the CPT solution and stirred for 30 min. 2,2'-dithiodiethanol (8.6 g, 55.8 mmol) was dissolved in 25 mL anhydrous THF, and this was added to the preparation and stirred overnight at room temperature. The mixture was washed with 50 mM HCl (100 mL x 2), H2O (100 mL x 2), and saturated brine (100 mL x 2). After the removal of the solvent by rotary evaporation, the product was purified by column chromatography (silica gel column, DCM: MeOH=50: 1, v/v) to get yellow solid (1.126 g, yield 74.2%). ^{1}H NMR (500 MHz, Chloroform-d) δ 8.42 (s, 1H), 8.23 (d, J = 8.5 Hz, 1H), 7.95 (d, J = 8.1 Hz, 1H), 7.85 (t, J = 8.3 Hz, 1H), 7.44 (s, 1H), 5.70 (d, J = 17.2 Hz, 1H), 5.39 (d, J = 17.2 Hz, 1H), 5.30 (s, 2H), 4.38 (s, 2H), 3.90 (s, 2H), 2.94 (d, J = 39.7 Hz, 4H), 2.28 (s, 1H), 2.17 (s, 1H), 1.01 (t, J = 7.5 Hz, 3H). HRMS (ESI) m/z: calculated for $C_{25}H_{24}N_2O_7S_2$, found $[M+H]^+$ =529.1517. The CPT-cc-OH was synthesized by following the same method as CPT-ss-OH preparation using 1,6-hexanediol instead of 2-hydroxyethyl disulfide. (1.058 g, yield 74.8%). ^{1}H NMR (500 MHz, Chloroform-d) δ 8.40 (d, J = 6.1 Hz, 1H), 8.24 (dd, J = 8.6, 5.6 Hz, 1H), 7.99 – 7.93 (m, 1H), 7.86 – 7.79 (m, 1H), 7.70 – 7.63 (m, 1H), 7.36 (s, 1H), 5.75 – 5.65 (m, 1H), 5.39 (d, J = 17.2 Hz, 1H), 5.30 (d, J = 3.0 Hz, 2H), 4.12 (s, 2H), 3.62 (s, 2H), 2.35 – 2.26 (m, 1H), 2.16 (dq, J = 14.8, 7.5 Hz, 1H), 1.72 – 1.65 (m, 2H), 1.54 (q, J = 6.9 Hz, 2H), 1.44 – 1.31 (m, 4H), 1.02 (dt, J = 15.0, 7.5 Hz, 3H). HRMS (ESI) m/z: calculated for $C_{27}H_{28}N_2O_7$ 492.1897, found $[M+H]^+$ =493.2250

1.3.2 Synthesis of CPT-ss-PLAs and CPT-cc-PLA$_{18}$

Reactions were performed under reduced humidity conditions (20% relative humidity) using dried N_2 gas within the glove box to obtain the desired conditions. CPT-ss-OH (200 mg, 0.379 mmol) and *D/L*- lactide (163.8 mg, 1.136 mmol) were dissolved in 25 mL of DCM in a 50 mL round bottom flask. While CPT-ss-OH and *D/L*- lactide in DCM under stirring, the DBU (86.3 mg, 0.568 mmol) solution was injected into the mixture. The mixture was left to stir for 10 minutes and then the reaction was quenched with excess glacial acetic acid. The crude product was purified by column chromatography (silica gel column, EA: Hexane=1: 3, v/v) to

get the solid CPT-ss-PLA_5 (196 mg, yield 58.3%). ^{1}H NMR (300 MHz, Chloroform-d) δ 8.33 (s, 1H), 8.15 (d, J = 8.5 Hz, 1H), 7.87 (d, J = 8.1 Hz, 1H), 7.77 (t, J = 7.7 Hz, 1H), 7.60 (t, J = 7.5 Hz, 1H), 7.27 (d, J = 4.2 Hz, 1H), 5.62 (d, J = 17.3 Hz, 1H), 5.31 (d, J = 17.3 Hz, 1H), 5.22 (s, 2H), 5.19 – 4.97 (m, 5H), 4.35 – 4.23 (m, 4H), 2.83 (dt, J = 15.5, 6.6 Hz, 4H), 2.21 (dq, J = 14.9, 7.5 Hz, 1H), 2.08 (dq, J = 14.6, 7.4 Hz, 1H), 1.58 – 1.40 (m, 15H), 0.93 (t, J = 7.5 Hz, 3H). The CPT- ss-PLA16 was synthesized by following the same method as CPT-ss-PLA_5 preparation using different ration of CPT-ss-OH and *D/L*-Lactide. (140.8 mg, yield 22.1%). ^{1}H NMR (300 MHz, Chloroform-d) δ 8.41 (s, 1H), 8.23 (d, J = 8.5 Hz, 1H), 7.95 (d, J = 8.1 Hz, 1H), 7.85 (t, J = 7.6 Hz, 1H), 7.68 (t, J = 7.5 Hz, 1H), 7.33 (s, 1H), 5.70 (d, J = 17.3 Hz, 1H), 5.39 (d, J = 17.3 Hz, 1H), 5.31 (s, 2H), 5.29 – 5.01 (m, 15H), 4.36 (q, J = 7.1 Hz, 4H), 2.91 (dt, J = 15.5, 6.5 Hz, 4H), 2.29 (dd, J = 14.1, 7.3 Hz, 1H), 2.16 (dd, J = 14.2, 7.3 Hz, 1H), 1.67 – 1.50 (m, 45H), 1.01 (t, J = 7.5 Hz, 3H). The CPT-ss-PLA_{36} was synthesized by following the same method as CPT-ss-PLA_5 preparation using different ration of CPT-ss-OH and *D/L*-lactide. (166.2 mg, yield 17.0%). ^{1}H NMR (500 MHz, Chloroform-d) δ 8.41 (s, 1H), 8.23 (d, J = 8.5 Hz, 1H), 7.95 (d, J = 8.1 Hz, 1H), 7.84 (t, J = 7.7 Hz, 1H), 7.68 (t, J = 7.5 Hz, 1H), 7.33 (s, 1H), 5.69 (d, J = 17.1 Hz, 1H), 5.39 (d, J = 17.2 Hz, 1H), 5.30 (d, J = 5.8 Hz, 3H), 5.25 – 5.01 (m, 36H), 4.40 – 4.27 (m, 5H), 2.93 (q, J = 6.4 Hz, 2H), 2.87 (t, J = 6.5 Hz, 2H), 2.28 (dq, J = 14.8, 7.4 Hz, 1H), 2.15 (dq, J = 14.7, 7.4 Hz, 1H), 1.56 (ddt, J = 10.0, 6.9, 2.6 Hz, 108H), 1.00 (t, J = 7.5 Hz, 3H). In the same way, the CPT-cc-PLA_{18} was synthesized. (148.6 mg, yield 20.0%). ^{1}H NMR (400 MHz, Chloroform-d) δ 8.40 (s, 1H), 8.21 (d, J = 8.6 Hz, 1H), 7.94 (d, J = 8.2 Hz, 1H), 7.88 – 7.79 (m, 1H), 7.67 (t, J = 7.5 Hz, 1H), 7.32 (s, 1H), 5.68 (d, J = 17.2 Hz, 1H), 5.38 (d, J = 17.3 Hz, 1H), 5.29 (s, 2H), 5.25 – 5.08 (m, 18H), 4.33 (tt, J = 12.4, 7.0 Hz, 1H), 4.05 (tt, J = 6.9, 4.4, 3.8 Hz, 4H), 2.29 (dt, J = 14.2, 7.4 Hz, 1H), 2.16 (dd, J = 14.1, 6.7 Hz, 1H), 1.69 – 1.61 (m, 4H), 1.60 – 1.54 (m, 54H), 1.00 (d, J = 7.5 Hz, 3H).

