Supporting Information

Photoresponsive prodrug-based liposomes for controllable release of anticancer drug chlorambucil

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Experimental section

1. General materials

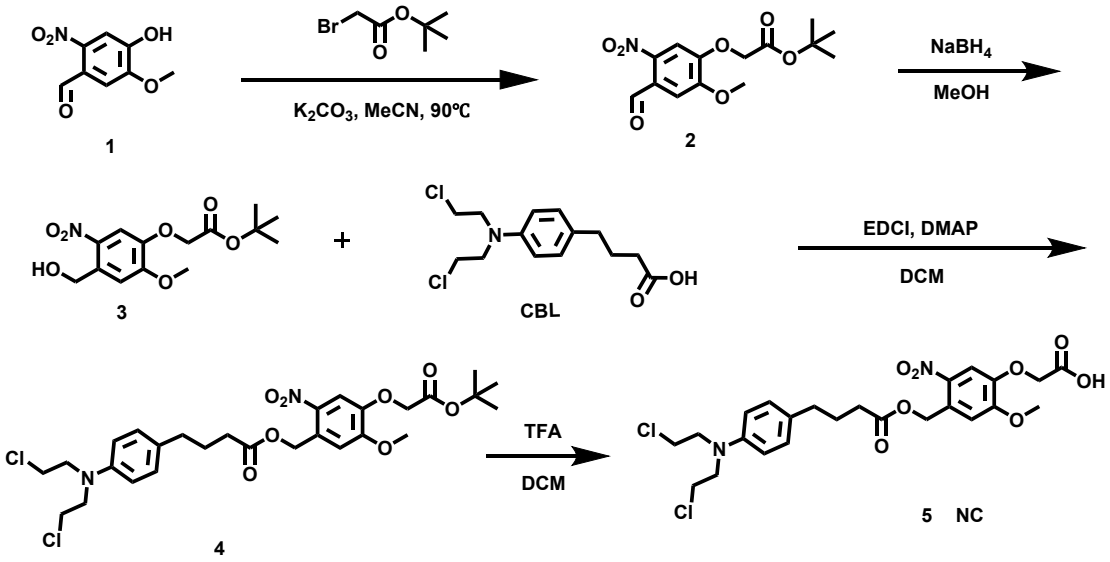
The raw material and reagents used in the experiment were purchased from commercial companies, and were used without further purification unless otherwise noted. Dry dichloromethane (DCM) was distilled from calcium hydride. All synthetic manipulations were performed under an atmosphere of argon gas. The single-arm (phospholipid1-undecanoyl-2-hydroxy-sn-glycero-3-phosphocholine, Lyso PC) was purchased from Avanti Polar Lipids, and the CCK-8 kit (Japan Tongren Institute of Chemistry) was purchased from Shanghai Biyuntian Biotechnology Co. Ltd. The cell culture medium used in this experiment was DMEM high-glucose medium supplemented with 10% FBS (V/V) and 1% Penicillin-Streptomycin (V/V), and the cells used were human cervical cancer cells (HeLa) and mouse fibroblasts (L929), which were both purchased from the cell bank of the Shanghai Academy of Sciences, Chinese Academy of Sciences.

2. Characterizations

Proton and carbon magnetic resonance spectra (^{1}HNMR, ^{13}CNMR) were recorded on a Bruker Avance 400/600MHz spectrometer, in which tetramethylsilane (TMS) was used as internal standard of chemical shift when recording ^{1}H NMR spectra. Mass spectra of the compounds were measured by a Micromass GCTTM and a Micromass LCTTM. UV absorption spectra were obtained by a Shimadzu UV-2550 UV-Vis spectrometer. The reversed-phase HPLC was monitored on an Agilent 1200 Series using BetaBasic-18 column. DLS data were obtained using Malvern Zetasizer Nano ZSP. TEM measurements were conducted on a JEM-1400 Transmission Electron Microscopy. Cell viability was measured by Synergy 2 Mulit-mode Microplate Reader (Biotek) using CCK-8 kit and all cytotoxicity data were averaged from six sets of data. All confocal luminescence images were performed with an A1R Nikon confocal microscope (CLSM) with 10× or 40× objective lens.

3. Synthesis of compounds

3.1 Synthesis of NC



Scheme S1. The synthesis procedure of **NC**.

Compound 1 was prepared as previously described.[1-3] Finally, the yellow solid compound 1 was obtained (3.0 g，87% yield). ^{1}HNMR (400 MHz, DMSO-d_6) δ(ppm): 11.11 (s, 1H), 10.16 (s, 1H), 7.50 (s, 1H), 7.36 (s, 1H), 3.94 (s, 3H).

Compound 2: To a solution of compound **1** (2.0 g, 10.0 mmol) in ACN (150 mL) was added K_2CO_3 (2.24 g, 16.0 mmol) and stirred at room temperature for 15 min. Then

tert-butyl bromoacetate (2.38 g, 12.0 mmol) was added and refluxed for 12 h at 90 ℃. Then the mixture was cooled to room temperature and K_2CO_3 was filtered. The solvent was removed under reduced pressure. The residue was purified by silica gel flash column chromatography (DCM/PE = 3/1) to afford the yellowish solid compound **2** (2.6 g, 83% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 10.46 (s, 1H), 7.51 (s, 1H), 7.44 (s, 1H), 4.73 (s, 2H), 4.04 (s, 3H), 1.5 (s, 9H). ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 187.62, 166.16, 153.49, 150.42, 143.21, 126.37, 110.41, 108.74, 83.52, 66.24, 56.75, 27.95. 56.75, 27.95. MS (ESI): m/z: Calcd. for $C_{14}H_{17}NO_7$ $[M+Na]^+$: 334.1. Found: 334.1.

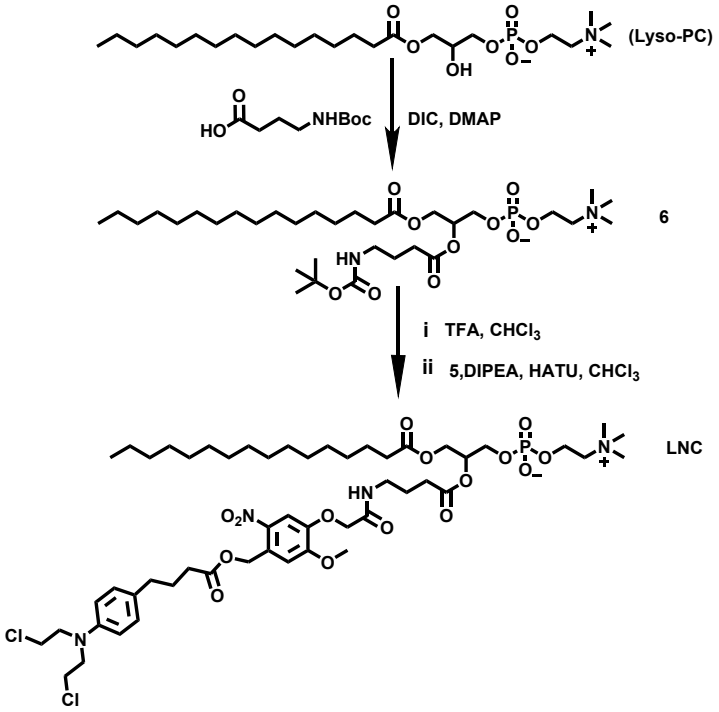
Compound 3: To a solution of compound **2** (2.6 g, 8.3 mmol) in methanol (100 mL) was slowly added $NaBH_4$ (0.63 g, 16.6 mmol) and stirred for 1 h at room temperature. The solvent methanol was removed under reduced pressure. then the mixture was redissolved by DCM (100 mL) followed by washing with water (100 mL × 2) and brine (100 mL). The combined organic layers were dried over anhydrous $NaSO_4$., filtered and concentrated. The residue was purified by silica gel flash column chromatography (DCM/MeOH = 100/1) to afford the milky yellow solid compound **3** (2.5 g, 95% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 7.59 (s, 1H), 7.41 (s, 1H), 5.59 (t, J =5.4 Hz, 1H), 4.83 (d，J =4.4 Hz, 2H), 4.79 (s, 2H), 3.92 (s, 3H), 1.43 (s, 9H). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm): 167.60, 153.83, 145.25, 138.22, 135.16, 110.20, 109.79, 81.90, 65.96, 60.24, 56.30, 27.80. MS (ESI): m/z: Calcd. for $C_{14}H_{19}NO_7$ $[M+Na]^+$: 336.1. Found: 336.1.

