Supporting Information

Pyridine-Appended Disulfidephospholipids Enable Exceptionally High Drug Loading and Stability as a Robust Liposomal Platform

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# These authors contributed equally

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Experimental Section

Materials

L-α-Glycerophosphocholine (GPC, purity ≥ 98%) and hydrogenated soybean lecithin (HSL, PC purity ≥ 98%) were purchased from Meryer Biochemical Technology Co., Ltd. (Shanghai, China). 11-Mercaptoundecanoic acid (purity ≥ 95%), triphenylmethyl chloride (TrCl, purity ≥ 97%), carbonyldiimidazole (CDI, purity ≥ 97%), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, purity ≥ 98%), trifluoroacetic acid (TFA, purity ≥ 99%) and 2,2'-dithiodipyridine (purity ≥ 98%) were obtained from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China). Camptothecin (CPT, purity ≥ 97%) was purchased from Yuanye Technology Co., Ltd. (Shanghai, China). Cholesterol (purity ≥ 97%), mPEG2000-DSPE and Cy5.5-DSPE-PEG2000 were purchased from Ruixi Biological Technology Co., Ltd (Shanxi, China). All other chemical solvents and reagents were supplied from local commercial sources and used as received. Fetal bovine serum (FBS), Roswell Park Memorial Institute-1640 medium (RPMI-1640), Dulbecco's Modified Eagle's medium (DMEM) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) were purchased from Beyotime Biotechnology Co., Ltd. (Shanghai, China). 4',6-diamidino-2-phenylindole (DAPI), L-glutathione (GSH) and penicillin-streptomycin liquid were purchased from Solarbio Life Sciences (Beijing, China).

Instructions

^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AV 500 MHz spectrometer in $CDCl_3$ as solvent (Bruker, MA, USA). High-resolution mass spectrometry (HRMS) was performed on a Bruker Apex IV FTMS spectrometer using ESI (electrospray ionization). Ultraviolet-visible (UV-vis) spectra was recorded on a UV-3600 spectrometer (Shimadzu, Japan). Dynamic light scattering (DLS) for size distribution and zeta-potential measurements was performed on a Zetasizer Nano ZS (Malvern, UK). Particle morphology was observed on the transmission electron microscopy (TEM, Tokyo, Japan) equipped with a 2 k x 2 k AMT CCD camera. Ultra-Performance Liquid Chromatography (UPLC) was used for analysis of CPT content eluting with methanol/water (40:60, v/v) mixtures, which is equipped with a VWD UV–VIS detector and a ZORBAX SB-C18 column (Analytical, 4.6 × 150 mm) (Agilent, CA, USA).

Synthesis of Pyr-SS-PC Lipids

Pyridine-appended disulfidephospholipids (Pyr-SS-PC) were synthesized by a three-step reaction as shown in Figure S1, and the experimental process and structure characterization were detailed in the following manner.

Synthesis of Tr-S-COOH Compound:[1] 11-Mercaptoundecanoic acid (1 g/4.58 mmol) was dissolved in 20 mL of anhydrous CH_2Cl_2, following dropwise addition of TrCl (2.55 g/9.16 mmol) solution at room temperature. After 6 h stirring, the resultant mixture was concentered and recrystallized twice with 200 mL of petroleum ether to obtain Tr-S-COOH as a white solid (2.01 g, Yield: 95.2%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.36-7.23 (m, Phenyl), 2.33 (t, H-10), 2.12 (t, H-1), 1.59 (m, H-2), 1.40-1.21 (m, H-3, 4, 5, 6, 7, 8, 9) ppm; ^{13}C NMR (125 MHz, $CDCl_3$): δ 177.13 (s, -COOH-), 145.34 (s, Phenyl-C-), 129.38 (m, Phenyl), 34.64 (s, C-1), 30.23 (m, C-3, 4, 5, 6, 7, 8, 9, 10), 24.81 (s, H-2) ppm. HRMS, ESI$^+$, m/z: Calcd for $C_{30}H_{36}O_2S$ [M-H]$^-$: 459.24; found 459.24.

Synthesis of Tr-S-PC Lipids: To a solution of Tr-S-COOH (0.46 g/0.99 mmol) in dimethyl sulfoxide (DMSO, 15 mL), 0.24 g/1.49 mmol of CDI was added and activated for 2 h at 35 °C. After that, DBU (0.10 g/0.39 mmol) and GPC (0.23 g /1.49 mmol) suspended in DMSO were further mixed and reacted overnight at 45 °C. The synthesized product was purified by silica gel column chromatography using CH_2Cl_2/CH_3OH (solvent A: CH_2Cl_2:CH_3OH, 5:1; solvent B: CH_2Cl_2:CH_3OH:H_2O 65:25:4) as the gradient elution. The acquired fractions were evaporated and vacuum dried to afford Tr-S-PC lipids (0.64 g, Yield: 56.4%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.40-7.34 (m, Phenyl), 5.19 (m, H-4''), 4.51-4.29 (m, H-4''), 3.99 (m, H-2''), 3.89 (m, H-3''), 3.77 (s, H-1''), 3.30 (s, -N$^+$(CH_3)$_3$), 2.25 (m, H-10, 10'), 2.10 (t, H-1, 1'), 1.53 (m, H-2, 2'), 1.30 (m, H-8, 8', 9, 9'), 1.24 – 1.04 (m, H-4, 4', 5, 5', 6, 6', 7, 7') ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ 174.55 (s, -C=O-), 145.34 (s, Phenyl-C-), 129.38 (m, Phenyl), 69.29 (s, C-3'', 4''), 54.72 (s, --N$^+$(CH_3)$_3$), 34.09 (d, C-1, 1'), 30.23 (s, C-9, 9' 10, 10'), 29.07-28.74 (m, C-4, 4', 5, 5' 6, 6', 7, 7', 8, 8'), 25.33 (s, C-2, 2') ppm. HRMS, ESI$^+$, m/z: Calcd for $C_{68}H_{88}NO_8PS_2$ [M+H]$^+$: 1142.57; found 1142.57.

Synthesis of Pyr-SS-PC Lipids: 0.2 g/0.17 mmol of Tr-S-PC lipids was dissolved in a mixture of TFA/CH_2Cl_2 (1:1, v/v) solution under N_2 environment, and subjected to deprotection for 4 h. After sufficient evaporation to remove TFA, 2,2'-dithiodipyridine (0.15 g/0.68 mmol) dissolved in 10 mL of dried CH_2Cl_2 was added into the system, further stirred at room temperature overnight. The crude product was chromatographed (CH_2Cl_2:CH_3OH:H_2O 65:25:4) on silica gel to provide the Pyr-SS-PC lipid (0.13 g, Yield: 86.3%). ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.5-7.02 (m, Pyridyl), 5.17 (m, H-1''), 4.39-3.78 (m, H-2'', 3'', 4''), 3.34 (m, -N$^+$(CH_3)$_3$), 2.26

(m, H-1, 1', 10, 10'), 1.65 (m, H-2, 2', 9, 9'), 1.54 (m, H-3, 3'), 1.33 (m, H-5, 5'), 1.24 (s, H-6, 6', 7, 7') ppm. ^{13}C NMR (125 MHz, $CDCl_3$): δ 174.55 (s, -C=O-), 149.8-120.3 (m, Pyridyl), 54.72 (s, $-N^+(CH_3)_3$), 38.85 (s, C-10, 10'), 33.92 (m, C-10, 10'), 28.68 (m, C-3, 3', 4, 4', 5, 5', 6, 6', 7, 7', 8, 8', 9, 9'), 24.83 (s, C-2, 2') ppm. HRMS, ESI^+, m/z: Calcd for $C_{40}H_{66}N_3O_8PS_2$ $[M+H]^+$: 876.35; found 876.35.

MD Simulations

Membranes composed of Pyr-SS-PC lipids were simulated using MD by GROMACS software, where each bilayer was consisted of 64 molecules of Pyr-SS-PC lipids in a 6.4 × 6.4 × 8.0 nm^3 box.[2] Starting with the standard united atom force field, the model of Pyr-SS-PC lipids was defined by replacing sn-1 and sn-2 tails of phosphatidylcholine with dithiodipyridine groups. The GROMACS Amber99sb-ildn force field coupled with TIP3P water model was used to build and analyze the final structures and the Lennard-Jones (LJ) parameters of lipid molecules were included in Lipid14 force field.[3] This system was simulated for 60 ns production runs in NPT ensemble with a coupling constant of 2 fs at a constant temperature of 293 K. For each run, the SHAKE algorithm and particle mesh Ewald method were used to determine bond lengths and long-range electrostatic interactions, respectively. A 12 angstrom cut-off was used for Van der Waals forces and Coulomb interactions. This simulation protocol has been shown to be reliable for membrane systems.[4]

Preparation of CPT@Pyr-SS-PC Lips

CPT-incorporating liposomes (CPT@Pyr-SS-PC Lips) composed of Pyr-SS-PC lipids were prepared based on the conventional dehydration-rehydration method.[5] Total lipids of Pyr-SS-PC, lecithin, cholesterol and DSPE-PEG_{2000} = 4:3:1:0.4 (molar ratio) and CPT at a molar ratio of 1:18 or 1:30 (drugs: lipids) were dissolved in the mixture of $CHCl_3$:CH_3OH (4:1, v/v) and evaporated under argon gas followed by further drying in a desiccator vacuum chamber. The film was re-hydrated in PBS (pH = 7.4) solution, with vigorous vortexing each 15 min for 1 h and then executed with 10 cycles of 30-s sonication on ice for 5 min. Nonencapsulated CPT was removed by centrifugation at 1400 × g for 10 min. Liposomes with mean hydrodynamic diameter ranging from 100 nm to 120 nm as well as low PDI (< 0.3) were finally achieved, as determined by DLS and TEM instruments by diluting liposomes suspensions to 0.1 - 0.5 mg/mL in distilled water. Traditional liposomes (CPT@PC Lips) as a positive control were prepared with the similar lipids composition through rehydration, vortexing and sonication as described above, except the addition of Pyr-SS-PC lipids.