1.3.3 Synthesis of PEG-b-PLA

The reactions were conducted under controlled conditions of reduced humidity (20% relative humidity) using dried N_2 gas within a glove box to ensure the desired reaction environment. Polyethylene glycol monomethyl ether 2000 (PEG2K) (500 mg, 0.25 mmol) and *D/L*-lactide (540.49 mg, 3.75 mmol) were dissolved in 10 mL of DCM in a 25 mL round-bottom flask. While stirring the PEG2K and *D/L*-lactide solution in DCM, DBU (57 mg, 0.375 mmol) was added via injection. The mixture was stirred for 10 minutes before quenching the reaction with excess glacial acetic acid. The resulting product was purified by precipitating the polymer from DCM into diethyl ether five times. Finally, the polymer was dried under vacuum to yield a white solid (986 mg, 94.8%). ^{1}H NMR (300 MHz, Chloroform-d) δ 5.18 – 5.13 (m, 30H), 3.62 (s, 180H), 3.36 (s, 3H), 1.56 (d, J = 7.1 Hz, 113H).

1.4 Preparation of CPT/PLANP and CPT-ss-PLA NPs

CPT was dissolved in DMSO at a concentration of 5 mg/mL. Next, PEG-b-PLA was added to 0.5 mL of the 10 mg/mL CPT solution. Subsequently, 40 μL of the mixture was added to 360 μL of PBS to prepare the CPT/PLANP. For the preparation of CPT-ss-PLA NPs, CPT-ss-PLA polymer prodrugs (10 mg) were dissolved in THF (1 mL) to prepare a stock solution at a concentration of 10 mg/mL. Similarly, PEG-b-PLA (10 mg) was dissolved in THF (1 mL) to create a stock solution at a concentration of 10 mg/mL. Different ratios of CPT-ss-PLA polymer

prodrugs and PEG-b-PLA were then mixed and added dropwise into 2 mL of pure water while stirring at room temperature for 20 minutes. The THF was evaporated under a nitrogen stream, and the solution volume was adjusted to 2 mL with pure water. This resulted in an aqueous solution of CPT-ss-PLA polymer prodrugs with a final concentration of 100 μg/mL. The diameters of the nanoparticles were measured using DLS without further purification.

1.5 *In vitro* drug release

The NPs used in the drug release study were formed with a ratio of 1:2 between CPT-ss-PLA polymer prodrugs and PEG-b-PLA. The release of CPT from CPT-ss-PLA NPs under 40 mM GSH was analyzed using HPLC. A GSH-buffered solution (40 mM) was prepared by dissolving GSH in phosphate-buffered saline (PBS) and adjusting the pH to 7.4 using diluted NaOH. To conduct the experiment, 100 μL of CPT-ss-PLA NPs stock solution (100 μg/mL) was thoroughly mixed with 900 μL of the GSH-buffered solution. For comparison, CPT release from CPT-ss-PLA NPs in PBS without GSH served as the control group. HPLC detection utilized a gradient elution of acetonitrile and water (50% CH_3CN for the first 5 minutes, followed by a gradient increase to 99% CH_3CN over 27 minutes) at a flow rate of 1 mL/min. CPT eluted at 4.0 minutes and was detected using a UV detector set at 370 nm.

1.6 Cell cultures

BxPC-3 and 4T1 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. PC-3 cells were maintained in F-12K medium (Kaighn's Modification of Ham's F-12 Medium) with 10% FBS and 1% penicillin-streptomycin. All cells were incubated in a humidified environment at 37 °C with 5% CO_2. For subculturing, the cells were detached using a trypsin solution for 3 minutes. The suspension was centrifuged at 5000 rpm for 3 minutes, and the supernatant was discarded. Fresh medium was added to neutralize any remaining trypsin. The cells were resuspended in serum-supplemented medium and returned to the humidified incubator at 37 °C and 5% CO_2 for continued culture.

1.7 *In vitro* cytotoxicity

The NPs used in the cytotoxicity study were formed with a ratio of 1:2 between CPT-ss-PLA polymer prodrugs and PEG-b-PLA. Different cancer cell lines, including BxPC-3, 4T1, and PC-3, were used to evaluate the in vitro cytotoxicity of CPT and CPT-ss-PLA NPs. The cells were seeded at a density of 3,000 cells per well and incubated for 24 h for attachment in 96-well plate. Culture medium was replaced with serial dilutions of CPT or CPT-ss-PLA NPs in fresh medium and incubated for 48 h. The medium was then replaced with 100 μL medium containing 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT). After 4 h incubation, the medium containing unreacted MTT was carefully removed, and the obtained blue formazan crystals were dissolved in 100 μL DMSO. The absorbance at 570 nm was measured by SpectraMax i3x. The cell viability was calculated based on the relative absorbance to untreated cells (100% cell viability) and control medium (no cells, 0% cell viability).