Compound 4: To a solution of Chlorambucil (CBL, 1 g, 3.2 mmol) in anhydrous DCM (20 mL) was added EDCI (0.95 g, 4.8 mmol) and stirred for 30 min at 0 ℃ in the dark. Then Compound **3** (1.5 g, 4.8 mmol) and DMAP (0.08 g, 0.7 mmol) were dissolved in anhydrous DCM (40 mL) and added to the above solution. After 24 h, the reaction mixture was washed with brine (20 mL × 2) and the combined organic layers were dried over anhydrous $NaSO_4$., filtered and concentrated. The residue was purified by silica gel flash column chromatography (DCM/PE = 3/1) to afford the yellowish solid compound **4** (1.2 g, 62% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.88 (s, 1H), 7.51 (s, 1H), 7.32 (d, J = 8.4 Hz, 2H), 6.9 (d, J = 8.4 Hz, 2H), 5.74 (s, 2H), 4.89 (s, 2H), 4.20 (s, 3H), 3.96 - 3.92 (m, 4H), 3.89

- 3.92 (m, 4H). 4H), 3.89 - 3.84 (m, 4H), 2.83 (t, J = 7.5 Hz, 2H), 2.68 (t, J = 7.4 Hz, 2H), 2.21 (p, J = 7.6 Hz, 2H), 1.74 (s, 9H). ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm)： 172.68, 166.71, 153.67, 146.36, 143.17, 139.63, 132.02, 129.75, 127.89, 113.33, 111.16, 110.03, 83.09, 66.16, 63.05, 56.36, 54.12, 39.85, 33.90, 33.40, 27.94, 26.56. MS (ESI): m/z: Calcd. for $C_{28}H_{36}Cl_2N_2O_8$ $[M+H]^+$: 599.2. Found: 599.2.

Compound 5 (NC): To a solution of Compound **4** (1g, 1.6 mmol) in DCM/TFA (20 mL, 4:1) was stirred for 1 h at room temperature in the dark. After the reaction was completed, TFA was removed as much as possible under reduced pressure by adding DCM several times and the residue was purified by silica gel flash column chromatography (DCM/MeOH = 25/1) to afford the yellow solid compound **5**. (0.8 g, 88% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ (ppm): 7.56 (s, 1H), 7.18 (s, 1H), 7.01 (d, J = 8.5 Hz, 2H), 6.65 (d, J = 8.5 Hz, 2H), 5.35 (s, 2H), 4.62 (s, 2H), 3.89 (s, 3H), 3.68 (t, J = 6.7 Hz, 8H), 2.47 (t, J = 7.5 Hz, 2H), 2.38 (t, J = 7.3 Hz, 2H), 1.79 (p, J = 7.5 Hz, 2H). ^{13}C NMR (151 MHz, DMSO-d_6) δ (ppm): 172.42, 169.80, 153.16, 146.96, 144.51, 139.62, 129.45, 129.32, 126.03, 112.01, 111.91, 109.49, 66.35, 62.67, 56.24, 52.22, 41.16, 33.23, 32.86, 26.62. HRMS (ESI): m/z: Calcd. for $C_{24}H_{28}Cl_2N_2O_8$ $[M+H]^+$: 543.1296. Found: 543.1300.

3.2 Synthesis of LNC



Scheme S2. The synthesis procedure of **LNC**.

Compound 6: To a solution of Boc-GABA-OH (205 mg, 1 mmol) in anhydrous DCM (5 mL) was added DIC (204 mg, 1.6 mmol) and DMAP (37 mg, 0.3 mmol), and stirred for 30 min at 0 °C. The above mixture was then added to a solution of Lyso PC (100 mg, 0.2 mmol) in anhydrous DCM (10 mL) and the reaction was carried out for 24 h at room temperature in the dark. Then the reaction mixture was filtered and washed with $CHCl_3$: MeOH (1:1, 50 mL). The solvent was removed under reduced pressure and the residue was purified by silica gel flash column chromatography (0 - 4% H_2O/25% MeOH/ $CHCl_3$) to afford the white solid compound **6** (100 mg, 73% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 5.20 (m, 1H), 4.32 (t, *J* = 16.8 Hz, 3H), 4.15 (t, *J* = 8.6 Hz, 1H), 3.96 (s, 2H), 3.80 (s, 2H), 3.35 (s, 9H), 3.12 (m, 2H), 2.35 (t, *J* = 7.4 Hz, 2H), 2.28 (t, *J* = 7.6 Hz, 2H), 1.78 (p, *J* = 7.2 Hz, 2H), 1.56 (m, 2H), 1.42 (s, 9H), 1.25 (s, 24H), 0.87 (t, *J* = 6.7 Hz, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 173.73, 173.12, 156.23, 78.84, 70.64, 66.07, 63.85, 62.64, 59.57, 54.24, 39.63, 33.98, 31.86, 31.35, 29.70, 29.67, 29.62, 29.58, 29.35, 29.30, 29.19, 28.38, 25.12, 24.79, 22.61, 14.04. MS (ESI): m/z: Calcd. for $C_{33}H_{65}N_2O_{10}P$ $[M+Na]^+$: 703.4. Found: 703.4.

Compound LNC: To a solution of compound **6** (100 mg, 0.15 mmol) in anhydrous $CHCl_3$ solution (3 mL) was added 450 μL TFA and stirred at room temperature overnight. After complete reaction, the reaction mixture was concentrated and redissolved in anhydrous $CHCl_3$ (1 mL) and DIPEA (33 μL, 0.2 mmol). Then compound **5** (120 mg, 0.225 mmol), HATU (112 mg, 0.3 mmol) and DIPEA (66 μL, 0.4 mmol) were dissolved in anhydrous $CHCl_3$ (2 mL) and stirred for 30 min at 0 °C, and then added to the above lipid solution. The reaction was carried out for 48 h at room temperature in the dark. After the reaction was completed, the solvent was removed under reduced pressure, and the mixture was diluted with MeOH/H_2O (V/V = 8/2, 45 mL) and further purified by reverse phase preparative HPLC (MeOH:H2O = from 70:30 to 100:0 in 15 min) to afford the yellow oily compound **LNC** (60 mg, 37% yield). ^{1}H NMR (400 MHz, $CDCl_3$) δ (ppm): 7.75 (s, 1H), 7.70 (t, J = 6.0 Hz, 1H), 7.06 (d, J = 8.6 Hz, 2H), 7.01 (s, 1H), 6.62 (d, J = 8.6 Hz, 2H), 5.46 (s, 2H), 5.21 (m, 1H), 4.63 (s, 2H), 4.37 – 4.28 (m, 3H), 4.14 (dd, J = 12.2, 6.8 Hz, 1H), 4.00 (dd, J = 12.4, 6.5 Hz, 2H), 3.95 (s, 3H), 3.78