Loading Efficiency and Cargo Release from CPT@Pyr-SS-PC Lips

Drug encapsulation efficiency (EE) and loading content (DLC) was determined using the ultracentrifugation method, according to previously reported.[6] CPT liposomes were centrifugated at 10,000 × g at 4 °C for 0.5 h and the amount of CPT in the supernatant was measured by UPLC (Agilent, CA, USA) at the absorbance of 365 nm using the standard curve in various concentration of CPT solution in menthol. Lipid concentration of tested liposomes was analysed by phosphate assay. The percentage of CPT incorporated in liposomes was calculated by the following formula:

EE% = (Weight of CPT in liposomes)/(Weight of CPT initial) × 100

DLC% = (Weight of CPT in liposomes)/(Weight of liposomes) × 100

For cargo release experiment, CPT@Pyr-SS-PC Lips (100 μg CPT/mL, 1 mL) were dialyzed against 20 mL of different appointed buffer (I: PBS (pH = 7.4); II: PBS (pH = 7.4) with 1 mM GSH; III: PBS (pH = 7.4) with 10 mM GSH) under sink condition. The release process was performed at 37 °C with constant shaking and protection form light. After various time intervals, aliquots of release medium were collected and assayed for CPT amount by UPLC, whereas the fresh medium was equivalently added for continued evaluation of CPT release. The release kinetics of CPT from liposomes were fitted to the developed release model previously to assess its possible release mechanism.[7]

Thermodynamic Stability Study

The thermodynamic stability in the presence of 10% FBS was measured by monitoring the hydrodynamic diameter and PDI of CPT@Pyr-SS-PC Lips (100 μg CPT/mL) within one month at 4 °C. In addition, stabilization of the active lactone form of CPT formulated in Pyr-SS-PC Lips was studied by comparison to the traditional CPT@PC Lips, which was co-incubated with human plasma at 37 ± 0.5 °C. Samples were withdrawn at 0, 2, 4, 6, 8, 12 and 24 h while the same volume of plasma solution (5% in PBS) was added. After being filtrated by 0.22 μm separate film (Thermo Scientific, MA, USA), 10 μL of the supernatant dissolved in menthol was immediately analyzed by UPLC. Quantification was completed using external standards of the lactone or carboxylate of CPT with a concentration range from 1.0 ng/mL to 1.0 μg/mL (R^2 = 0.998). The ratio of lactone form of CPT in formulated liposomes was calculated as follows:

Ratio of Lactone Form = $(C_{\text{lactone form}})/(C_{\text{lactone form}} + C_{\text{carboxylate form}}) \times 100\%$

Cell Culture

Human breast cancer cells MCF-7 and murine mammary carcinoma cell line 4T1 were purchased from the Cell Culture Center of the Institute of Basic Medical Science, Chinese Academy of Medical Science. Cells were cultured in DMEM and RPMI-160 containing 10% FBS and antibiotics (50 units/mL penicillin and 50 units/mL streptomycin), respectively at 37 °C with 5% CO_2.

Cellular Uptake

CPT itself was used as the fluorescence probe for the real-time monitoring of cellular internalization study. MCF-7 cells were plated at a density of 5×10^4 per well on glass bottom dishes (Cat.# 150680, Thermo Scientific, MA, USA) and cultured at 37 °C for 24 h. CPT@Pyr-SS-PC Lips (2 μg/mL CPT) were incubated with MCF-7 cells for 40 min and 60 min, respectively. After washed with PBS buffer three times, cells were treated with Lyso-Tracker Green (2 mL, 50 nM) for 30 min to mark late endosomes and lysosomes, fixed with 4% paraformaldehyde and stained with DAPI solution (Beyotime, Shanghai, China) for imaging under CLSM (FV3000, Olympus, Japan). To quantify the cellular uptake of CPT@Pyr-SS-PC Lips, MCF-7 cells were seeded in 6-well plates at 1×10^6 cells/well and incubated with liposomes at a CPT concentration of 4 μg/mL for predetermined time intervals. After that, the cells were washed with PBS buffer and detached by 0.025% trypsin/EDTA for data collection using a BD FACS flow cytometer (BD FACSCanto, NJ, USA).

Internalization Mechanism

For colocalization study with endocytic markers, the blank Pyr-SS-PC Lips were labelled with Cy5.5 lipophilic dyes at a ratio of 0.02:1 (Cy5.5:lipids), wherein the procedures described above were followed to incorporate Cy5.5 dyes into the lipid bilayer of liposomes (abbreviated as Cy5.5@Pyr-SS-PC Lips). 5×10^4 per well of MCF-7 cells were seeded on glass bottom dishes (Cat.# 150680, Thermo Scientific, MA, USA) and grown overnight. The cells were then incubated with Cy.5.5@Pyr-SS-PC Lips for 20 min at 37 °C, fixed and permeabilized with 0.1% Triton X-100. Following block with dilution buffer, the samples were treated with primary antibody clathrin or caveolin-1 (1:100, Beyotime, Shanghai, China) and sequential secondary antibody (1:500, goat anti-rabbit IgG (H+L), Alexa Fluor 488 antibody) for 2 h at r.t. Nuclei were counterstained with DAPI (Beyotime, Shanghai, China). The fluorescence images were observed by CLSM (FV3000, Olympus, Japan). To quantify the extent of colocalization, Manders' overlap coefficients (MOC) were used by monitoring > 50 cells at time point.

Endocytosis Inhibition

The effect of temperature block on cellular uptake was investigated by pre-incubating MCF-7 cells for 1 h at 4 °C, and subsequently treated with CPT@Pyr-SS-PC Lips for another 1 h at 4 °C. Moreover, the endocytosis inhibitors of chlorpromazine (CPZ, 25 μg/mL) and nystatin (50 μg/mL) were pretreated with MCF-7 cells for 1 h at 37 °C to inhibit clathrin- or caveolin-mediated entry pathway. The inhibitor solution was replaced by freshly prepared Cy5.5@Pyr-SS-PC Lips and further incubated for 2 h. Cells without pretreatment of endocytosis inhibitors were compared as a control. The cellular uptake of liposomes was determined by examining Cy5.5 fluorescence using spectrofluorometer, and normalized according to the fluorescent intensity of control group.

Cell Viability

Two cell lines MCF-7 and 4T1 that were seeded 5×10^3 cells per well in a 96-well plate were selected for *in vitro* cytotoxicity test of CPT@Pyr-SS-PC Lips. CPT in free or conventional and Pyr-SS-PC Lips was added as indicated in media with 10% FBS. After 24 h and 48 incubation at 37 °C, media was abandoned and cell viability was determined by MTT assay. The IC50 values (half-maximal inhibitory concentration) of different formulations was calculated with nonlinear regression fit to a sigmoidal dose-response curve using Graphpad prism 7.0 software (Version 7.0, La Jolla, CA, USA).

Cell Apoptosis and Cell Cycle

MCF-7 cells were plated at a density of 5.0×10^5 cells/well and cultured at 37 °C. On the following day, the cells were treated with free CPT, CPT@Pyr-SS-PC Lips and CPT@PC Lips (equiv. 4 μg CPT/mL) for 48 h. MCF-7 cells without the treatment were used as a control. For quantitative analysis of apoptosis, 5 μL of Annexin V-FITC and 10 μL of PI solution were added into the treated cell samples according to the manufacturer's instructions. For cell cycle determination, cells were collected and fixed with 70% ethanol overnight. The fixed cells were incubated with PI solution containing 0.1 mg/mL RNase A for 30 min in the dark. Both cell apoptosis and cycle were detected by BD Biosciences FACSCanto II Analyzer (BD FACSCanto, NJ, USA) and 2×10^4 events per sample were counted.

Cell Migration

A wound healing assay was conducted to assess the anti-migratory effect of CPT@Pyr-SS-PC Lips against MCF-7 cells.[8] The wounds were built by scratching the confluent monolayer of MCF-7 cells with a 10 μL sterile pipet in the middle of 6-well plate. CPT@Pyr-SS-PC Lips in media at a CPT concentration of 4 μg/mL were

added to the scratched monolayers. Images of the wound area were taken using a light microscope at 0 h, 24 h and 48 h of CPT incubation timepoints. The ratio of cell migration from different treated groups were normalized in terms of wound area by ImageJ software.

Western Blotting

The procedures for western blotting analysis were performed as described by Mahmood et. al.[9] The obtained protein samples after treated with above CPT formulations for 48 h were separated by 10% SDS-PAGE, transferred to PVDF membrane (0.22 μm, Millipore Co., Bedford, MA), and blocked with 5% defatted dry milk in TBS-Tween 20 (0.1%, v/v) and then incubated with primary caspase 3 (1:5000, ab32351, Abcam, MA, USA), Bax (1:1000, ab32503, Abcam, MA, USA) and Bcl-2 (1:2000, ab182858, Abcam, MA, USA) antibodies overnight at 4 °C. Following several washes, the membrane was probed by HRP-conjugated anti-rabbit IgG (1:1000, A0208, Beyotime, Shanghai, China). β-Actin was used as the loading control. Protein bands were detected using Chemiluminescent HRP Substrate (Themo Scientific, USA) S13 according to the manufacture's protocol and analyzed using the ChemiDocTM MP Imaging System (Bio-Rad, USA).

Animals

Animal study protocols were all approved by the Animal Use and Care Committee of Pharmacy, Yantai University. Balb/c nude mice (female, 18-20 g) obtained from Charles River Laboratories (Beijing, China) were used for experiments. MCF-7 cells (5×10^6 per mice) were resuspended in serum-free media and subcutaneously administered into the right flank of mice to establish *in vivo* xenograft tumor model.

Hemolysis Analysis

Whole blood as a gift from affiliated hospital of Yantai University was stored in heparinized tube and × 1000 g centrifuged for 20 min at 4 °C. The obtained hemocyte was diluted to 2% erythrocyte suspension prior to next experiments. Serial concentrations of CPT@Pyr-SS-PC Lips were hatched with 2% erythrocyte suspension at 37 °C for 4 h in an incubator. Subsequently, samples were centrifuged for 10 min at 1000 g and the absorbance at 540 nm was measured using UV/Vis spectrometer (Shimad, Japan). Deionized water and normal saline (0.9%) were chosen as positive control with 100% hemolysis and negative control with 0% hemolysis, respectively. A% hemolysis ratio (< 5%) was considered as nontoxic. Percent hemolysis rate was calculated using the standard formula as:

Hemolysis Rate (%)= (Ae-An)/(Ap-An)×100%

Ae means the absorbance of experimental groups, wherein, Ap and An are the absorbance of the respective positive and negative control groups.

Pharmacokinetics Study

When tumors reached to ~ 120 mm^3 in size, free CPT, CPT@PC Lips and CPT@Pyr-SS-PC Lips at a dose of 5 mg CPT/kg were intravenously injected into mice (n = 10) via the tail vein. At the designated time points, 100 μL of plasma samples were collected after blood being centrifuged × 3000 g for 10 min and mixed with 100 μL of phosphoric acid (0.15 M). CPT was extracted with 800 μL of acetonitrile. This performance produced the total amount of free CPT and CPT incorporated into liposomes as a lactone form. The CPT concentration was measured by UPLC using a mobile phase consisting of a 35/65 (v/v) mixture of acetonitrile and 5 mM KH_2PO_4 solution at a flow rate of 1.0 mL/min. The half-life ($t_{1/2}$) were calculated using the bootstrap method.

***Ex vivo* Optical Imaging and Biodistribution**

To track the behavior of Pyr-SS-PC Lips *in vivo*, optical imaging and ex vivo biodistribution were investigated in Balb/c nude mice bearing MCF-7 tumors (n = 6). The free Cy5.5 and conventional Cy5.5@PC Lips were employed as positive controls. Successive images were captured at 1 h, 2 h, 4 h, 6 h, 8 h and 24 h after post-injection *i.v.*, using an IVIS Lumina II system (Lumina 3, PerkinElmer, CA) with excitation wavelength of 650 nm and emission wavelength of 700 nm. For ex vivo biodistribution analysis, mice treated with above Cy5.5-labelled liposomes were sacrificed at 6 h and 24 h post-injection. Major orangs and tumor tissues were harvested and imaged with same operation.