1.8 Animal procedures

All animal studies were ethically approved by NUS Institutional Animal Care and Use Committee (approval number: R23-0488). Female BALB/c nude mice aged 6–8 weeks were purchased from Gempharmatech and inoculated with 5×10^6 BxPC-3 cells in right flank region. Once the tumor volume reached approximately 100 mm^3, the mice were randomly divided into six experiment groups. Each group received intravenous administration of the respective formulations via the tail vein on days 0, 3, 6, 9, and 12. Tumor volume and body weight were measured every two days throughout the study. Mice were humanely euthanized if the tumor size exceeded 2 cm in its largest dimension.

2. SUPPROTING FIGURES AND TABLES

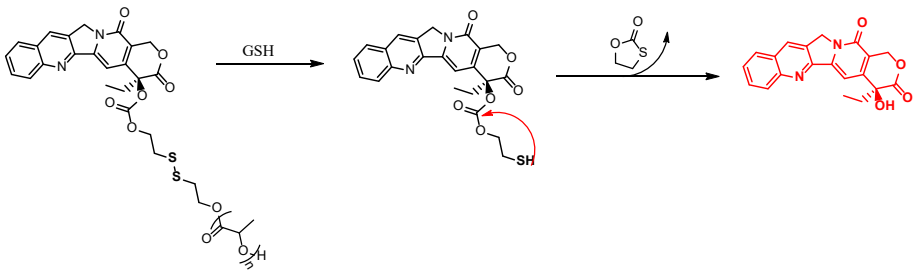

Figure S1. The mechanism of GSH triggered drug release.

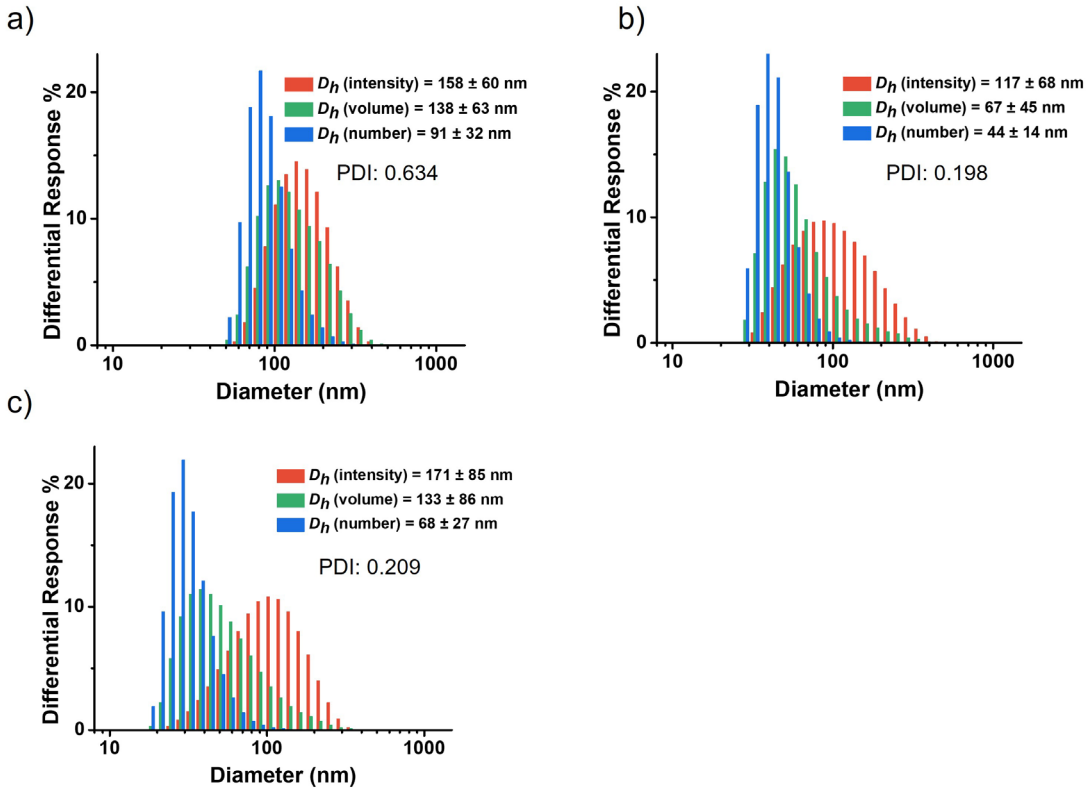

Figure S2. Characterizations of CPT-ss-PLA_5 (a), CPT-ss-PLA_{36} (b) and CPT-cc-PLA_{18} (c) by dynamic light scattering

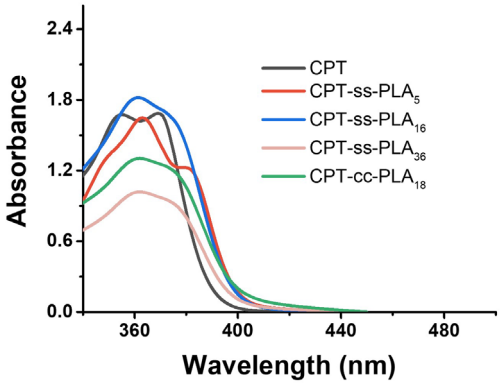

Figure S3. UV-vis spectrum of CPT (black line), CPT-ss-PLA_5 (red line), CPT-ss-PLA_{16} (blue line), CPT-ss-PLA_{36} (pink line) and CPT-cc-PLA_{18} (green line)

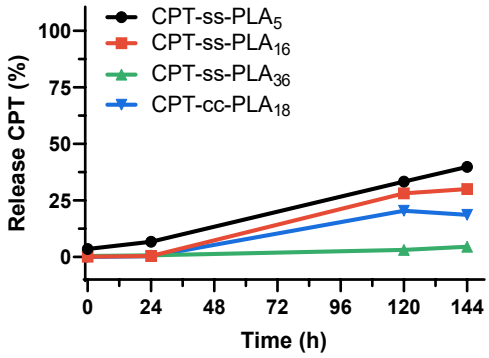

Figure S4. Intact CPT release in the presence of PBS.