(s, 2H), 3.75 –3.66 (m,4H), 3.65 – 3.59 (m, 4H), 3.34 (m, 11H), 2.58 (t, J = 7.5 Hz, 2H), 2.41 (m ,4H), 2.27 (t, J = 7.7 Hz, 2H), 1.94 (p, J = 7.5 Hz, 2H), 1.87 (p, J = 7.7 Hz, 2H), 1.56 (m, 2H), 1.25 (s, 24H), 0.87 (t, J = 6.8 Hz, 3H). ^{13}C NMR (151 MHz, $CDCl_3$) δ (ppm): 173.96, 173.17, 173.02, 168.01, 154.30, 146.52, 144.78, 140.08, 130.57, 130.01, 128.95, 112.52, 111.83, 111.28, 71.02 (d, J = 6.9 Hz), 68.91, 66.49 (d, J = 6.9 Hz), 64.53 (d, J = 5.2 Hz), 63.30, 62.76, 60.13 (d, J = 4.9 Hz), 56.73, 54.72, 53.89, 40.88, 38.69, 34.35, 34.26, 33.83, 32.25, 31.77, 30.05, 30.00, 29.91, 29.69, 29.52, 27.06, 25.17, 25.01, 23.02, 14.46. HRMS (ESI): m/z: Calcd. for $C_{52}H_{83}Cl_2N_4O_{15}P$ $[M+H]^+$: 1105.5043. Found: 1105.5049; $[M+Na]^+$: 1127.4862. Found: 1127.4873.

4. Supplemental figures

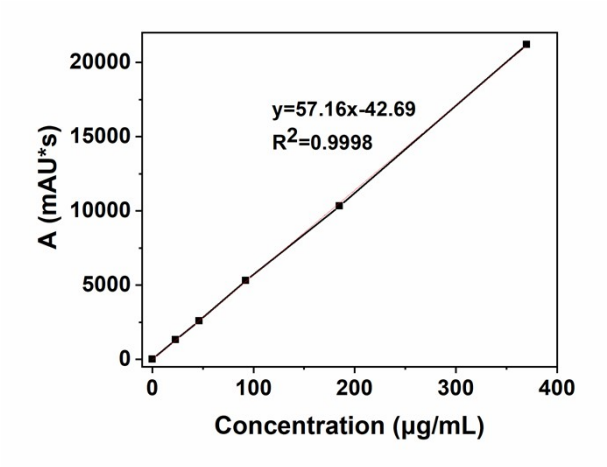


Fig. S1 The standard curve of drug CBL by HPLC analysis.

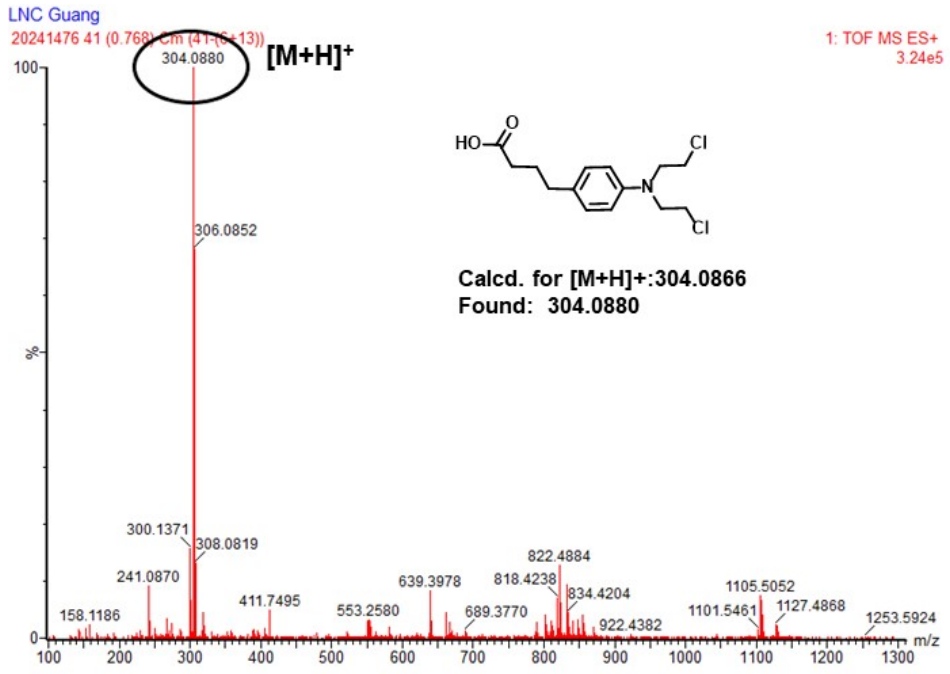


Fig. S2 HRMS (ESI) spectrum for photolytic products **(CBL)**.

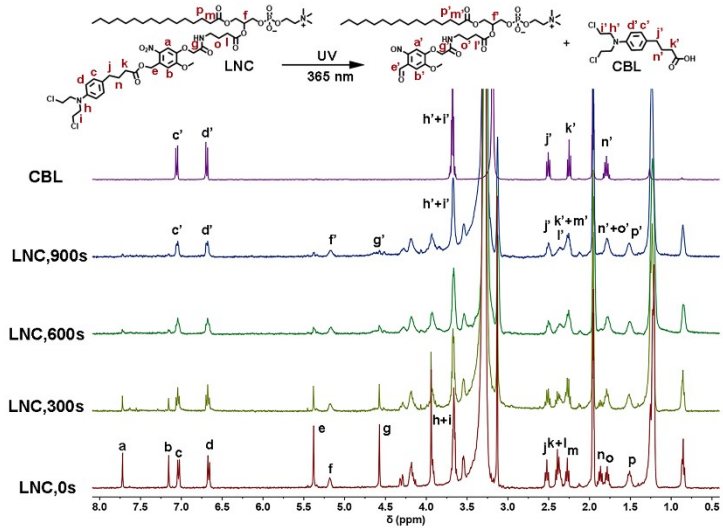


Fig. S3 ^{1}H NMR spectra of **LNC** in mixed solution of CD_3CN/D_2O (V/V= 9/1, 3 mM) upon irradiation of a 365 nm LED light (10 mW cm^{-2}) for varying durations. The NMR spectra of CBL in CD_3CN/D_2O (V/V= 9/1, 3 mM) was also provided for comparison.

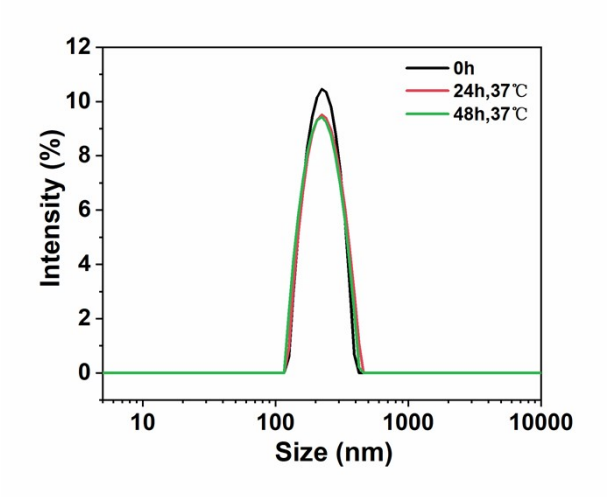


Fig. S4 The stability of **LNC** liposomes at 37°C in the dark determined by DLS analysis for varying durations (0 h, 24 h, 48 h).