Antitumor Evaluation *in vivo*

Tumor-bearing mice with ~ 100 mm^3 in size were randomly assigned to the following treatment groups (n = 5): 0.9% saline (control), free CPT: 1.25 mg/kg, CPT@PC Lips: 1.25 mg/kg and CPT@Pyr-SS-PC Lips: 1.25 mg/kg. The dosage of each groups was given via tail vein administration every other day, while the tumor volume and body weight of mice were recorded simultaneously for an additional 20 days by the end of experiment. Tumor volume was calculated as V (mm^3) = 1/2 × length (mm) × width $(mm)^2$.

Histology and Immunohistochemical Analysis

On the first day after last injection, mice were sacrificed and major organs and tumor tissues were collected for further histology and immunohistochemical analysis.

Specifically, the isolated major organs and tumors were fixed in 10% formalin, embedded in paraffin and stained with hematoxylin and eosin (H&E) for histological examination under a light microscope. Tumor tissues were stained by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) with an *In Situ* Cell Death Detection Kit for apoptosis analysis. Moreover, the other immunohistochemistry (IHC) of tumor tissues were assessed by labelling with cleaved caspase 3 and proliferation (Ki-67) antibodies to study the extent of cell apoptosis induced from CPT and CPT@Pyr-SS-PC Lips. The procedures for immunostaining were conducted following the same way in previous literatures.[10]

Statistical Analysis

The quantitative data were presented as mean ± S.D. Student's t-test or one-way analysis of variance (ANOVA) was performed to the statistical significance within the data at 95% confidence levels. For survival analyses, the log-rank (Mantel–Cox) test was performed to assess differences between the median survival times of relevant groups. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ and ns, no significant difference.

Supplementary Figures

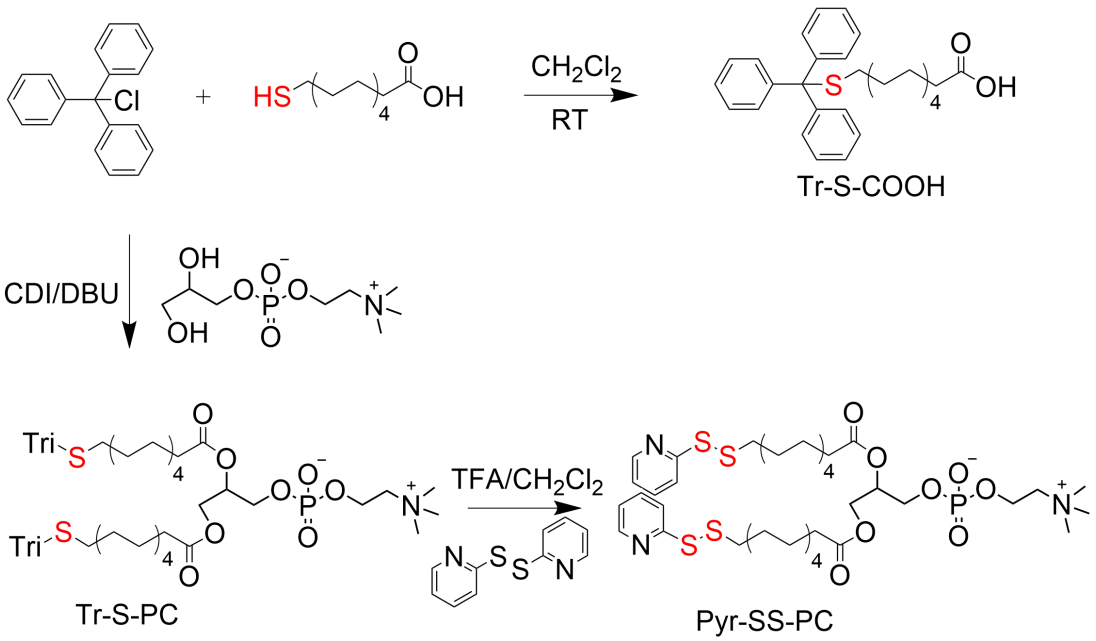


Figure S1. Three-steps for synthesis of Pyr-SS-PC lipids.

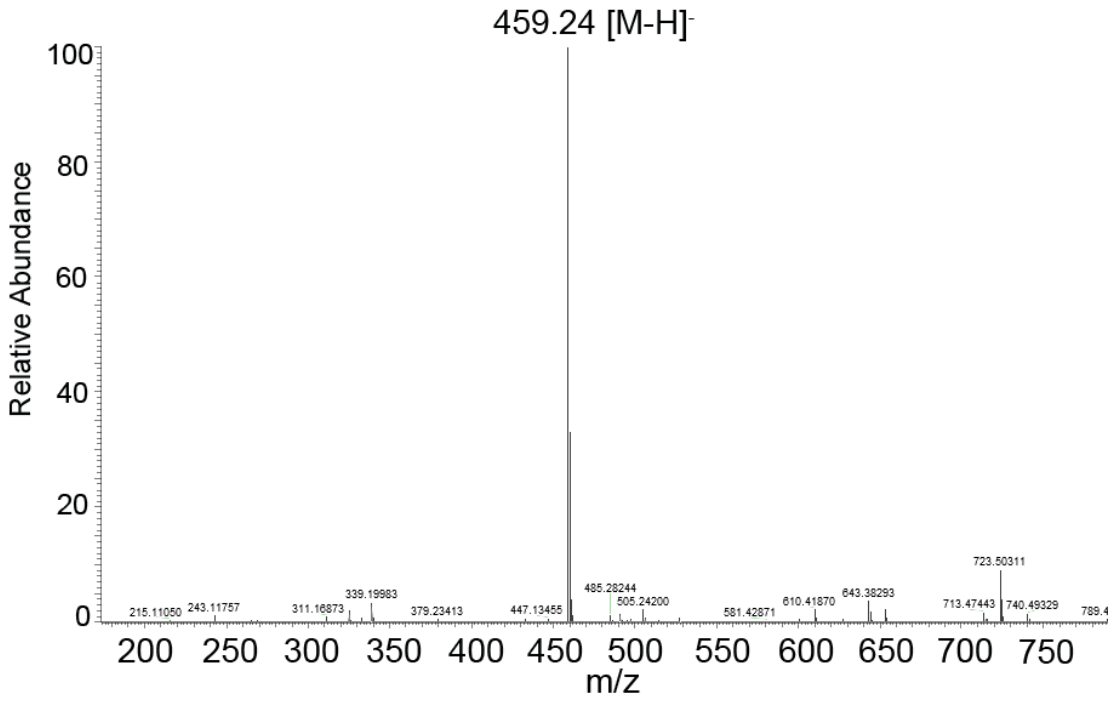
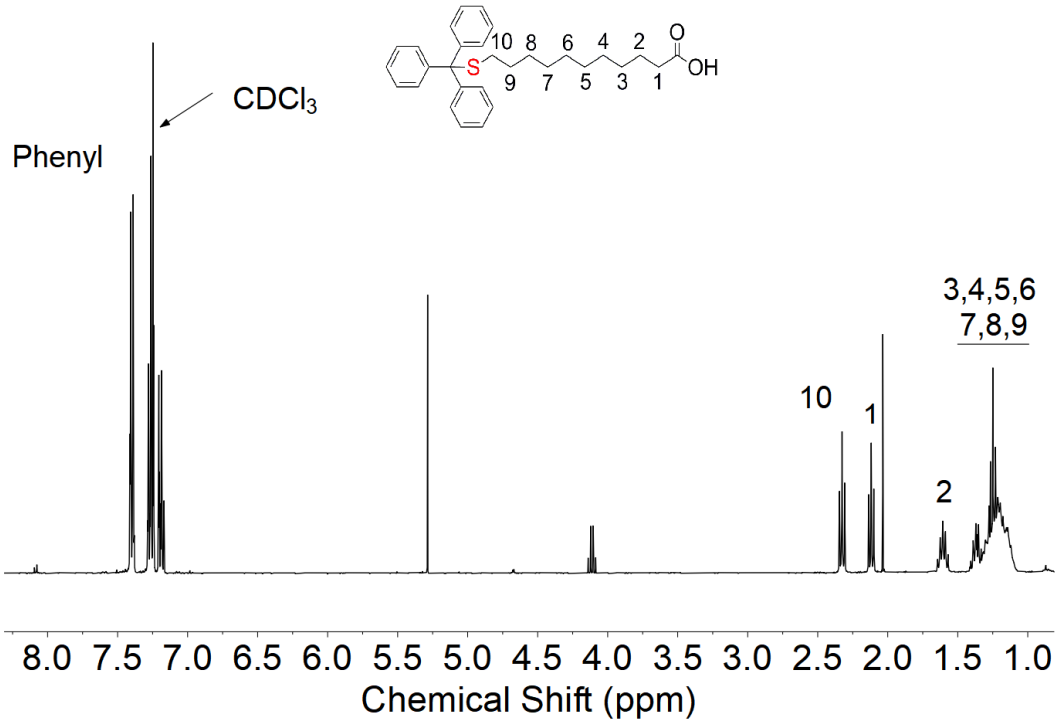


Figure S2. HRMS profile of Tr-S-COOH compound. The *m/z* value of Tr-S-COOH was 459.24 (*m/z*, M-H$^-$), which was consistent with the calculated value.



