Supporting Information

Dendritic phospholipids-based drug delivery system

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Materials

H-Lys-OMe·2HCl, Boc-Lys(Boc)-OH, 1-hydroxybenzotriazole hydrate (HOBT), 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), were purchased from GL Biochem. Ltd. (Shanghai, China). *N*, *N*-diisopropylethylamine (DIPEA) and trifluoroacetic acid (TFA) were purchased from J&K Scientific (Beijing, China). Doxorubicin hydrochloride (DOX·HCl) was purchased from Hisun Pharmaceutical (Zhejiang, China). 1, 2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) was purchased from CordenPharma (Swizerland). All other solvents were purchased from Tianjin Kemiou Chemical Reagent Co., Ltd. (Tianjin, China). Roswell Park Memorial Institute (RPMI)-1640 medium, penicillin, streptomycin and fetal bovine serum (FBS) were purchased from Hyclone (USA). 4T1 and BGC823 cell line (mouse breast cancer cell) were purchased from Chinese Academy of Science Cell Bank for Type Culture Collection (Shanghai, China).

Methods

The molecular weight of each compound was tested by matrixassisted laser desorption ionization time of flight mass spectroscopy (MALDI-TOF MS, Bruker Autoflex III) or electrospray ionization time-of-flight mass spectrometry (ESI-TOF MS, Waters Q-TOF Premier). Dynamic light scattering (DLS) experiments were performed

on a Brookhaven Zetasizer Nano ZS at 25 °C. Each measurement was performed in triplicate and an average value was reported. UV-Vis absorbance spectra were measured by a UV3100 (Shimadzu, Japan). Morphology and structure of the obtained nanocarriers were observed with a transmission electron microscope (TEM, JEM-100S, JEOL, Japan). The cell viability was detected by MTT [3-(4',5'-dimethylthiazol-2'-yl)-2,5-diphenyltetrazolium bromide] assay, and the absorbance of each sample was measured at 570 nm using a Tecan microplate reader. The qualitative and quantitative of cellular uptake were observed by Confocal Laser Scanning Microscopy (CLSM, LSM 710, Zeiss, Germany) and Fluorescence Activated Cell Sorting (Accuri C6, BD Biosciences, USA).

Synthesis of dendritic phospholipids

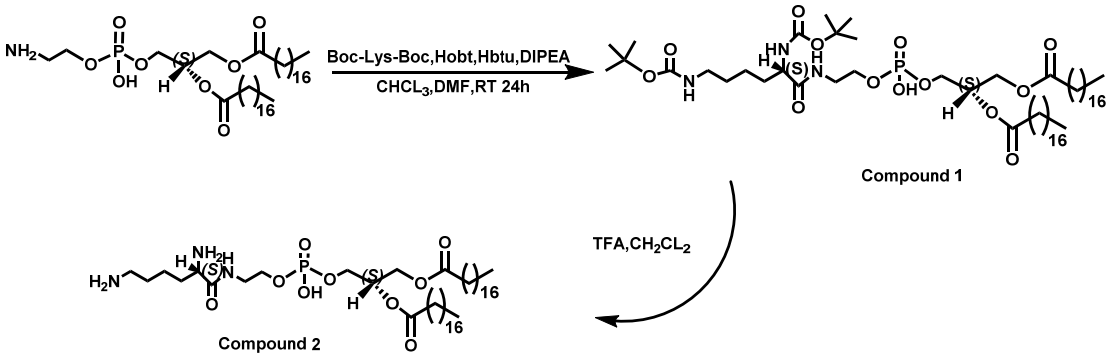

Figure S1. Synthesis procedure of DPL-1.

DSPE (3.00 g, 4.00 mmol), Boc-L-Lys(Boc)-OH (1.66 g, 4.80 mmol), Hobt (0.64 g, 4.80 mmol) and Hbtu (1.82 g, 4.80 mmol) was dissolved in anhydrous trichloromethane (50 mL) and anhydrous DMF (10 mL) under nitrogen atmosphere. DIPEA (4.00 mL, 28.00 mmol) was added to the above mixed solution under stirring at 0℃. The solution was stirred under nitrogen for another 24 h at room temperature. Then, the mixture was washed with saturated $NaHCO_3$, $NaHSO_4$, and NaCl solution for several times. The mixture was dried with $MgSO_4$ for 2 h. After the removal of solvents, the mixture was purified by silica gel column chromatography (DCM/MeOH, 12/1, v/v) to obtain **Compound 1** (yield: 83%). **Compound 1** was dried in vacuum and dissolved in anhydrous dichloromethane (DCM)/TFA (1:1, 20 mL) for 4 h to put off

tert-butyl groups. The mixture was concentrated, and the product was treated with anhydrous diethyl ether to obtain **Compound 2** (yield: 91%).