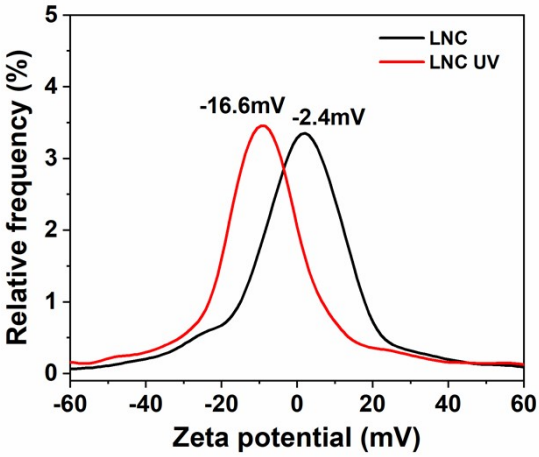


Fig. S5 Changes in zeta potential of **LNC** liposomes before (**LNC**) and after irradiation (**LNC UV**).

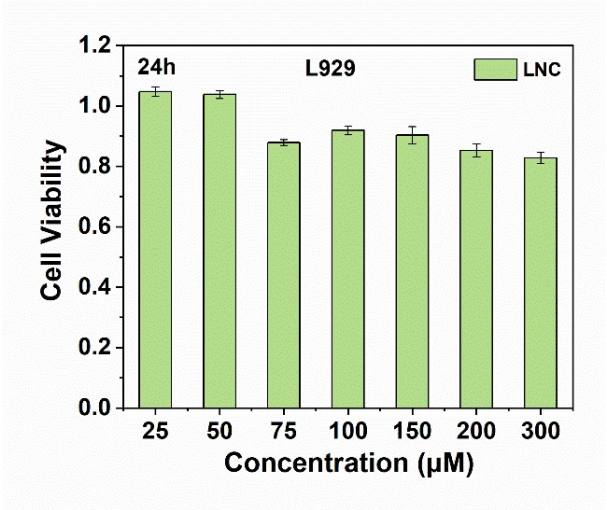


Fig. S6 Cell viability of L929 at different concentrations of **LNC** liposomes determined by CCK-8 assay at 24 h.

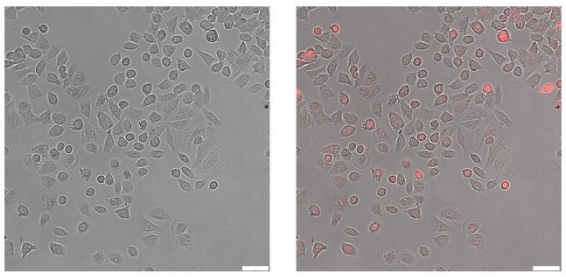


Fig. S7 The confocal images of HeLa cells after incubation with NR-loaded **LNC** liposomes (100 μM) for 4 h. The scale bars are 50 μm (dark field on the left and bright field on the right).

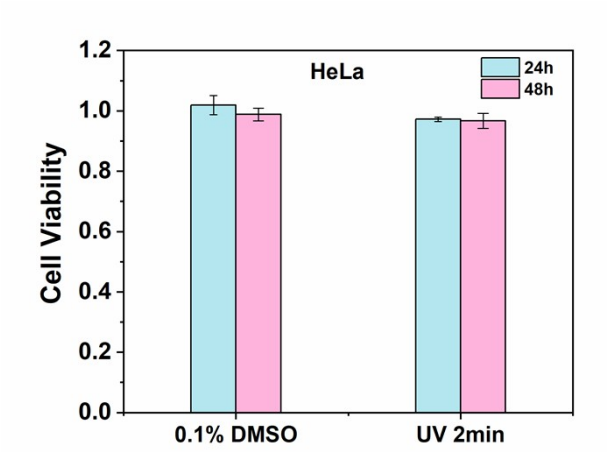


Fig. S8 Cell viability of 0.1% DMSO and UV irradiation (365 nm, 10 mW cm^{-2}, 2 min) determined by CCK-8 assay at 24 h and 48 h.

Table S1. IC_{50} (μM) values of liposomes towards HeLa Cells after 24 h and 48 h incubation.

		IC_{50}	
Cells	Compound	24 h	48 h
HeLa	CBL	＞300.00	281.43 ± 1.36
	LNC	Not determined	Not determined
	LNC UV	72.25 ± 4.96	42.90 ± 0.14

5. NMR and MS characterizations for compounds

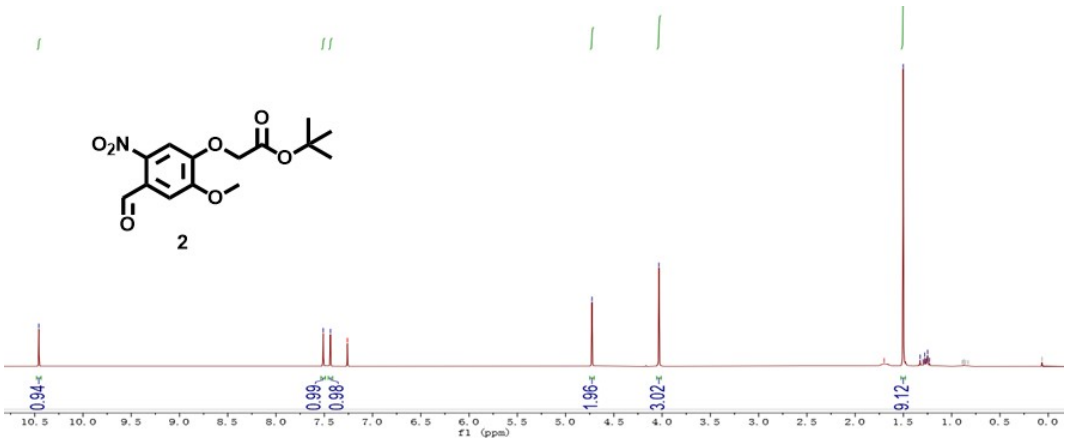


Fig. S9 ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound **2**.

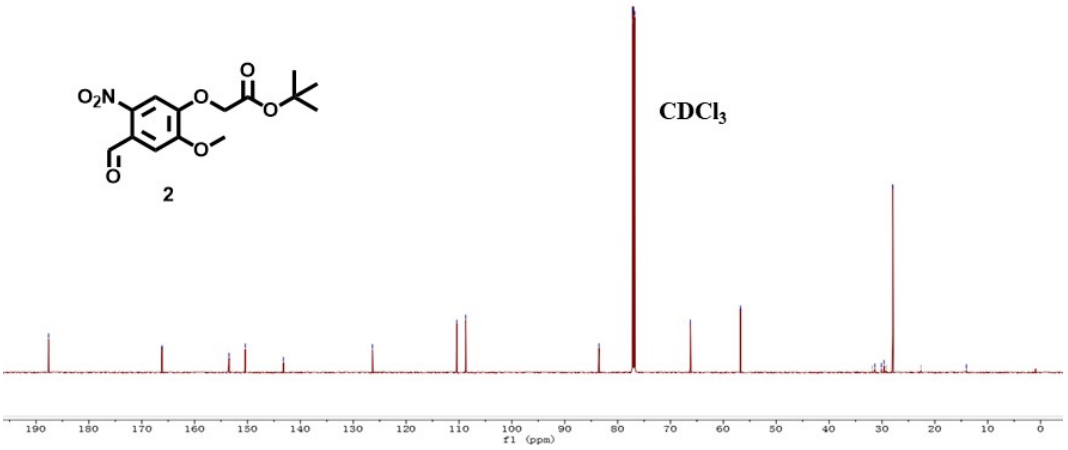


Fig. S10 ^{13}C NMR spectrum (151 MHz, $CDCl_3$) of compound **2**.