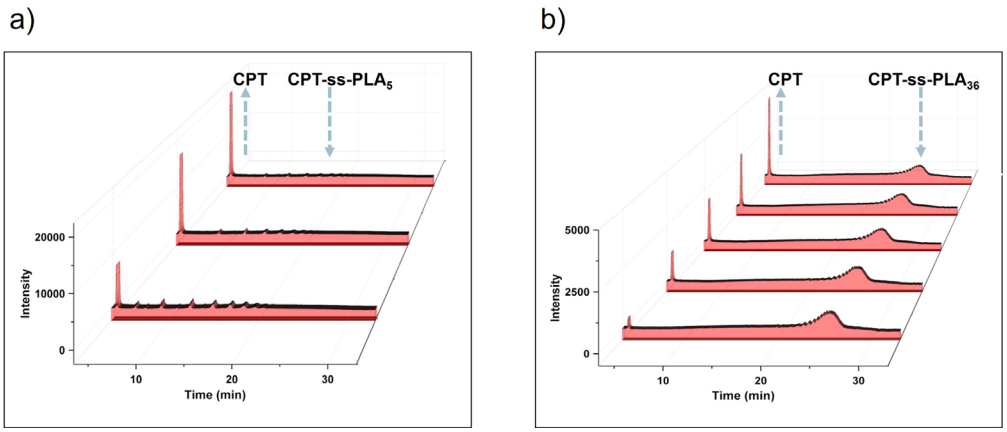

Figure S5. HPLC traces of intact CPT release from prodrug CPT-ss-PLA_5 (a) and CPT-ss-PLA_{36} (b) in PBS containing 40 mM GSH at different time points.

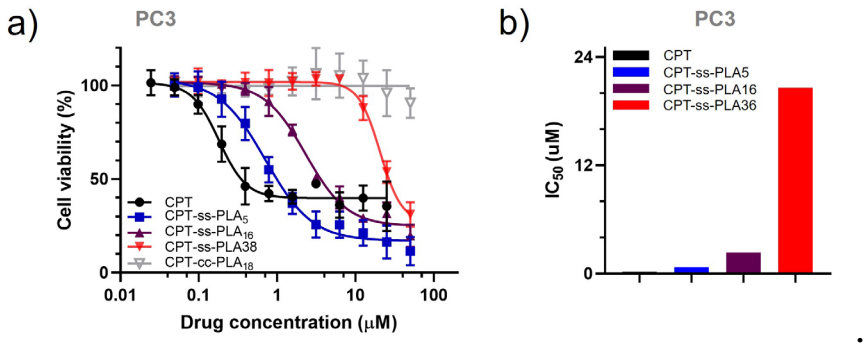

Figure S6. (a) In vitro cytotoxicities of CPT and CPT prodrugs against PC-3 cancer cells. (b) Ratio of IC_{50} values against the PC-3 cancer cells vs different CPT or CPT-ss-PLA NPs after 48 h incubation.

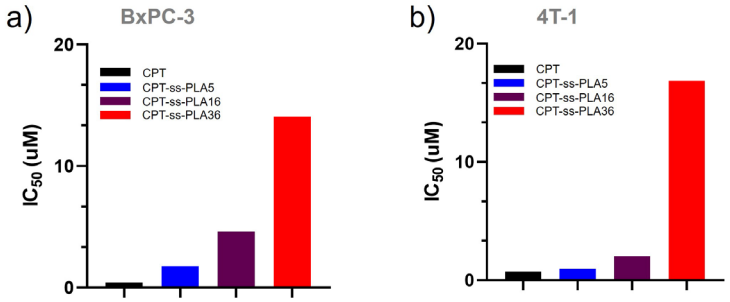

Figure S7. IC_{50} values against the BxPC-3 (a) and 4T1 (b) cancer cells vs different CPT or CPT-ss-PLA NPs after 48 h incubation.

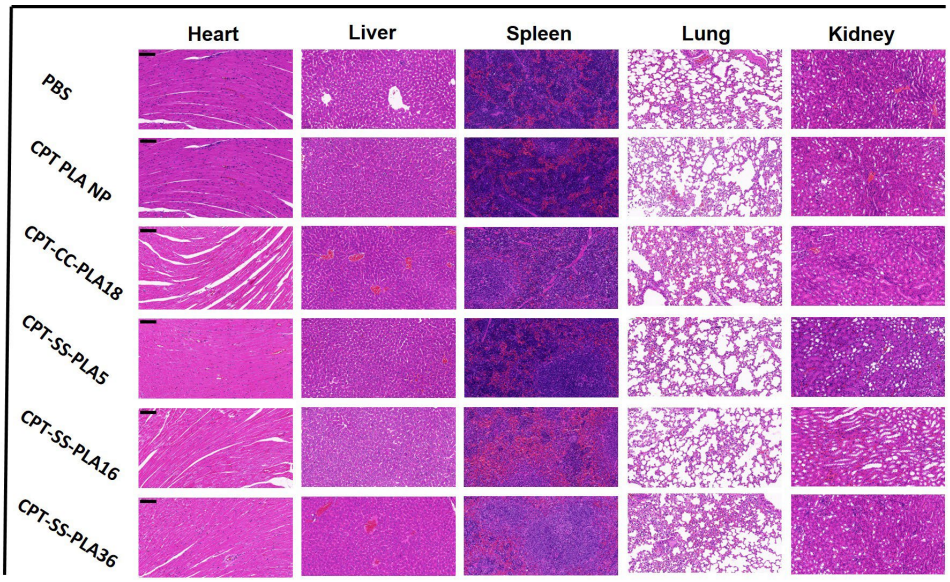

Figure S8. H&E staining images of the major organs (heart, liver, spleen, lung, and kidney) from the mice after injection of PBS, CPT PLA NP, CPT-cc-PLA_{18}, CPT-ss-PLA5, CPT-ss-PLA_{16} or CPT-ss-PLA_{36}. (Scale bar is 120 μm)