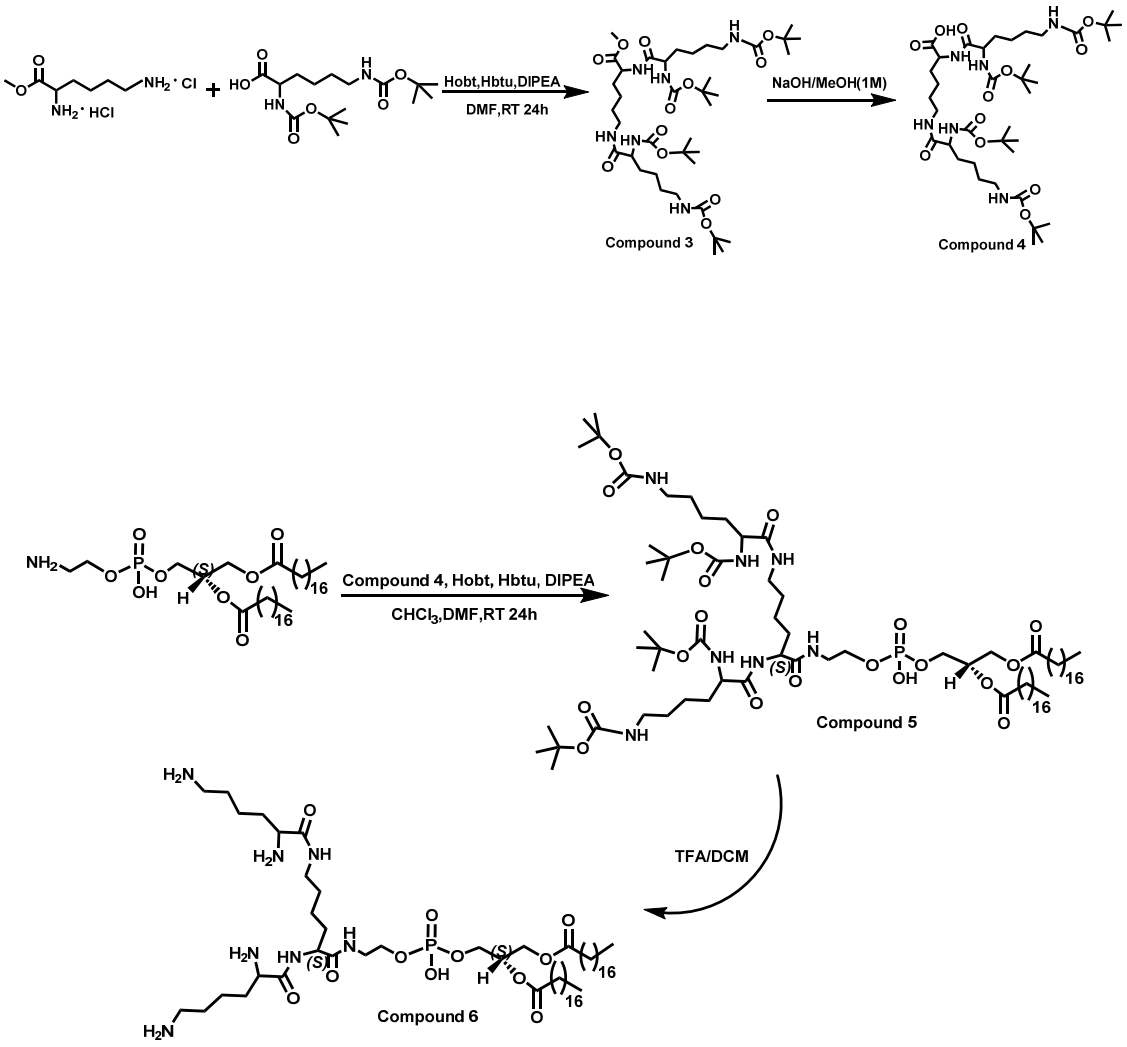

Figure S2. Synthesis procedure of DPL-2.