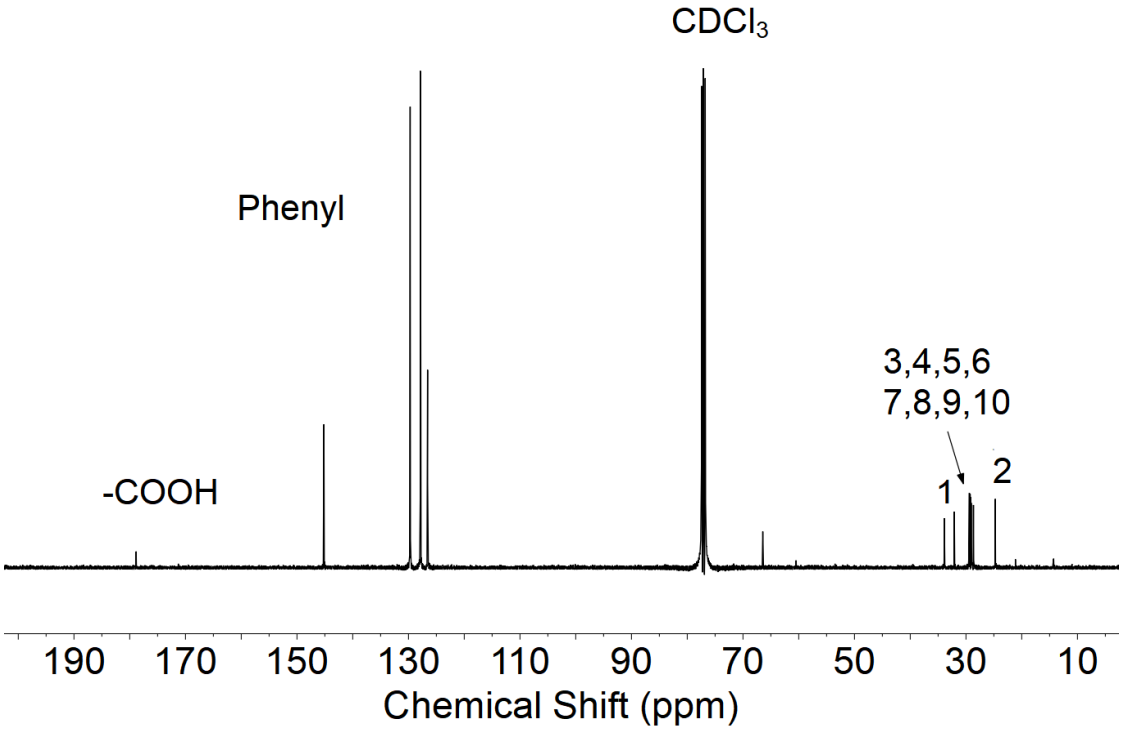


Figure S3. ^{1}H NMR and ^{13}C NMR spectra of intermediate Tr-S-COOH compound. a) ^{1}H NMR spectrum of Tr-S-COOH in $CDCl_3$; b) ^{13}C NMR spectrum of Tr-S-COOH in $CDCl_3$.

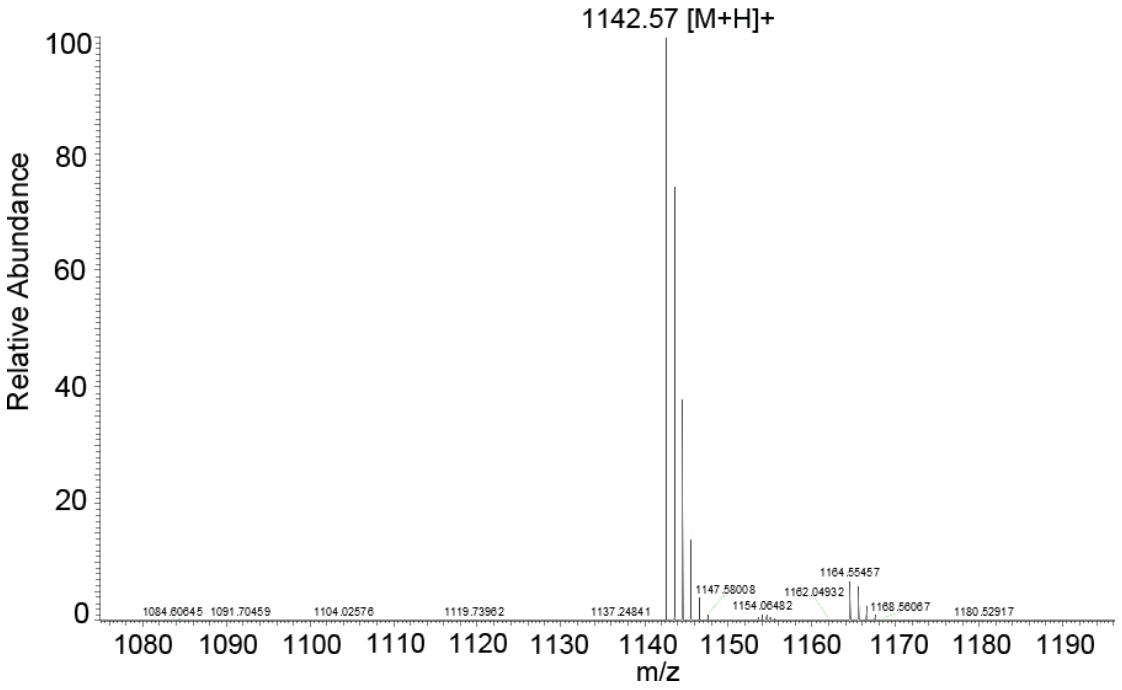


Figure S4. HRMS profile of Tr-S-PC compound. The *m/z* value of Tr-S-PC was 1142.57 (*m/z*, $M+H^{+}$), which was consistent with the calculated value.

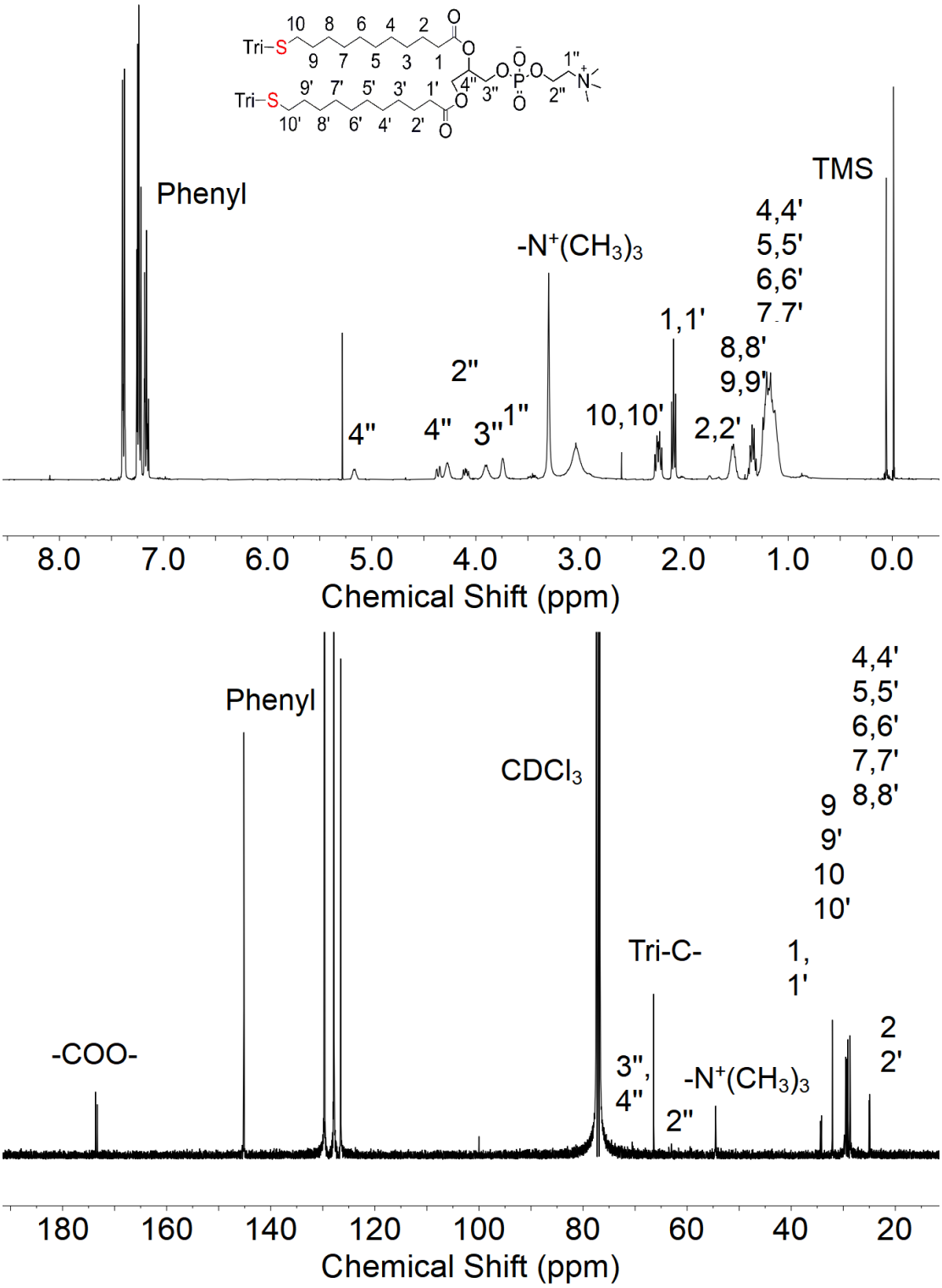


Figure S5. ^{1}H NMR and ^{13}C NMR spectra of Tr-S-PC compound. a) ^{1}H NMR spectrum of Tr-S-PC in $CDCl_3$; b) ^{13}C NMR spectrum of Tr-S-PC in $CDCl_3$.

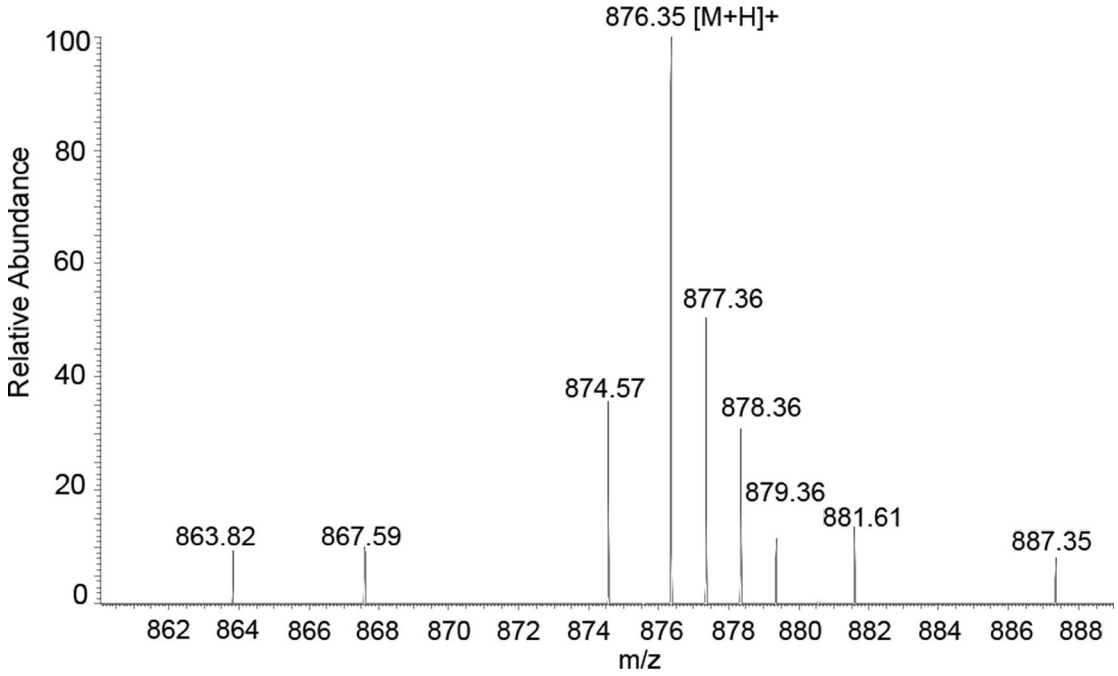
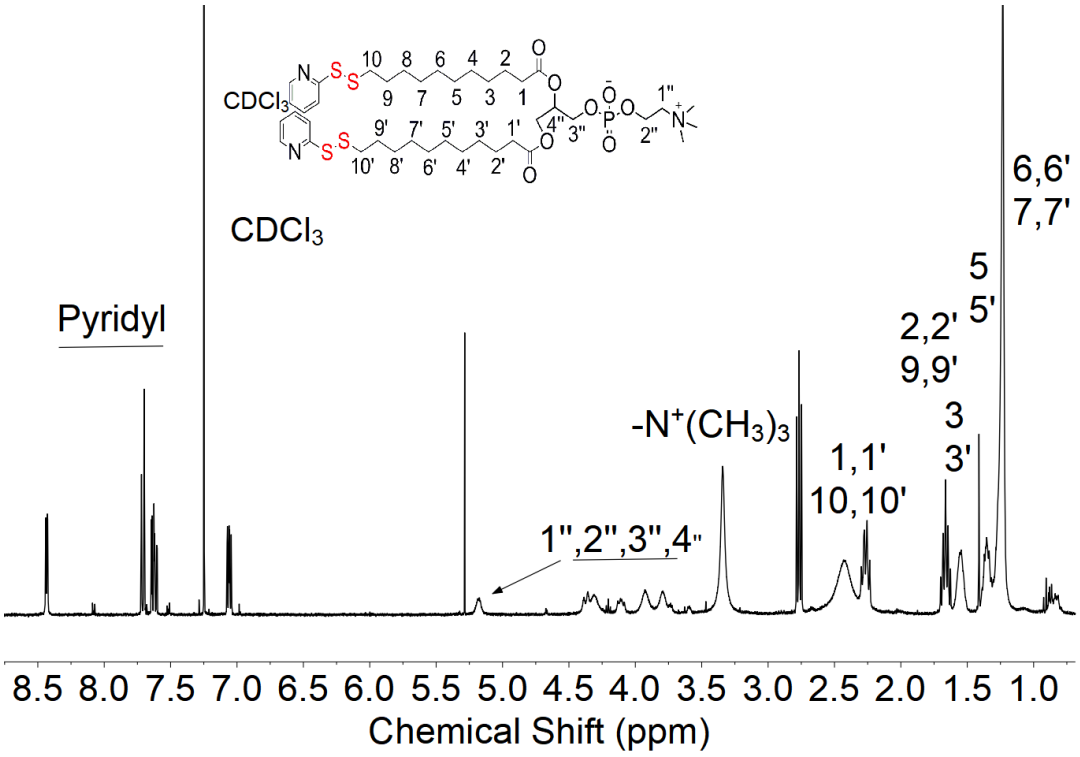