Table S1. Molecular weights of polymers calculated by ^{1}H NMR and GPC

Polymer-drug Conjugates	DP[a]	Mn[b] (Da)	GPC		
			Mn (Da)	Mw (Da)	PDI
CPT-ss-PLA$_5$	5	888	1119	1258	1.07
CPT-ss-PLA$_{16}$	16	1681	2330	2490	1.07
CPT-ss-PLA$_{36}$	36	3122	3260	3461	1.06

[a] DP (degrees of polymerization): Determined from ^{1}H NMR spectrum

[b] Determined from ^{1}H NMR spectrum

Table S2. The IC_{50} values of CPT-ss-PLA NPs

IC_{50} values (μM)[a]	4T1	PC-3	BxPC-3
CPT	0.74	0.18	0.41
CPT-ss-PLA$_5$	0.97	0.71	1.78
CPT-ss-PLA$_{16}$	2.05	2.31	4.62
CPT-ss-PLA$_{36}$	16.85	20.60	14.06
CPT-cc-PLA$_{18}$	N/A	N/A	N/A

[a] Numbers in parentheses are corresponding IC_{50} values calculated by GraphPad Prism 8

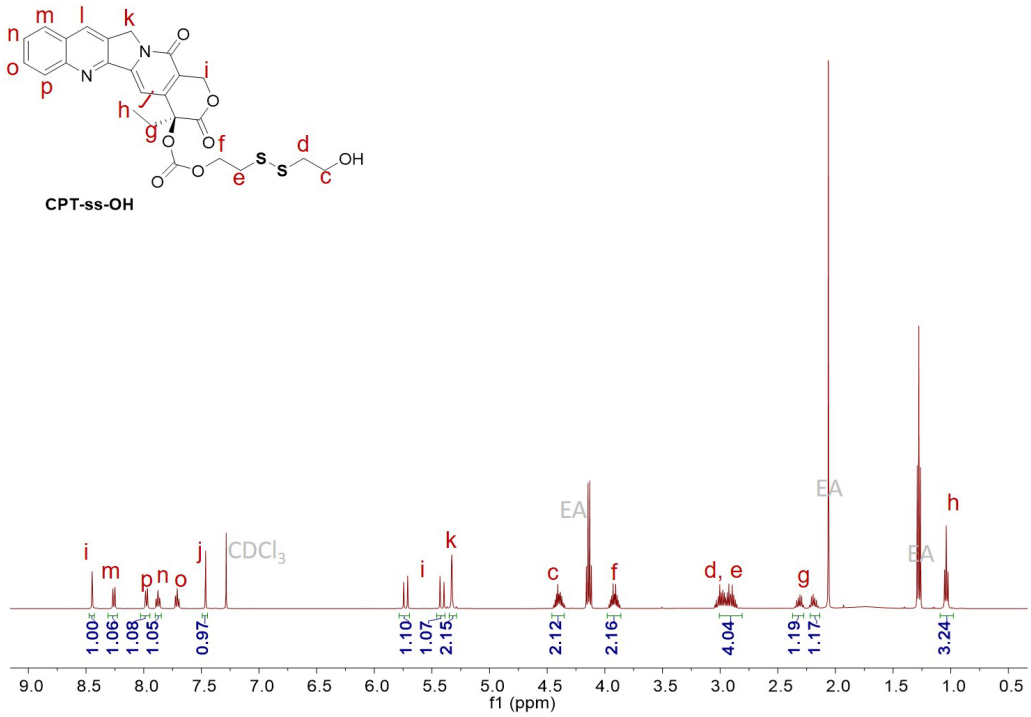

Figure S9. The ^{1}H NMR spectrum of CPT-ss-OH in $CDCl_3$