H-Lys-OMe.2HCl (1.00 g, 4.3 mmol), Boc-L-Lys(Boc)-OH (4.46 g, 12.9 mmol), Hbtu (4.89 g, 12.9 mmol) and Hobt (1.68 g, 12.9 mmol) were dissolved in anhydrous DMF (25 mL) in nitrogen atmosphere. DIPEA (5.7 mL, 34.4 mmol) was added in the ice-water bath. This reaction mixture was stirred at room temperature for 48 hours. Then, the mixed solution was washed with saturated $NaHCO_3$, $NaHSO_4$, and NaCl solution for several times. The mixture was dried with $MgSO_4$ for 2 h. After the removal

of solvents, the mixture was purified by silica gel column chromatography (DCM/MeOH, 12/1, v/v) to obtain **Compound 3** (yield: 85%).

Compound 3 (4.00 g, 3.4 mmol) was treated with NaOH in 50 mL MeOH (1 mol/L) for 4 hours to expose carboxyl groups. After the removal of MeOH, the mixture was dissolved in H_2O and adjusted to neutral pH value. **Compound 4** could be extracted by DCM and dried with $MgSO_4$ for 2 h (yield: 74%).

DSPE (0.75 g, 1.00 mmol), **Compound 4** (1.81 g, 1.5 mmol), Hobt (0.27 g, 2 mmol) and Hbtu (0.57 g, 2 mmol) was dissolved in anhydrous trichloromethane (25 mL) and anhydrous DMF (3 mL) under nitrogen atmosphere. DIPEA (2.00 mL, 14.00 mmol) was added to the above mixed solution under stirring at 0℃. The solution was stirred under nitrogen for another 48 h at room temperature. The mixture was washed with saturated NaHCO3, NaHSO4, and NaCl solution for several times. The mixture was dried with MgSO4 for 2 h. After the removal of solvents, the mixture was purified by silica gel column chromatography (DCM/MeOH, 12/1, v/v) to obtain **Compound 5** (yield: 81%).

Compound 5 was dried in vacuum and dissolved in anhydrous dichloromethane DCM/TFA (1:1, 10 mL) for 4 h to put off tert-butyl groups. The mixture was concentrated, and the product was treated with anhydrous diethyl ether to obtain **Compound 6** (yield: 92%).