Figure S6. HRMS profile of Pyr-SS-PC compound. The *m/z* value of Pyr-SS-PC was 876.35 (*m/z*, $M+H^+$), which was consistent with the calculated value.



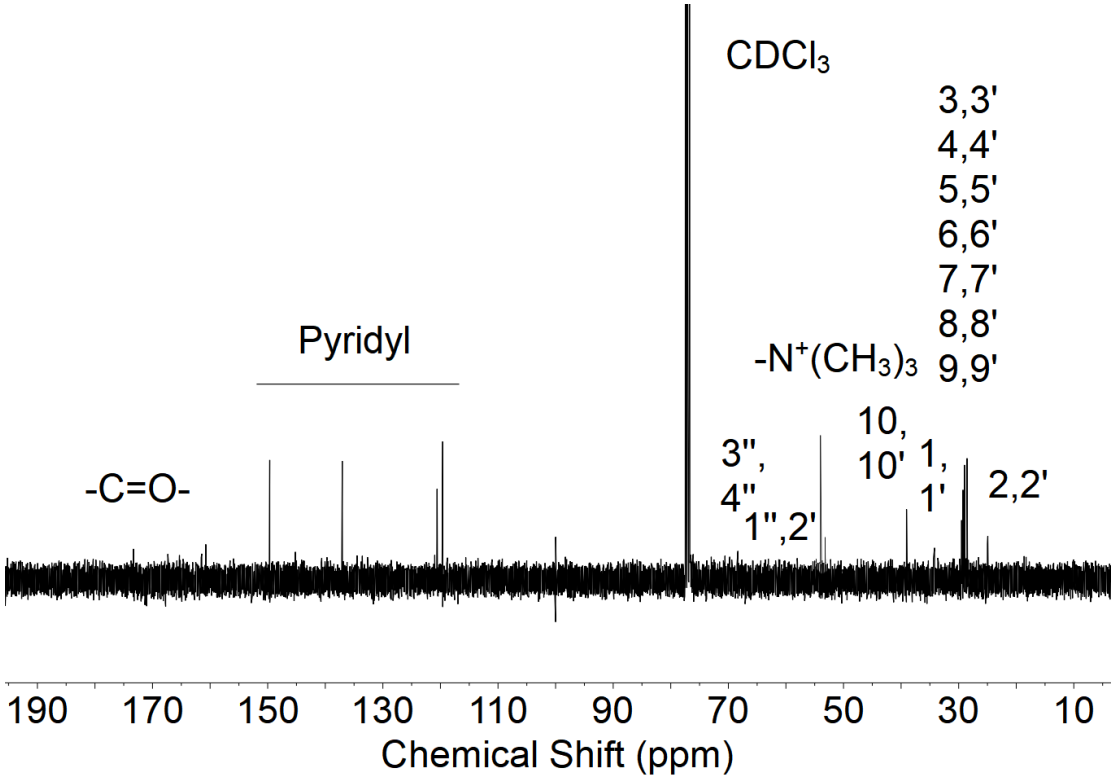


Figure S7. ^{1}H NMR and ^{13}C NMR spectra of Pyr-SS-PC lipids. a) ^{1}H NMR spectrum of Pyr-SS-PC in $CDCl_3$; b) ^{13}C NMR spectrum of Pyr-SS-PC in $CDCl_3$. The separated appearance of pyridyl protons at 7.5-7.02 ppm and tert-butyl protons at 3.34 ppm from PC material demonstrated the successful synthesis of Pyr-SS-PC lipids.

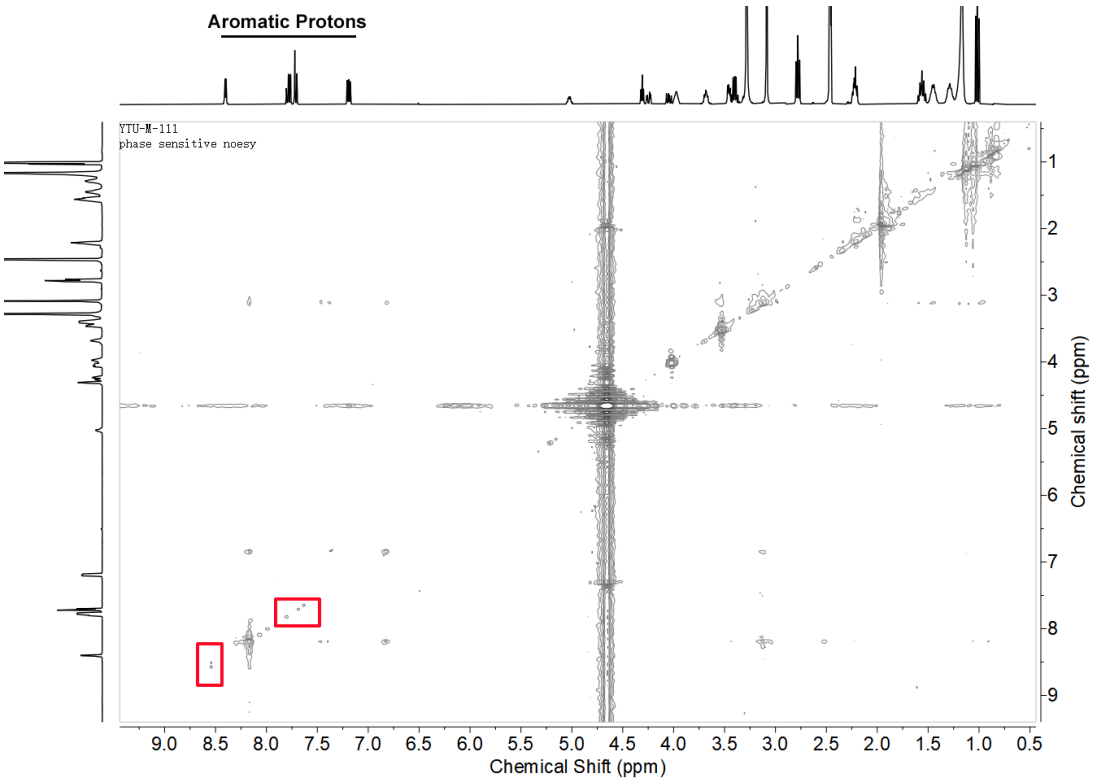


Figure S8. ^{1}H NMR 2D nuclear Overhauser effect spectroscopy (NOESY) spectra of lyophilized powder of CPT@Pyr-SS-PC Lips in DMSO-d_6. The cross peaks between of Pyr-SS-PC lipids and CPT were labeled in the red box.

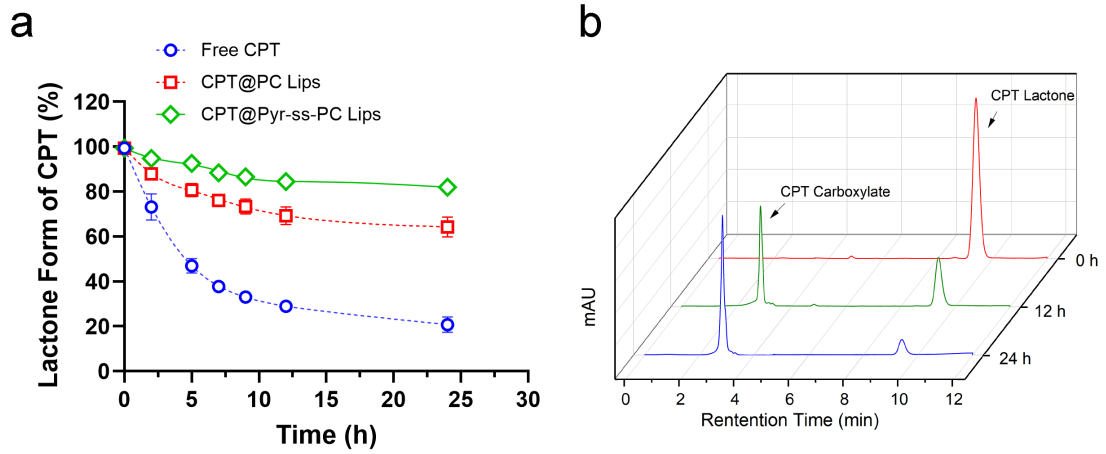


Figure S9. a) Stability of lactone form of CPT incorporated in Pyr-SS-PC Lips in physiological environment (PBS, pH 7.4) containing 10% FBS at 37 °C. The molar ratio of CPT and Pyr-SS-PC lipids at 1:18 was used and each point of the plot is the result of an average of three independent samples (n = 3); b) Ring-opening profile of lactone to carboxylate form of free CPT for 24 h incubation, as demonstrated by HPLC.