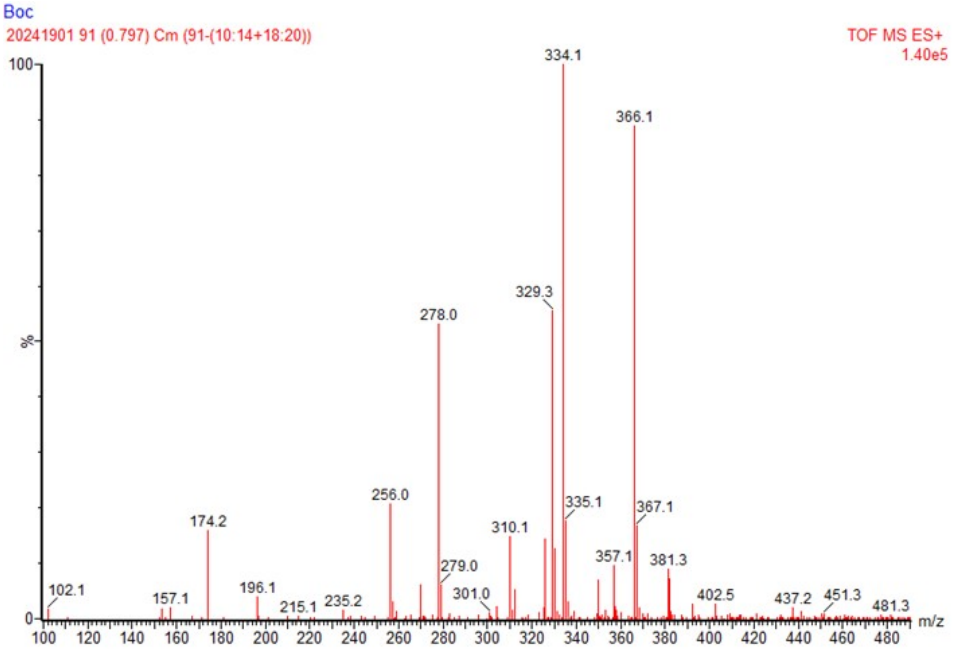


Fig. S11 MS (ESI) spectrum recorded for compound **2**.

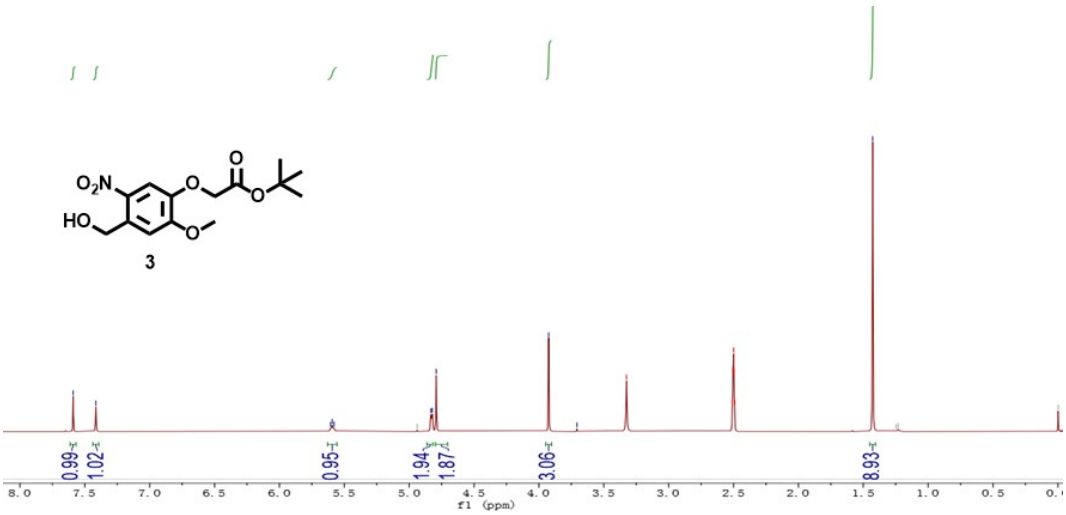


Fig. S12 ^{1}H NMR spectrum (400 MHz, DMSO-d_6) of compound **3**.

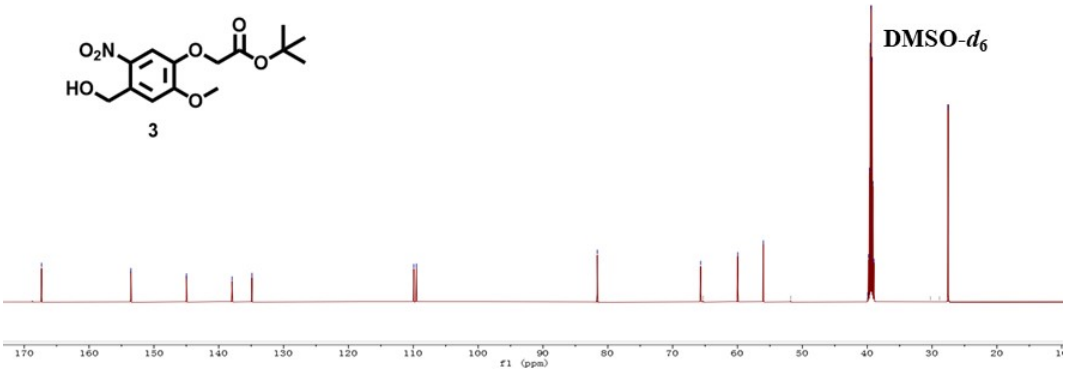


Fig. S13 ^{13}C NMR spectrum (151 MHz, DMSO-d_6) of compound **3**.

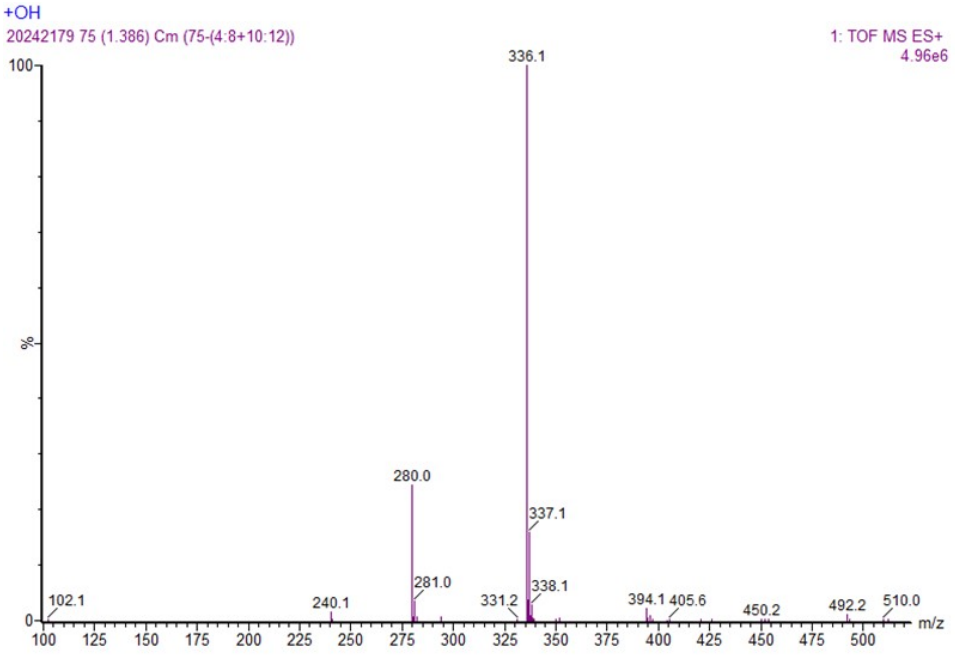


Fig. S14 MS (ESI) spectrum recorded for compound **3**.