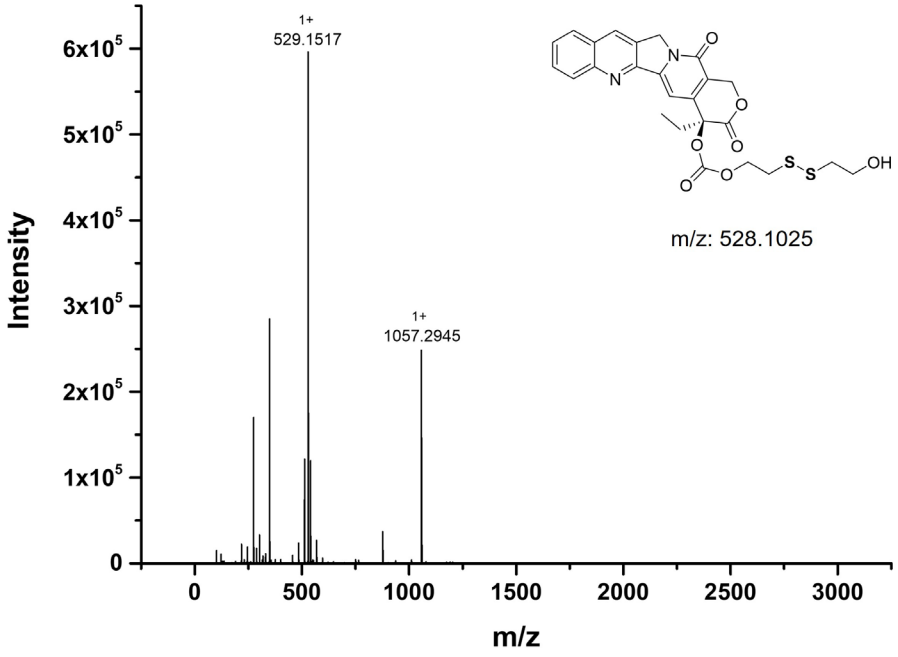

Figure S10. High-resolution mass spectrum of CPT-ss-OH

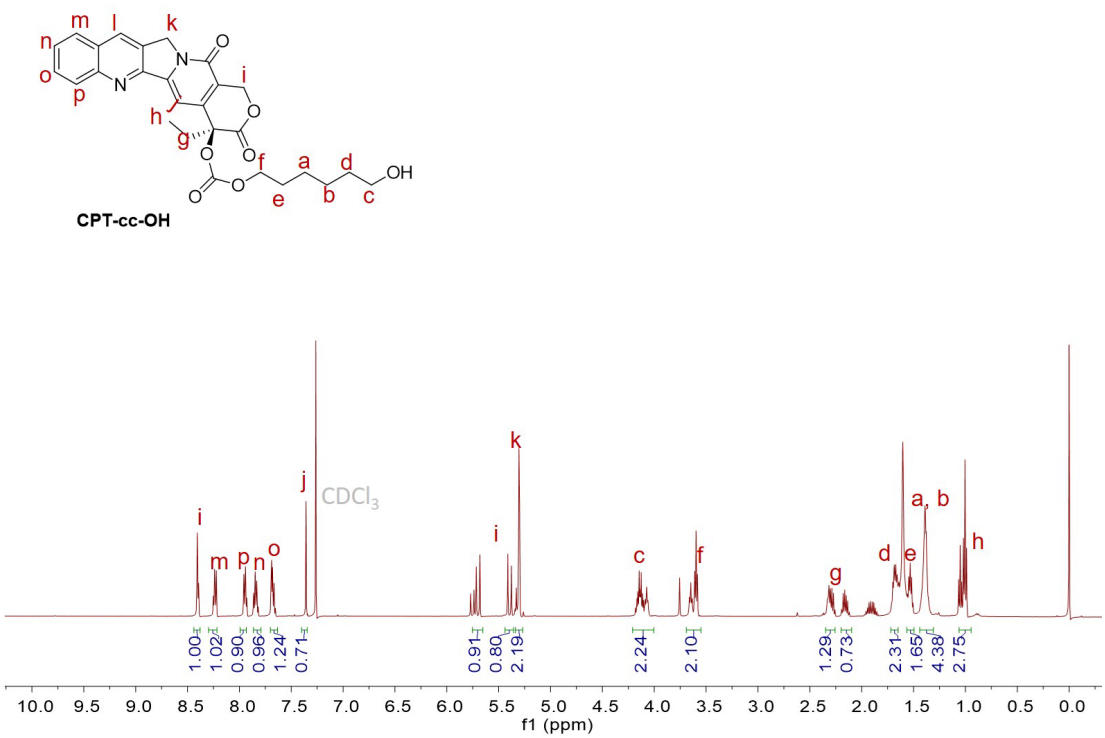

Figure S11. The ^{1}H NMR spectrum of CPT-cc-OH in $CDCl_3$

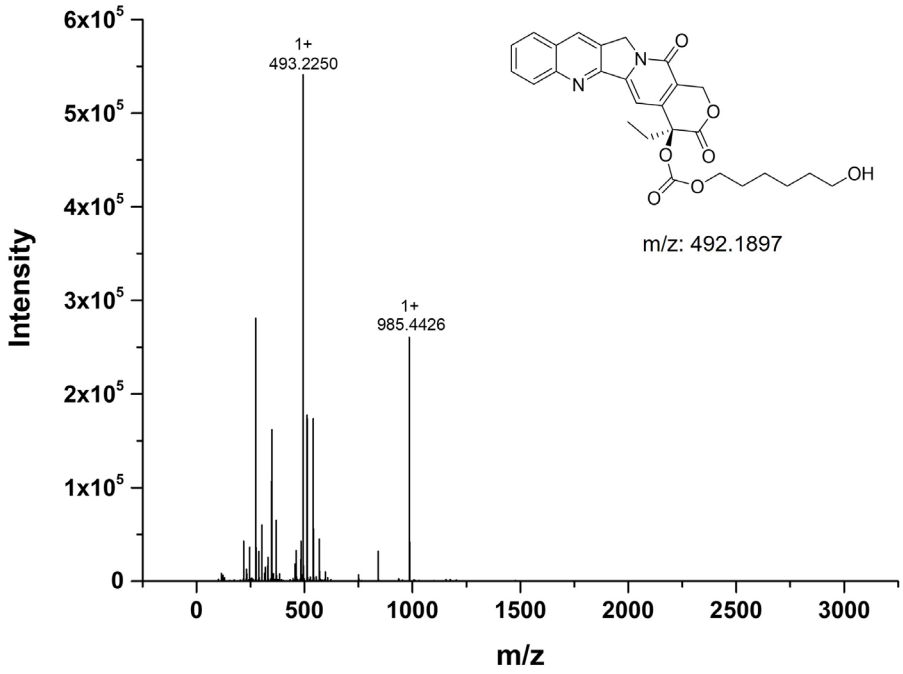

Figure S12. High-resolution mass spectrum of CPT-cc-OH

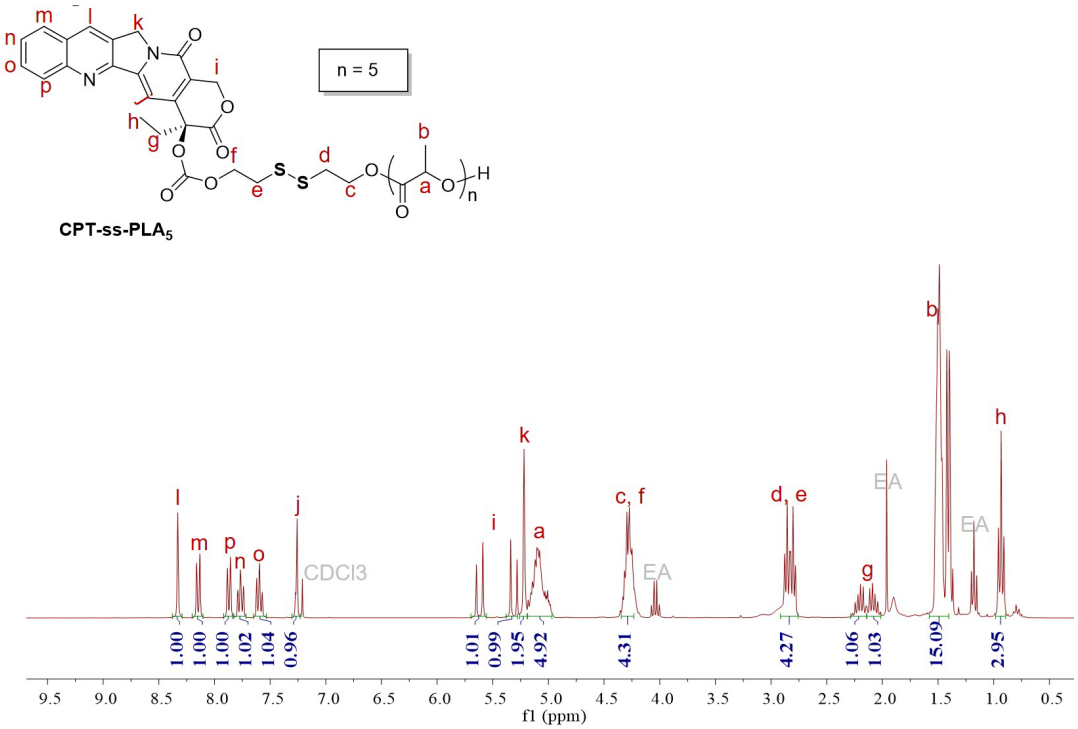

Figure S13. The ^{1}H NMR spectrum of CPT-ss-PLA_5 in $CDCl_3$

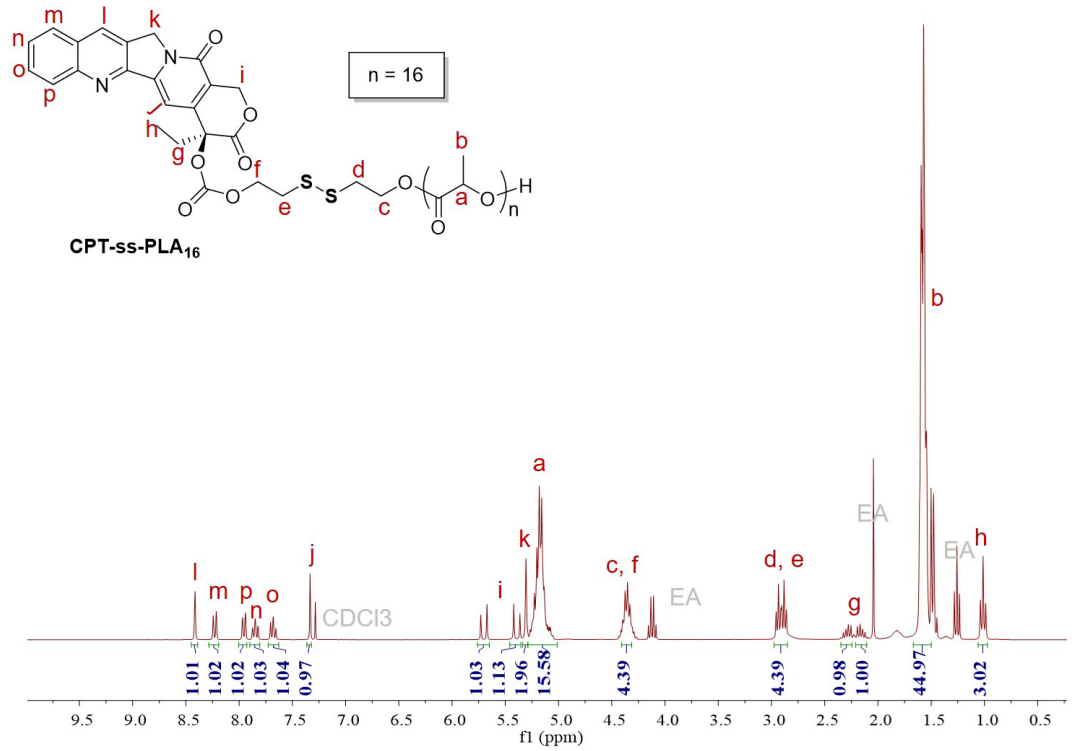