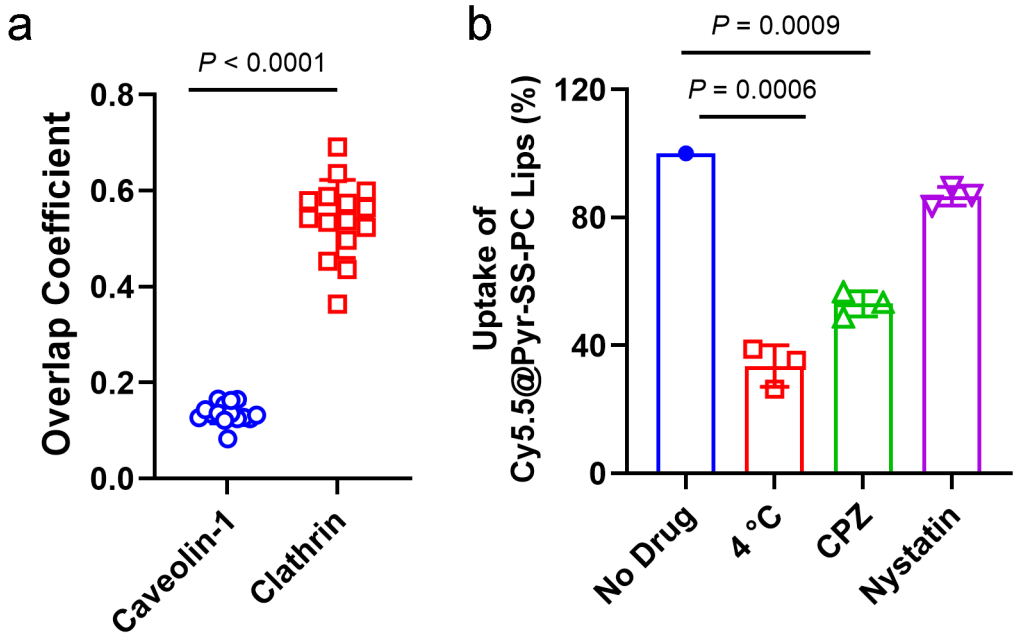


Figure S10. a) Quantification of Cy5.5@Pyr-SS-PC Lips colocalized with clathrin and caveolin-1 signals for 20 min incubation. Manders' overlap coefficients (MOC) was used to evaluate their overlap coefficients by monitoring > 50 cells of each

sample; b) Inhibition of energy-dependent uptake at 4 °C, caveolin-dependent uptake by 50 μg/mL nystatin and clathrin-dependent uptake by 25 μg/mL chlorpromazine (CPZ). The internalization of Cy5.5@Pyr-SS-PC Lips was confirmed by measuring the intensity of Cy5.5 fluorescence. Data were given as mean ± S.D (n = 3). P values represent differences between the 2 illustrated groups, analyzed by Student's t-test.

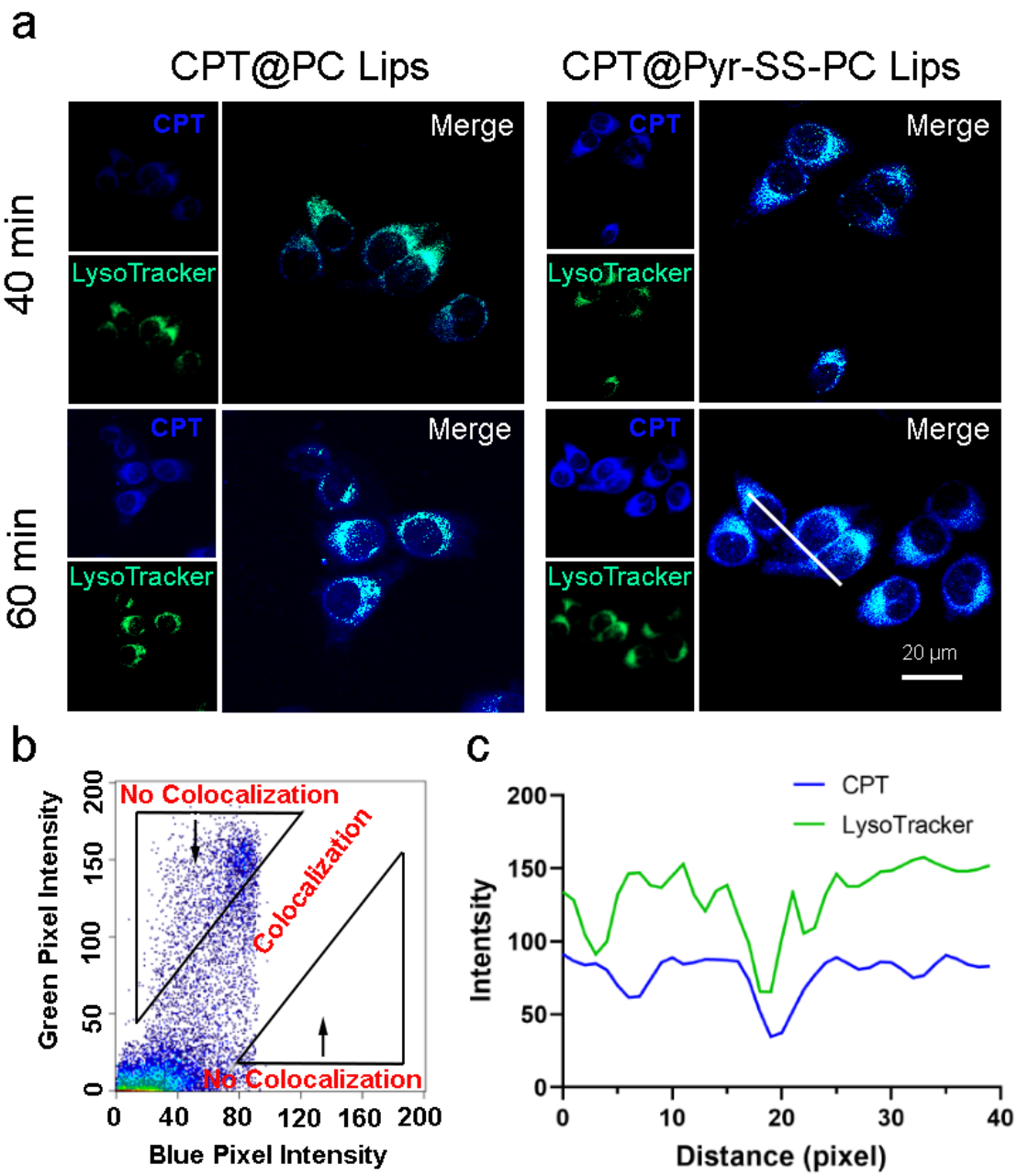


Figure S11. a) Intracellular trafficking of CPT@Pyr-SS-PC Lips through lysosomes. MCF-7 cells were incubated with CPT@Pyr-SS-PC Lips (blue) for 40 min and 60 min at 37 °C. Lysosomes were labelled with LysoTracker Green. Scale bar = 20 μm; b) Images of the colocalization of CPT (blue) and lysosomes (green) and scatter plot.

The non-colocalizing pixels from scatter plot are indicated by black triangles. Pearson's correlation coefficient was quantified by counting 10 cells in three independent experiments; c) Plots of pixel intensity along the white line of merged images.

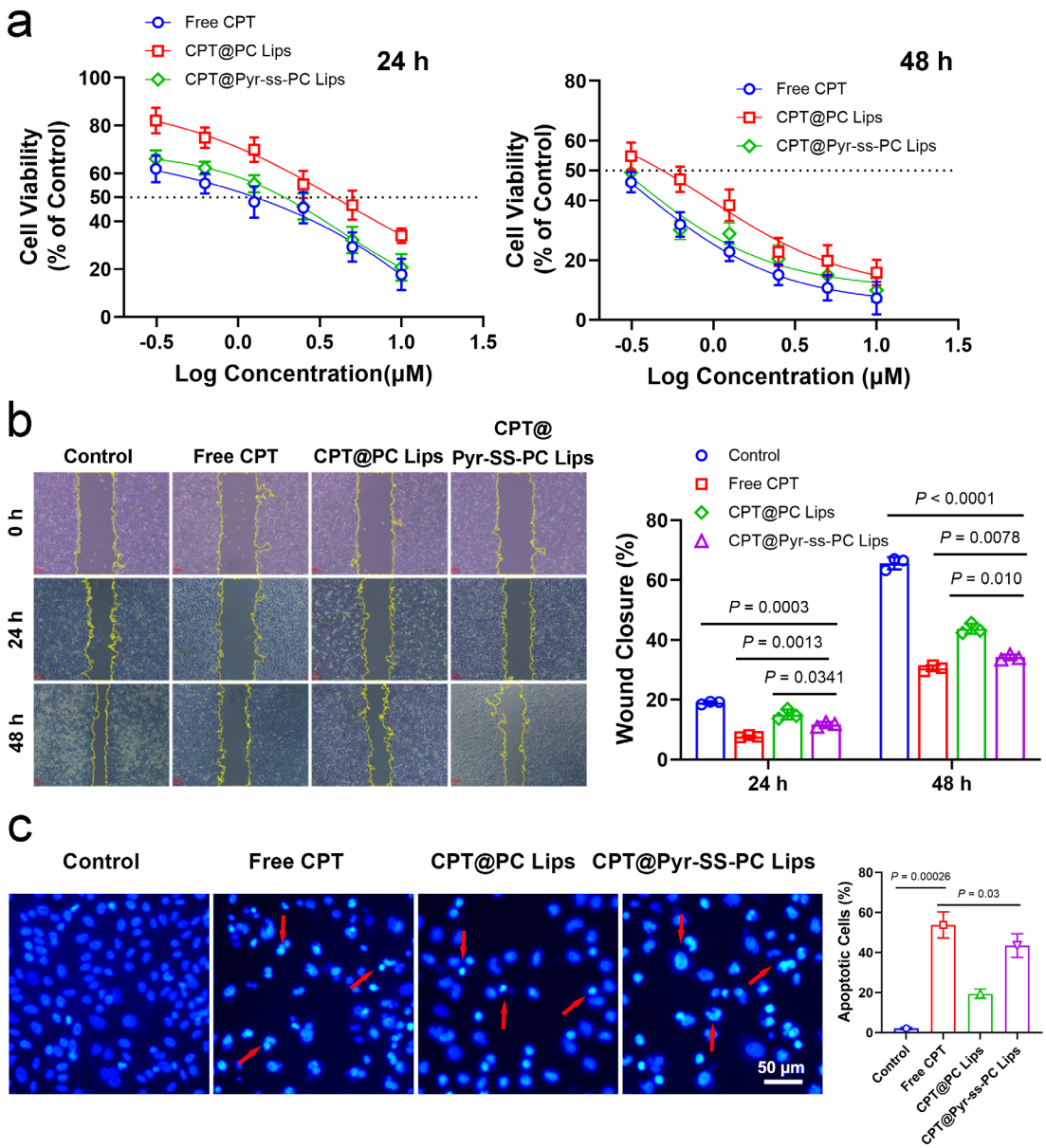


Figure S12. a) *In vitro* cytotoxicity of CPT@Pyr-SS-PC Lips to 4T1 cells for 24 h and 48 h incubation, determined by MTT assay; b) Wound healing assay in MCF-7 cells and quantitative analysis of scratch wound healing after 48 h treatment with CPT@Pyr-SS-PC Lips; c) Apoptosis observed by Hoechst 33258 staining (100 ×). After MCF-7 cells were treated with different CPT formulations (4 μg/mL), the morphological changes of cell apoptosis and the percent apoptotic cells were recorded

by Hoechst staining. Arrows represent apoptotic cells. Data are expressed as mean ± S.D. of three independent experiments. *P* values represent differences between the 2 illustrated groups, analyzed by Student's t-test.