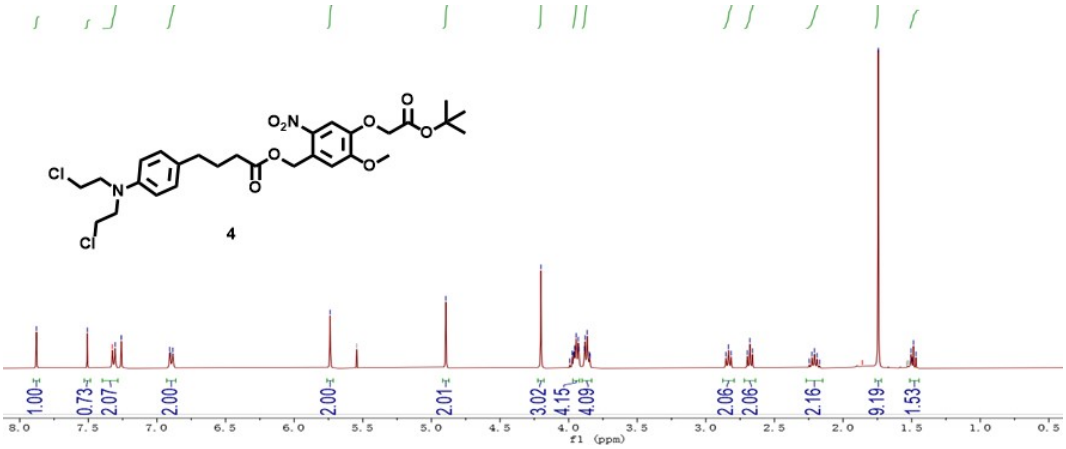


Fig. S15 ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound **4**.

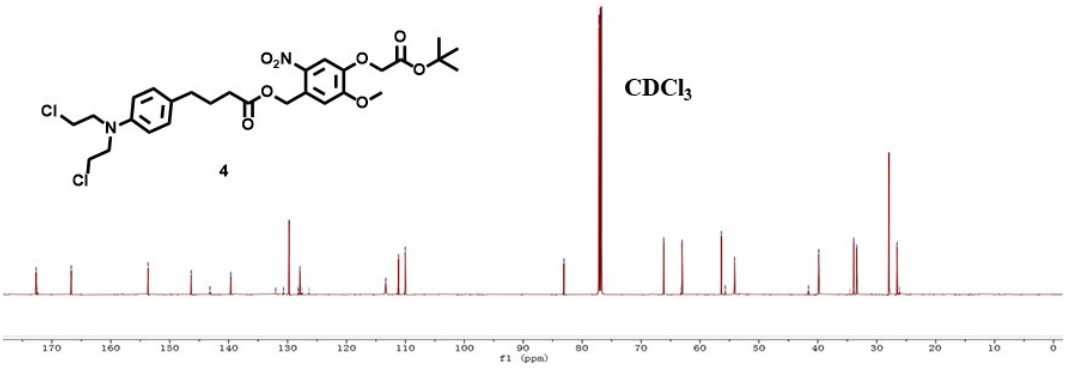


Fig. S16 ^{13}C NMR spectrum (151 MHz, $CDCl_3$) of compound **4**.

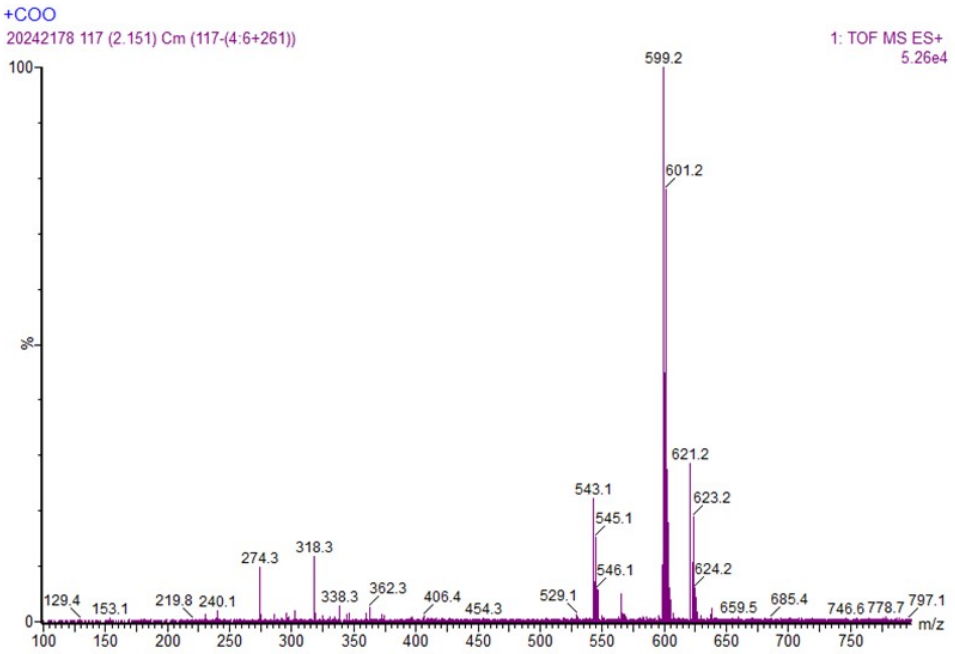


Fig. S17 MS (ESI) spectrum recorded for compound **4**.

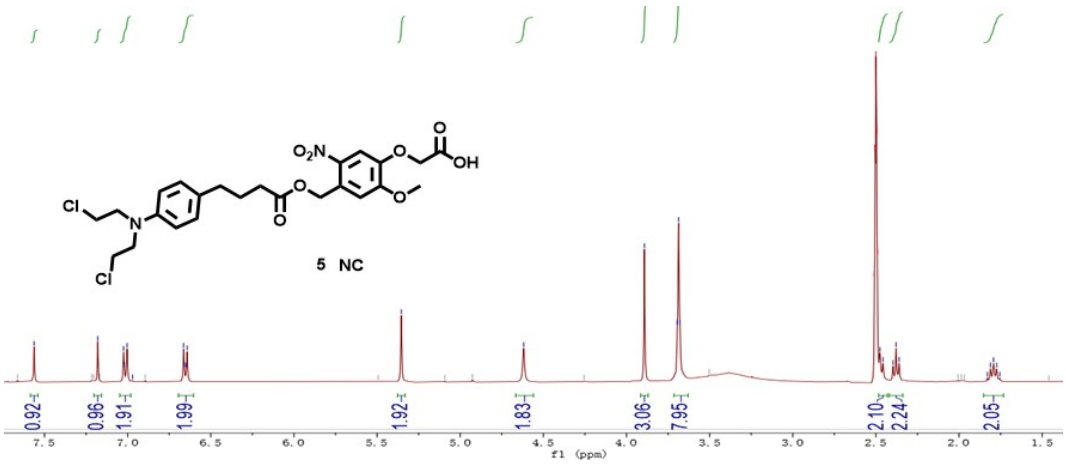


Fig. S18 ^{1}H NMR spectrum (400 MHz, DMSO-d_6) of compound **5**.