Figure S14. The ^{1}H NMR spectrum of CPT-ss-PLA_{16} in $CDCl_3$

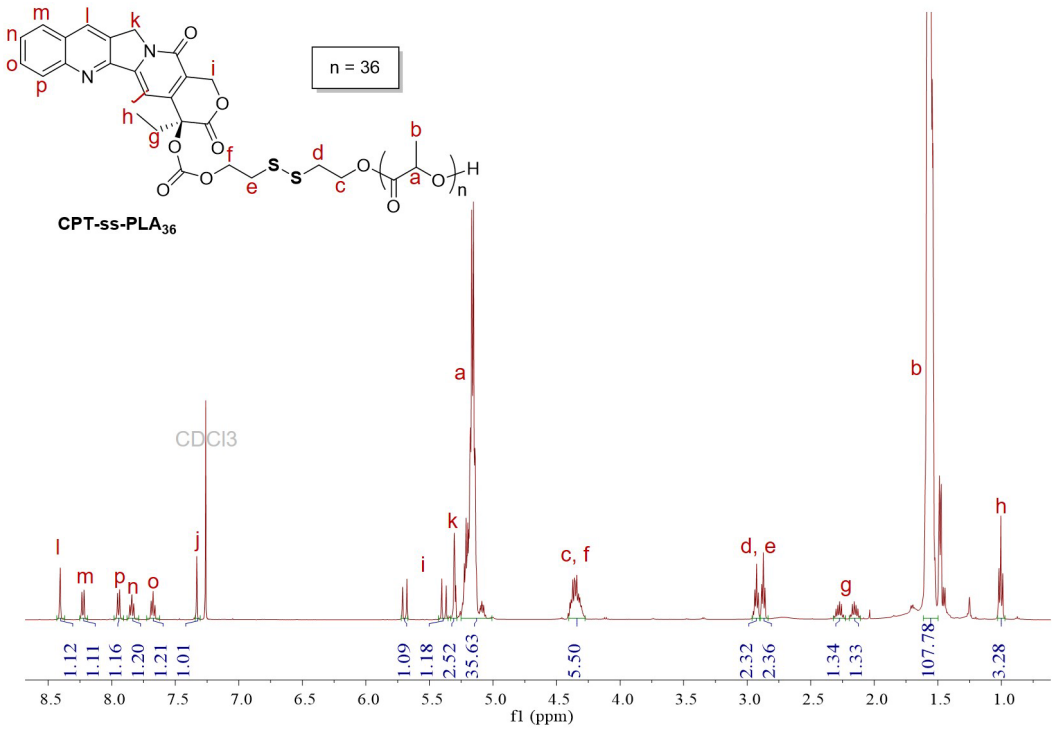

Figure S15. The ^{1}H NMR spectrum of CPT-ss-PLA$_{36}$ in $CDCl_3$

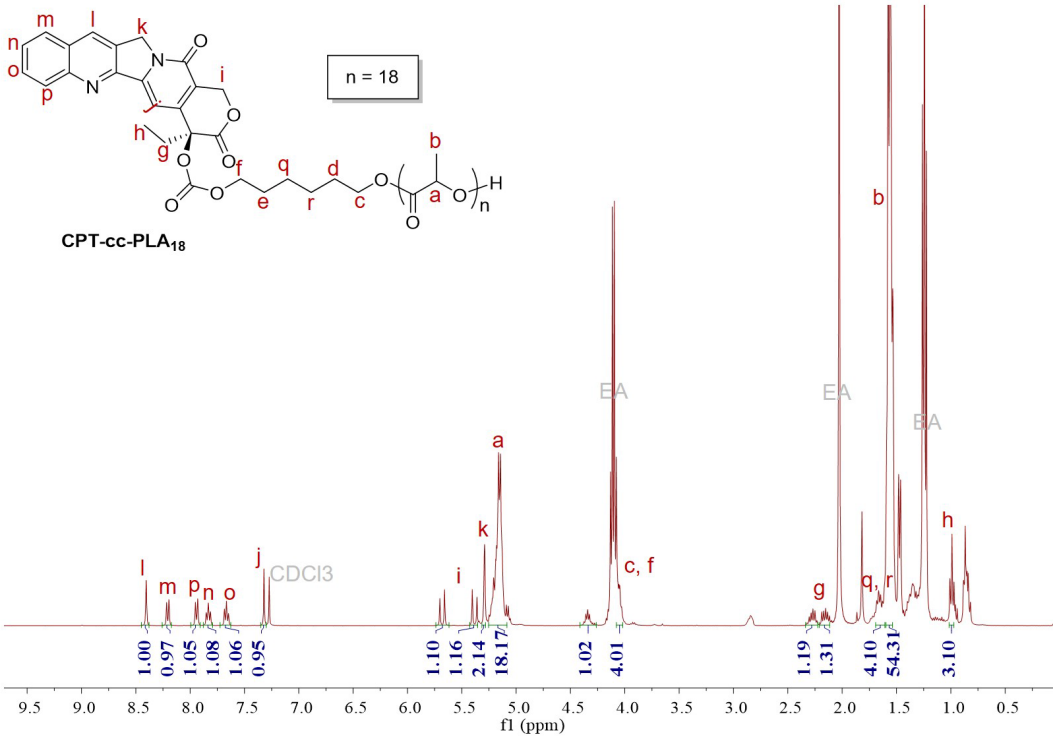

Figure S16. The ^{1}H NMR spectrum of CPT-cc-PLA$_{18}$ in $CDCl_3$

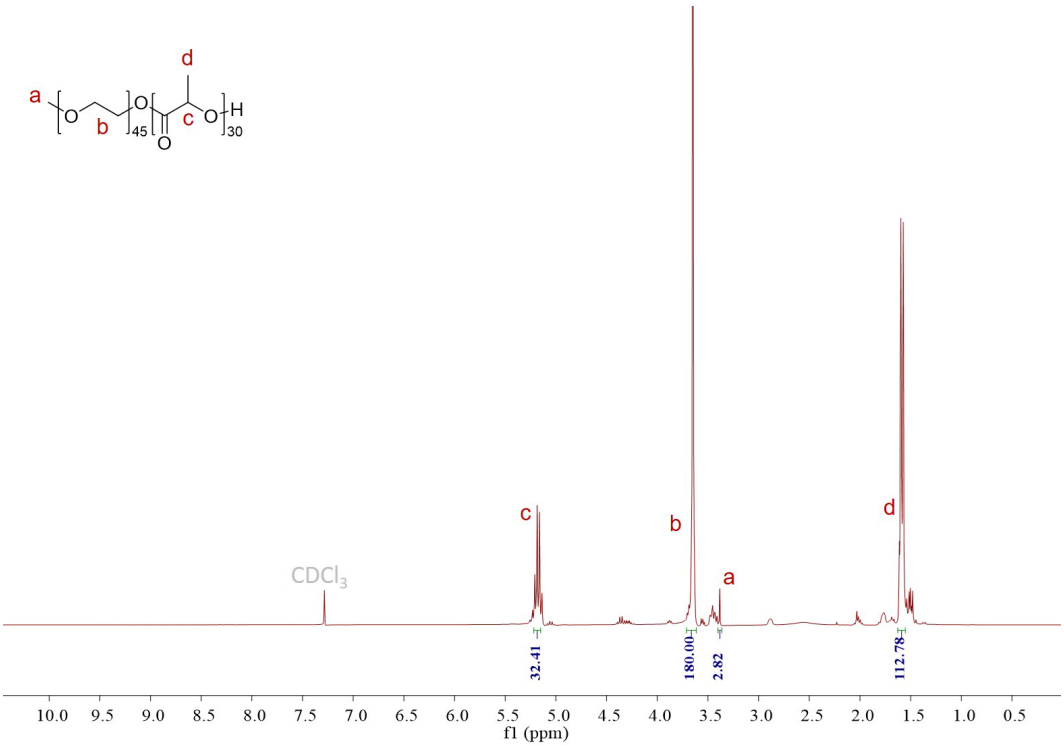

Figure S17. The ^{1}H NMR spectrum of PEG-b-PLA in $CDCl_3$