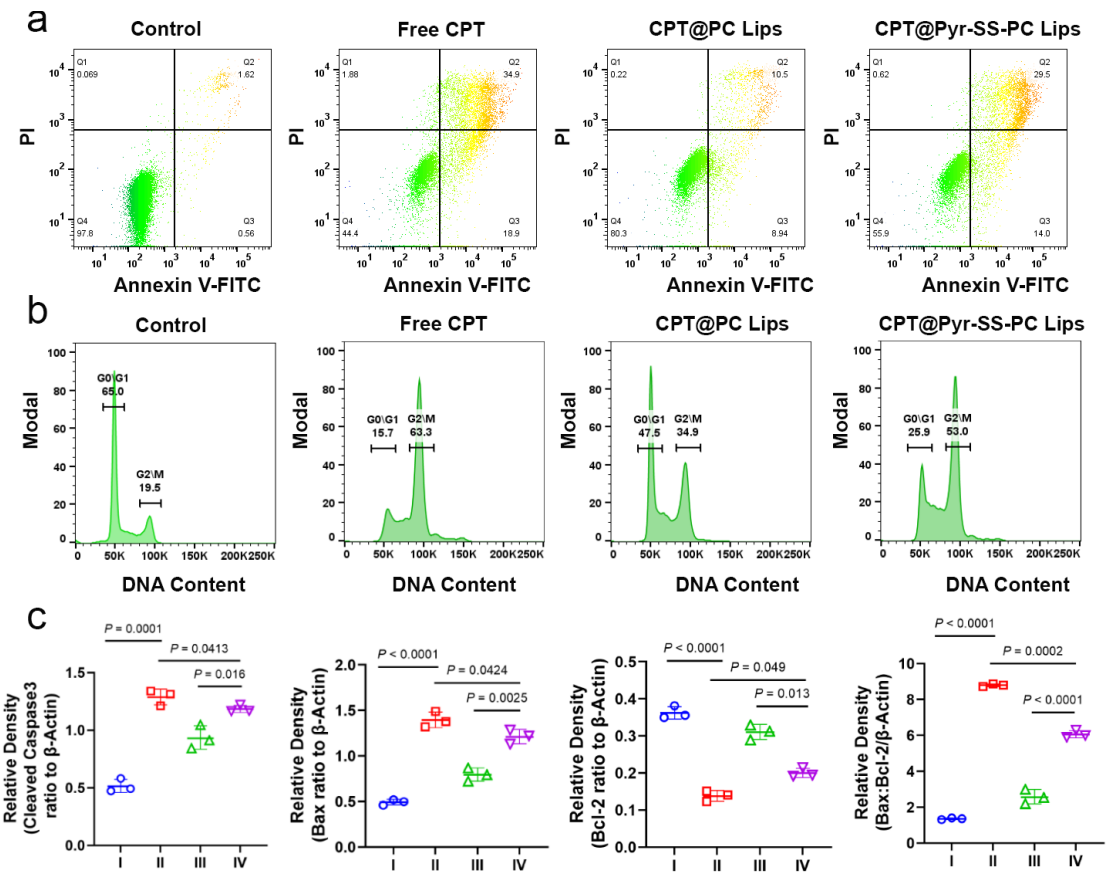


Figure S13. Flow cytometry analysis of a) apoptosis with Annexin V-PI staining and b) cell cycle distribution of MCF-7 cells after 48 h culture on the different CPT substrates; c) The densitometric quantification of cleaved caspase-3, Bax, Bcl-2 and Bax/Bcl-2 ratio in CPT@Pyr-SS-PC Lips-induced apoptosis, 48 h after administration in MCF-7 cells. β-actin was used as an internal control. Data are represented as mean ± S.D. $n = 4$ per group. *P* values represent differences between the 2 illustrated groups, analyzed by Student's t-test.

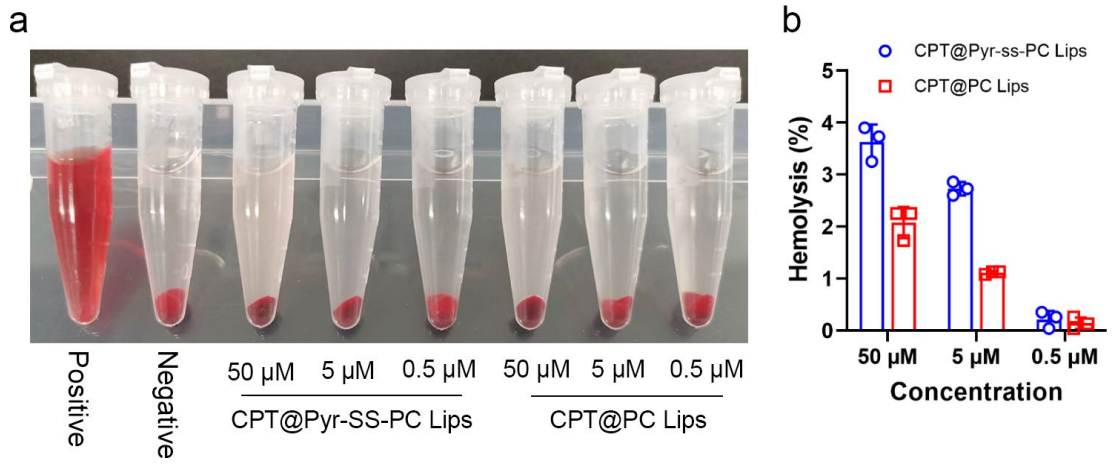


Figure S14. Hemolysis of CPT@Pyr-SS-PC Lips. a) Images of tubes containing red blood cells (RBCs) incubated with different concentrations of liposomes (after centrifugation). 0.9% saline (0% hemolysis) and distilled water (100% hemolysis) was set as negative and positive controls; b) Hemolysis rate (%) of tested liposomes and the value less than 5% was considered safe for *in vivo* administration.

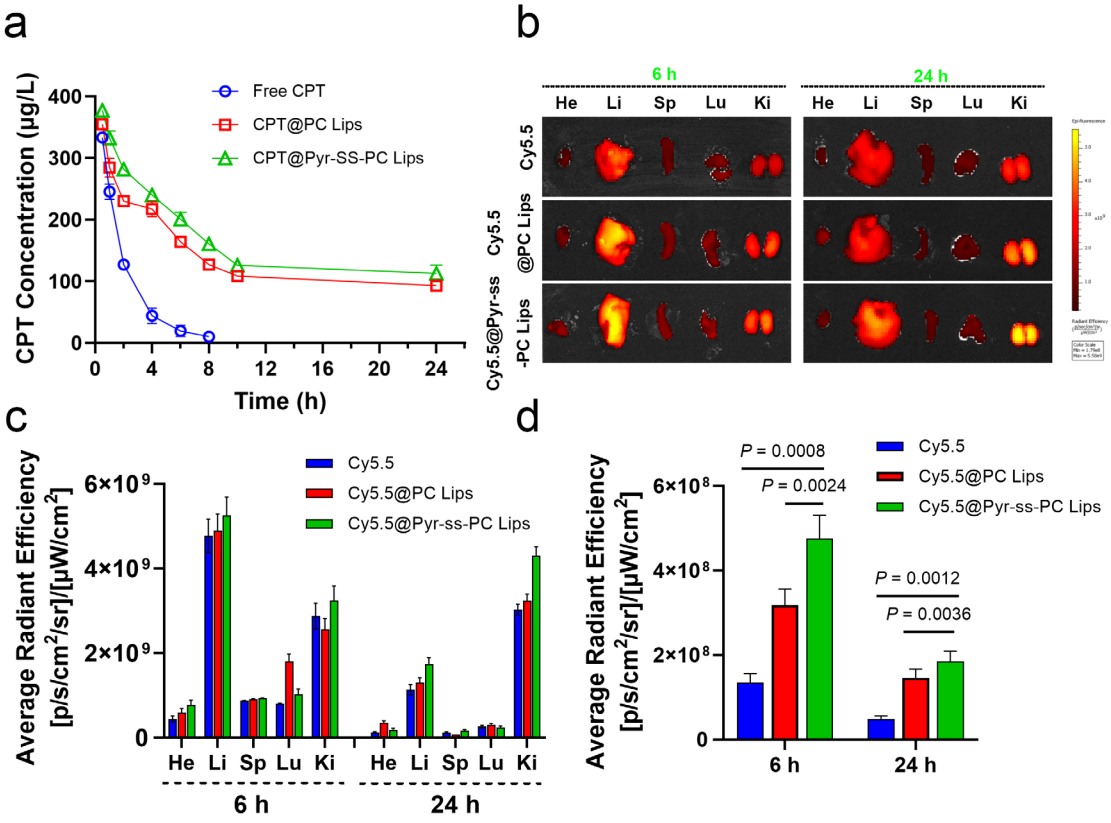


Figure S15. a) *In vivo* pharmacokinetics of free CPT, CPT@Pyr-SS-PC Lips and CPT@PC Lips (5 mg CPT/kg) after *i.v.* administration in MCF-7 tumor-bearing Balb/c nude mice (n = 10 per group) at 0.25 h, 0.5 h, 1 h, 2 h, 4 h, 6 h, 8 h, 10 h and 24 h of post-injection; b) Fluorescence images of mice treated with Cy5.5 labelled

Lips and c) quantitative biodistribution of major organs (He: heart; Li: liver; Sp: spleen; Lu: lung; Ki: Kidney) excised 6 h and 24 h administration; d) Accumulation analysis of fluorescence intensity in tumor sites of each groups. All data are presented as mean ± S.D. $n = 6$ per group. P values represent differences between the 2 illustrated groups, analyzed by Student's t-test.