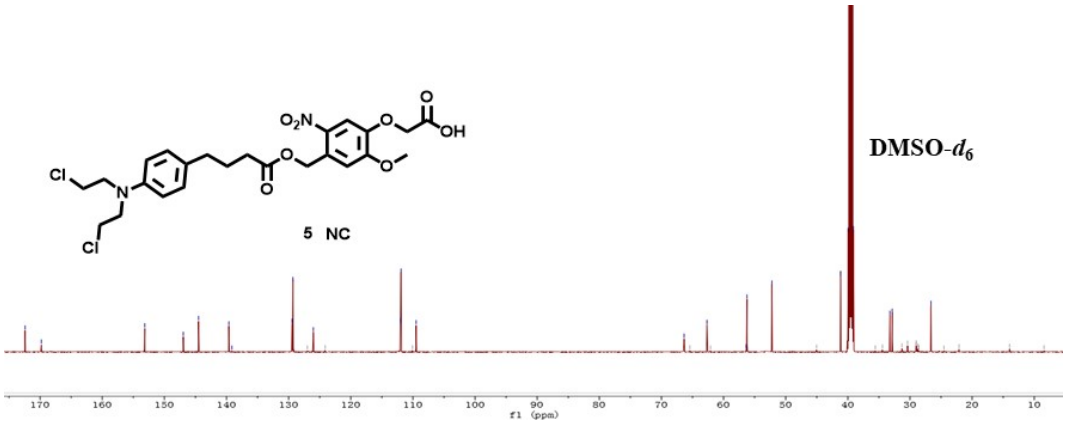


Fig. S19 ^{13}C NMR spectrum (151 MHz, DMSO-d_6) of compound **5**.

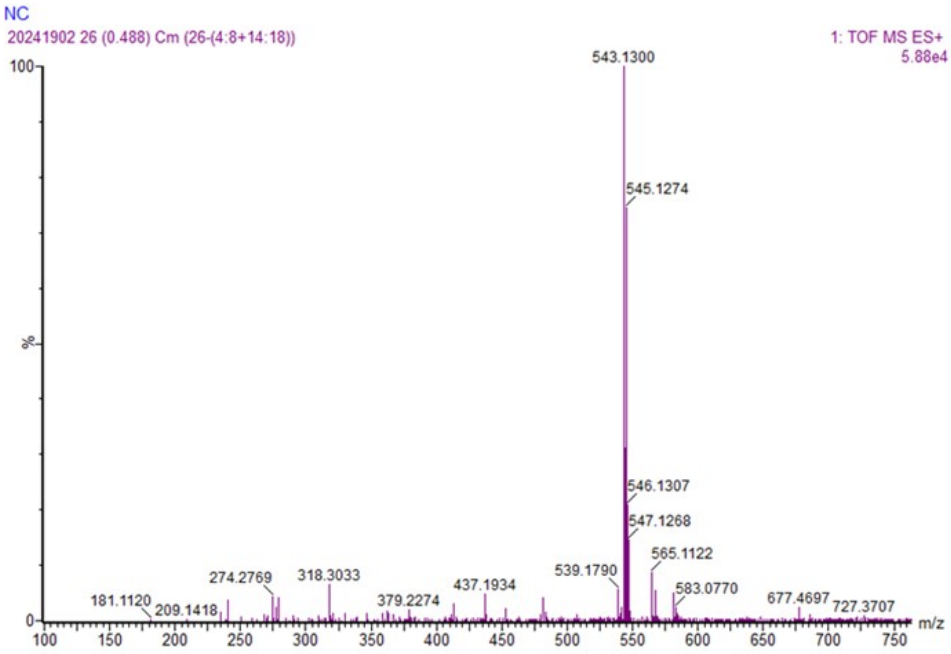


Fig. S20 HRMS (ESI) spectrum recorded for compound **5**.

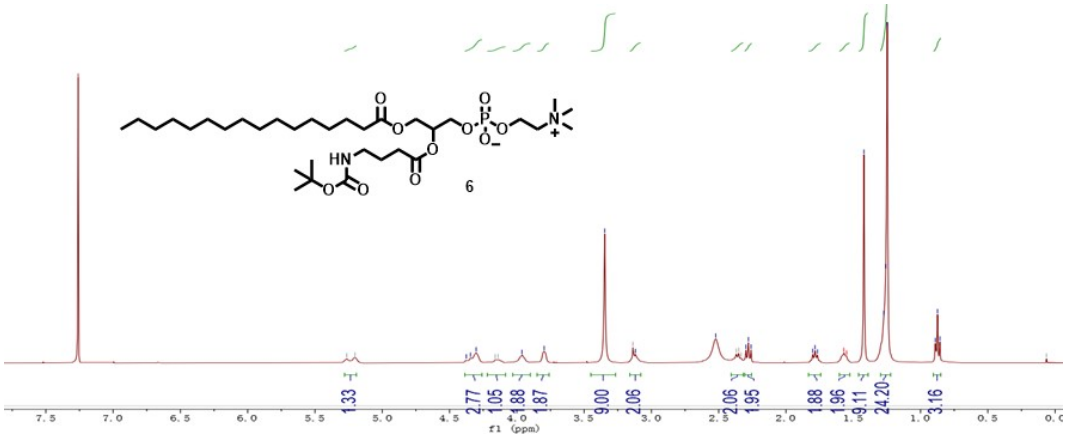


Fig. S21 ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound **6**.

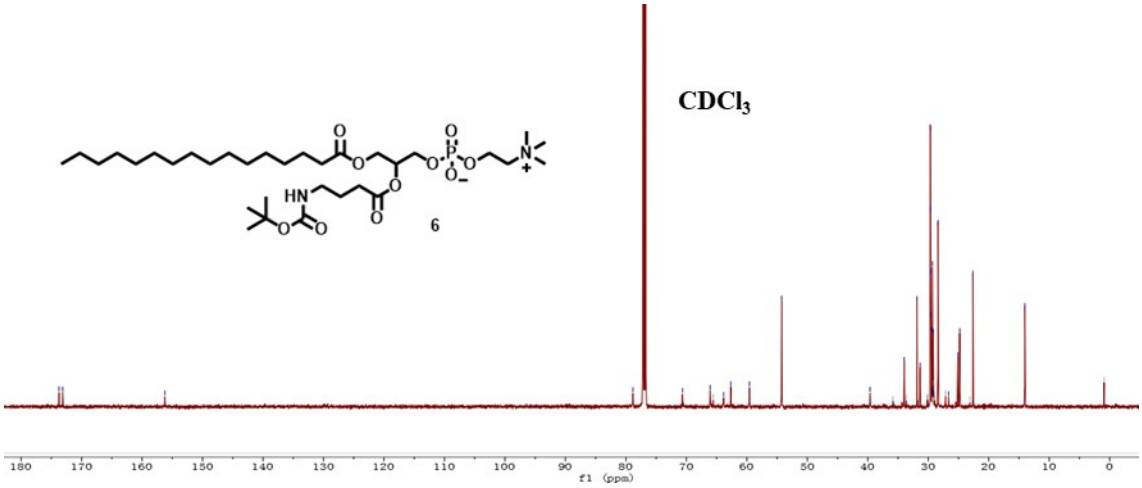


Fig. S22 ^{13}C NMR spectrum (151 MHz, $CDCl_3$) of compound **6**.