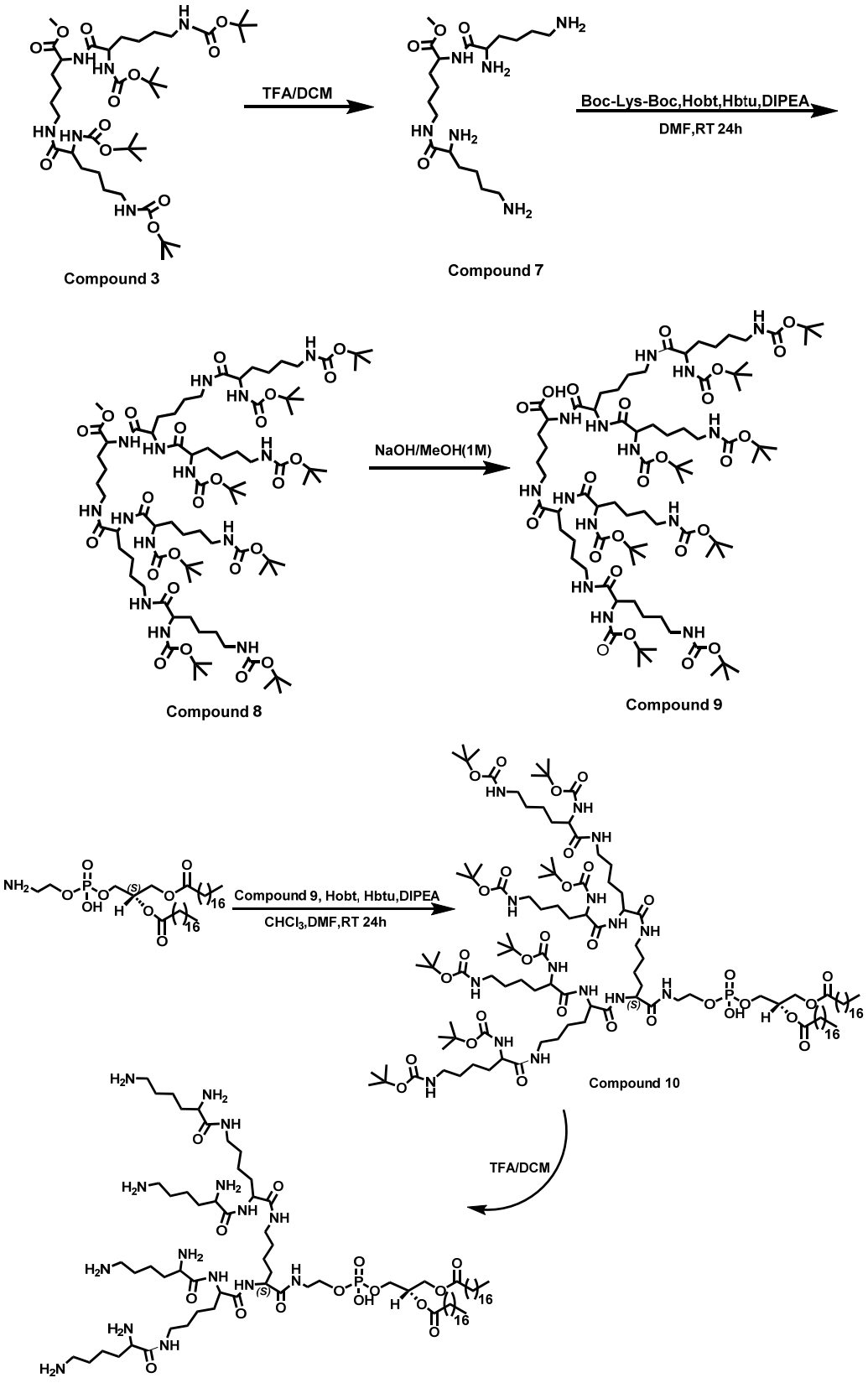

Figure S3. Synthesis procedure of DPL-3.

Compound 3 was dried in vacuum and dissolved in anhydrous dichloromethane (DCM)/TFA (1:1, 10 mL) for 4 h to put off tert-butyl groups. The mixture was concentrated, and the product was treated with anhydrous diethyl ether to obtain **Compound 7** (yield: 92%).

H-Lys-OMe.2HCl (0.93 g, 4.0 mmol), **Compound 7** (387.5, 12.9 mmol, Hbtu (4.89 g, 12.9 mmol) and Hobt (1.68 g, 12.9 mmol) were dissolved in anhydrous DMF (25 mL) in nitrogen atmosphere. DIPEA (5.7 mL, 34.4 mmol) was added in the ice-water bath. This reaction mixture was stirred at room temperature for 48 hours. Then, the mixed solution was washed with saturated $NaHCO_3$, $NaHSO_4$, and NaCl solution for several times. The mixture was dried with $MgSO_4$ for 2 h. After the removal of solvents, the mixture was purified by silica gel column chromatography (DCM/MeOH, 12/1, v/v) to obtain **Compound 8** (yield: 85%).

Compound 8 (4.00 g, 2.3 mmol) was treated with NaOH in 40 mL MeOH (1 mol/L) for 4 hours to expose carboxyl groups. After the removal of MeOH, the mixture was dissolved in H_2O and adjusted to neutral pH value. **Compound 9** could be extracted by DCM and dried with $MgSO_4$ for 2 h (yield: 74%).

DSPE (1.5 g, 2.00 mmol), **Compound 9** (5.14 g, 3 mmol), Hobt (0.52g, 4 mmol) and Hbtu (1.14 g, 4 mmol) was dissolved in anhydrous trichloromethane (50 mL) and anhydrous DMF (6 mL) under nitrogen atmosphere. DIPEA (4.00 mL, 28.00 mmol) was added to the above mixed solution under stirring at 0℃. The solution was stirred under nitrogen for another 48 h at room temperature. The mixture was washed with saturated $NaHCO_3$, $NaHSO_4$, and NaCl solution for several times. The mixture was dried with $MgSO_4$ for 2 h. After the removal of solvents, the mixture was purified by silica gel column chromatography (DCM/MeOH, 12/1, v/v) to obtain **Compound 10** (yield: 81%).

Compound 10 was dried in vacuum and dissolved in anhydrous dichloromethane (DCM)/TFA (1:1, 10 mL) for 4 h to put off tert-butyl groups. The mixture was concentrated, and the product was treated with anhydrous diethyl ether to obtain **Compound 11** (yield: 92%).

Preparation and characterization of nanocarriers

Dendritic phospholipids (or with hydrophobic DOX) was dissolved in almost 50 μL of methanol/ chloroform (v:v=1:1), and then injected into 1 mL of PBS buffer under fast string. After removal of the residual solvent, dendritic phospholipids would spontaneously self-assemble into nanocarriers. The unencapsulated DOX was removed using centrifugation at 1000 rpm for 10 min. DLS was used to determine the size and zeta potential of DPNs in PBS (pH = 7.4) at 25 °C. Copper grids were dipped into the fresh SPA solutions to produce the TEM samples. Until the solvent evaporated off, the samples were observed using transmission electron microscopy. The drug-loading capacity was calculated using the following equation:

Dox loading capacity $=W_{drug}/W_{DPLs}\times 100\%$

where W_{drug} is the weight of drug and W_{DPLs} is the total weight of the drug and DPLs (DPL-1 to 3).