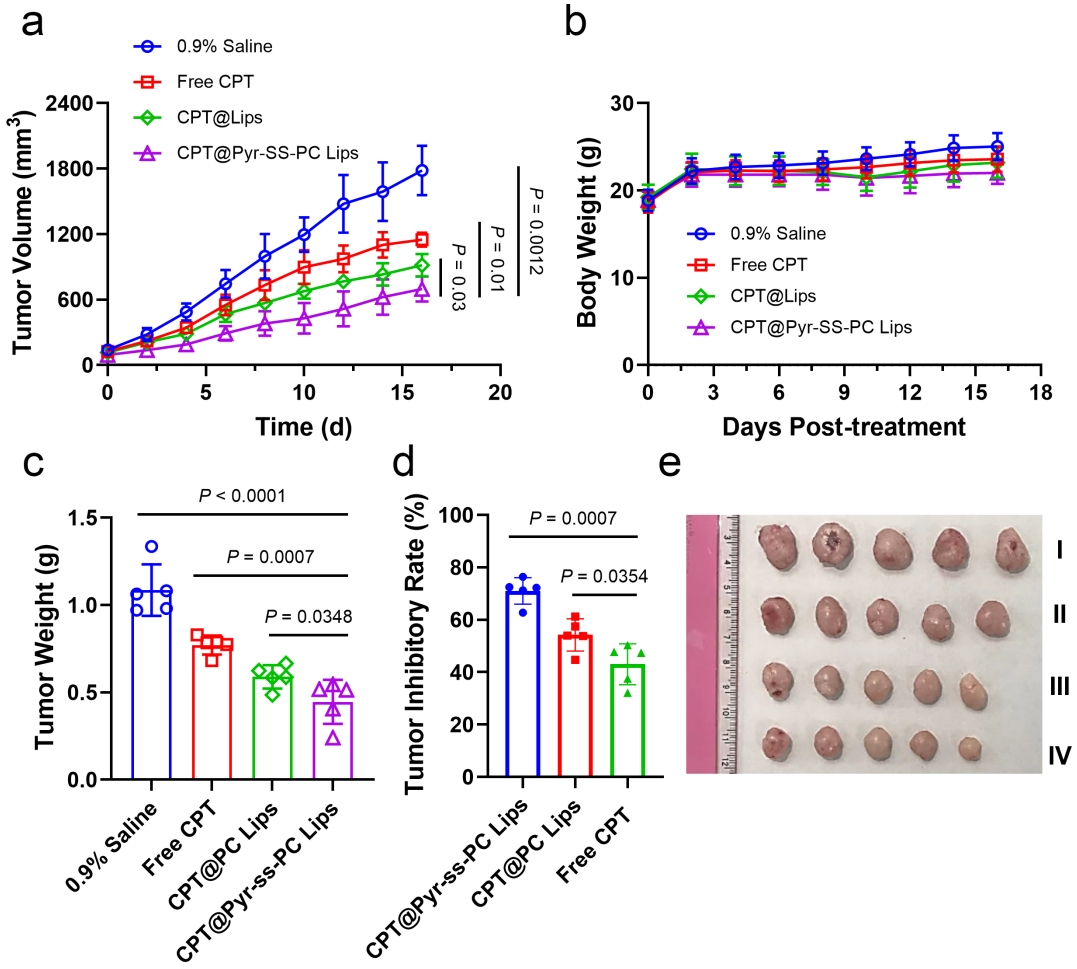


Figure S16. a) The growth curves of tumors and b) body weight changes of mice bearing MCF-7 tumors after *i.v.* injection of 0.9% saline, free CPT, CPT@PC Lips and CPT@Pyr-SS-PC Lips every other day for 16 days (n = 5 for each group); c) Average weight of tumors, d) Tumor inhibition rate (TIR%) and e) images of tumors harvested from mice treated with I) 0.9% saline, II) free CPT, III) CPT@PC Lips and IV) CPT@Pyr-SS-PC Lips. Data are represented as mean ± S.D. P values represent differences between the 2 illustrated groups, analyzed by Student's t-test.

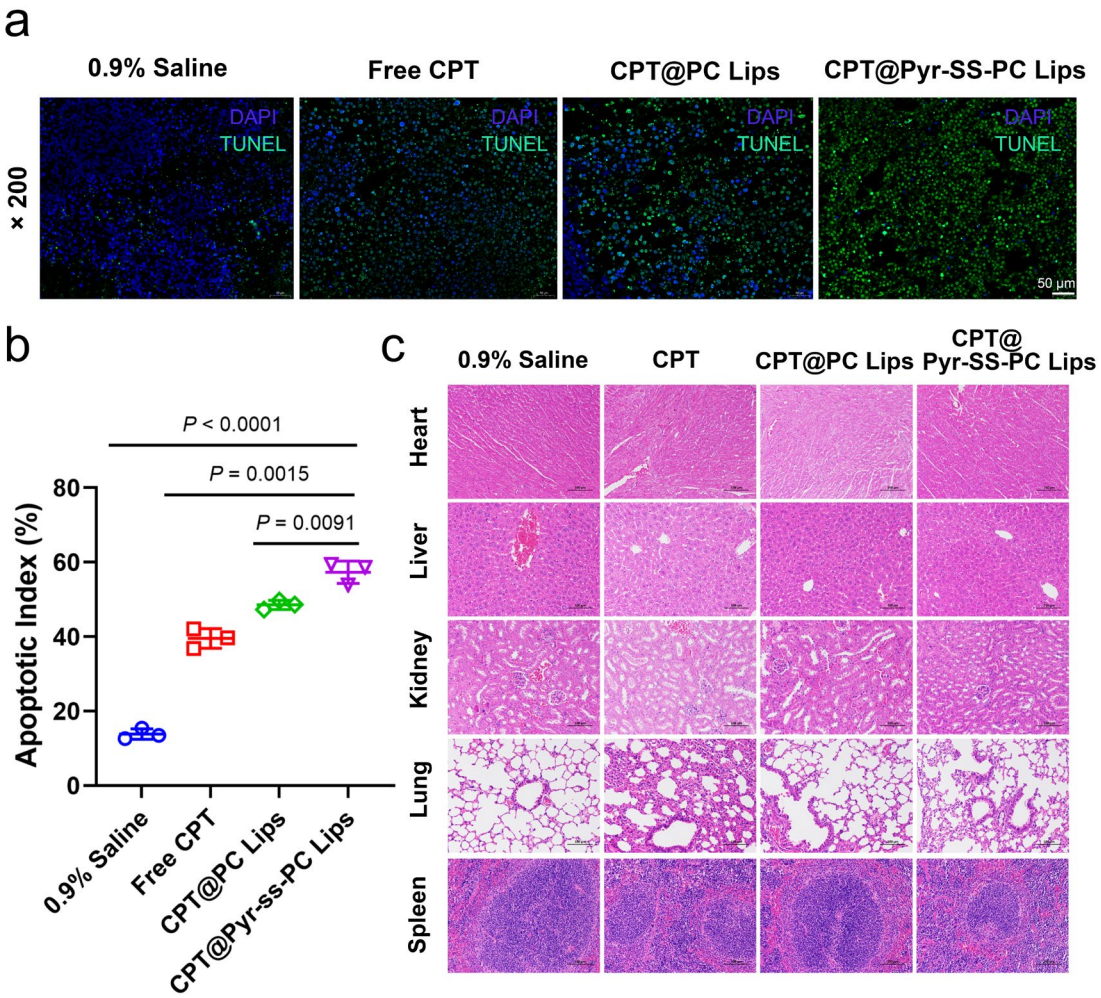


Figure S17. a) Representative apoptosis of MCF-7 cells in tumors analyzed by TUNEL assay (green). Nuclei were stained with DAPI (blue). Original magnification, 200 ×; b) Apoptotic index (%) counting at least 200 cells in three different fields of different treated groups; c) H&E examination of major organs of the MCF-7 tumor-bearing nude mice after anticancer treatment with various drug formulations. Scale bar = 100 μm. Data are represented as mean ± S.D. *P* values represent differences between the 2 illustrated groups, analyzed by Student's t-test.

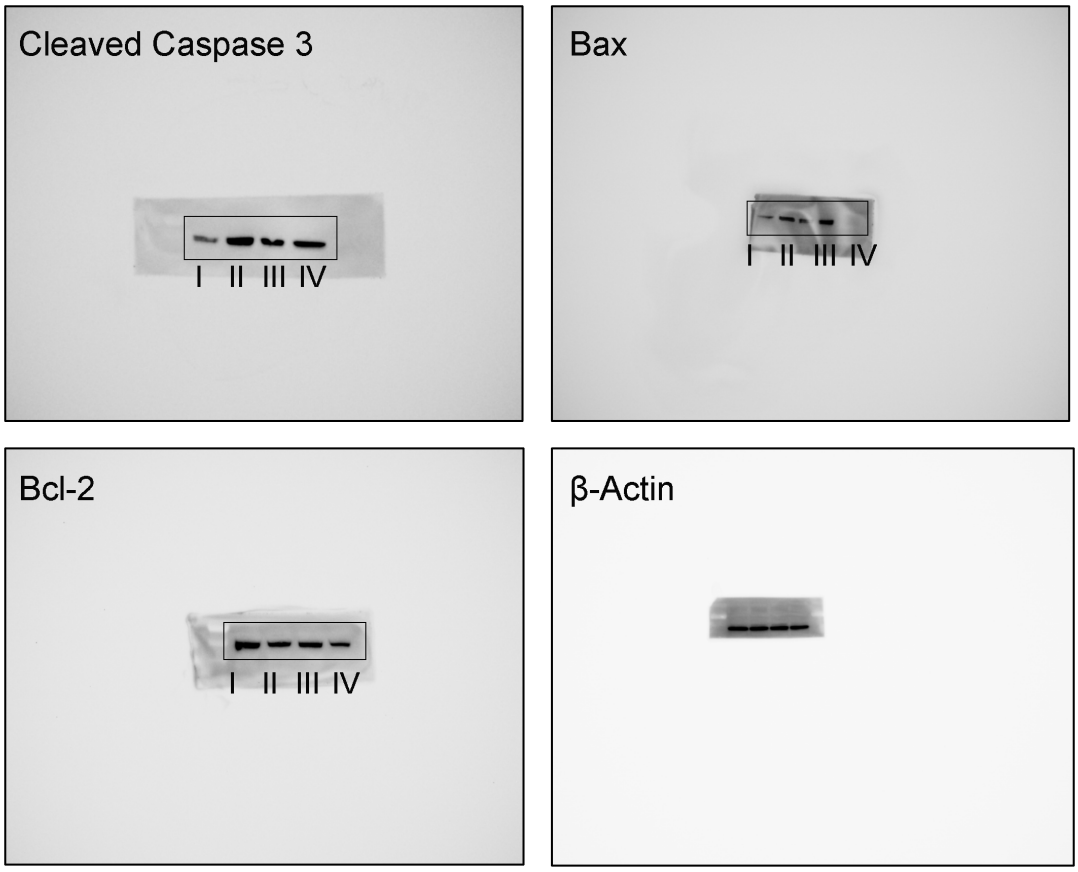


Figure S18. Source data for Figure 3d. I) Blank, II) free CPT, III) CPT@PC Lips and IV) CPT@Pyr-SS-PC Lips.

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