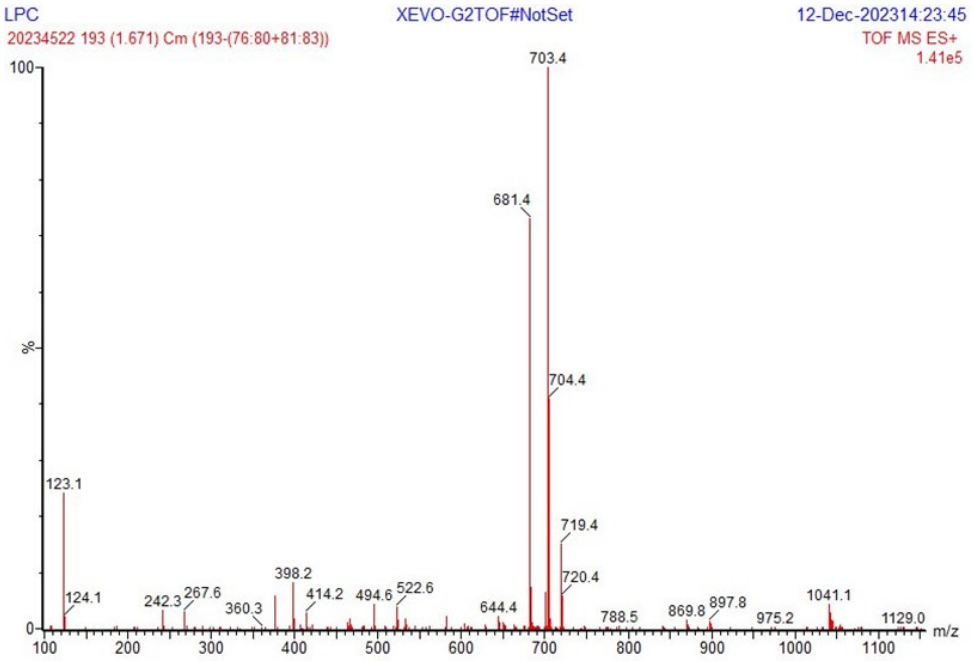


Fig. S23 MS (ESI) spectrum recorded for compound **6**.

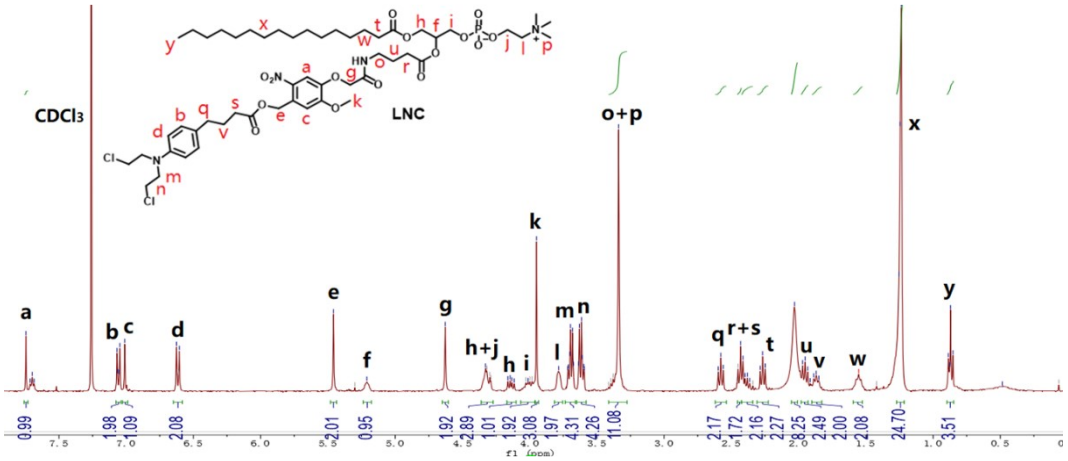


Fig. S24 ^{1}H NMR spectrum (400 MHz, $CDCl_3$) of compound **LNC**.

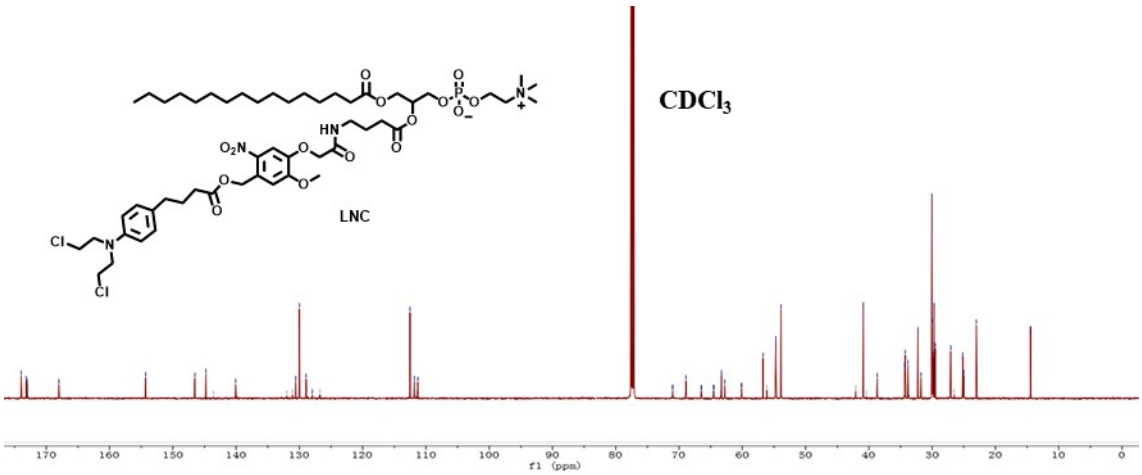


Fig. S25 ^{13}C NMR spectrum (151 MHz, $CDCl_3$) of compound **LNC**.

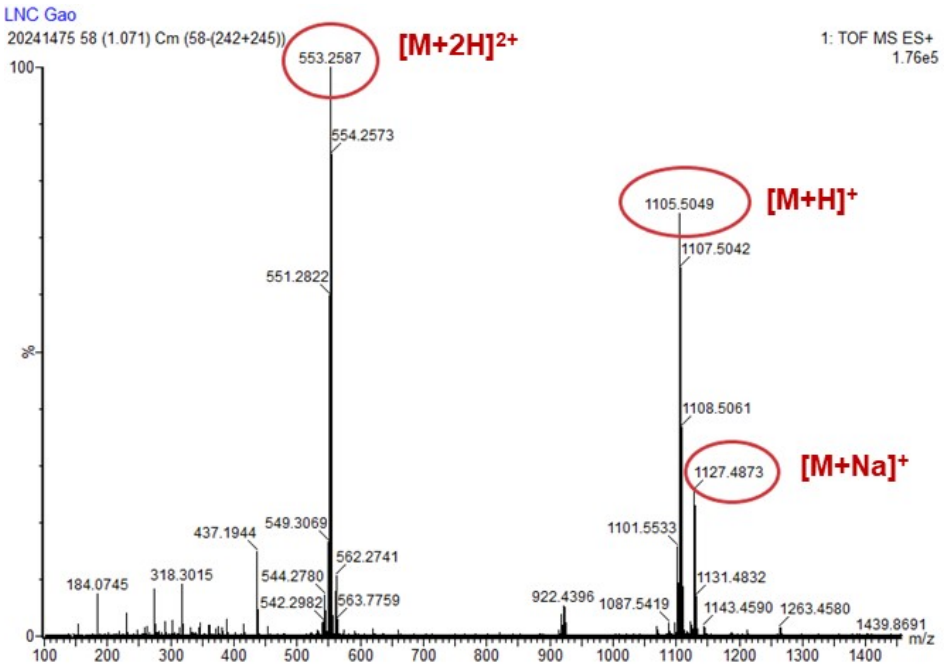


Fig. S26 HRMS (ESI) spectrum recorded for compound **LNC**.

6. References

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