Analyses of dendritic lipopeptides by Circular Dichroism (CD)

The CD experiments were conducted on a Chirascan spectrometer (Applied Photophysics Ltd., Leatherhead, UK). The samples of DPN-2 and DPN-3 were prepared at concentrations of 0.05 mg/ml at pH 7.4. The solution was placed in the sample cell with a path length of 1.0 cm. The analysis was calculated by CDNN software.

In vitro drug release from DOX-loaded nanocarriers

1mL of DOX-loaded nanocarriers was put into a dialysis bag (MWCO 7 kDa) and suspended into 5 mL of 0.01 M PBS, at pH 7.4 and 5.0 respectively, with agitation about 200 rpm at 37 ^{o}C and shielded from light. Periodically, withdrawn the release medium outside of the dialysis and added another 5 mL fresh PBS. A fluorescence spectrometer (RF-5301PC, Shimadzu, Japan) with excitation at 480 nm and emission at 590 nm was used to determine the concentration of DOX and a standard curve was initially obtained. The cumulative amount of DOX released from nanoparticles was plotted against time.

In vitro cell cytotoxicity

MTT assays were used to evaluate the cell cytotoxicity of the DOX-loaded nanoparticles and free DOX. A density of 5×10^3 BGC823 and 4T1 cells were seeded into a 96-well plate and subsequently incubated at 37 ^oC overnight. Next day, the culture medium was exchanged with 200 μL fresh 1640 mediums including free DOX and DOX-loaded nanoparticles at 1, 2, 4, 8, 16, 32 μg/mL, respectively. The blank nanoparticles were also added into a series of wells at a gradient concentration to evaluate the cytocompatibility. Three wells with 200 μL culture medium alone were used as control and three wells containing cells without drug were used as reference since it has the maximum of cell viability. After incubation for 24 h and washing twice with PBS, then added 180 μL fresh medium with 20 μL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL) in each well, and incubated for another 4 h. A microplate reader (Huadong, DG-5031, NJ) was used to measure the absorbance of every well at 570 nm. Cell viability was calculated by the following formula:

Cell viability (%) = (Abs of test cells) / (Abs of control cells) ×100%,

Where Abs is absorbance.

***In vitro* cellular uptake**

BGC823 cells and 4T1 cells were cultured in DMEM medium, which was changed every two days until 80% coverage was reached. Subsequently, the cells were digested and 1×10^5 cells were seeded into a 6-well plate chambering cover-glass and incubated for overnight. Then 200 μL DOX-loaded nanopartilces were added into and incubate for another 4 h at 37 ^oC, followed by washing to wipe off any free DOX-loaded nanopartilces. Afterwards, the cells were fixed in 4% paraformaldehyde for 8-10 min, washed, dyed with Hoechst 33258 and washed again. The coverslips were observed with an ×63 oil immersion lens through a confocal laser scanning microscopy (CLSM; LSM 710, Zeiss, Germany).

Quantification of cellular uptake of BGC823 cells and 4T1 cells were also measured by flow cytometry (Accuri C6, BD Biosciences, USA). BGC823 cells and

4T1 cells were cultured in DMEM medium, which was changed every two days until 80% coverage was reached. Subsequently, the cells were digested and 1×10^5 cells were seeded into a 6-well plate chambering cover-glass and incubated for overnight. Then 200 μL DOX-loaded nanopartilces were added into and incubate for another 4 h at 37 ^{o}C, followed by washing to wipe off any free DOX-loaded nanopartilces. Afterwards, the cells were washed with cold PBS three times, and the cells were harvested by trypsinization and centrifugation at 1000 rpm for 5 min. The cells were resuspended in 1 mL of PBS buffer (pH 7.4). The fluorescence intensity of the cells was examined using flow cytometry.

Penetration of DOX-loaded nanoparticles in 4T1 multicellular spheroids (MCs)

4T1 MCs were formed similar to SY5Y MCs which prepared in our group before. When the MCs grow to a diameter of about 150 μm, they were harvested for experiment. For each sample, 2 mL medium contain about 20 spheroids were transferred to a 10 mL centrifuge tube. Then 500 μL free DOX and DOX-loaded nanoparticles, which has equal concentration of DOX, were added into the tube and allowed to incubated at 37 ^{o}C. At predetermine time points, samples were picked out, medium was discarded, and after washed with PBS, MCs were then observed by CLSM.

Characterizations of dendritic phospholipids

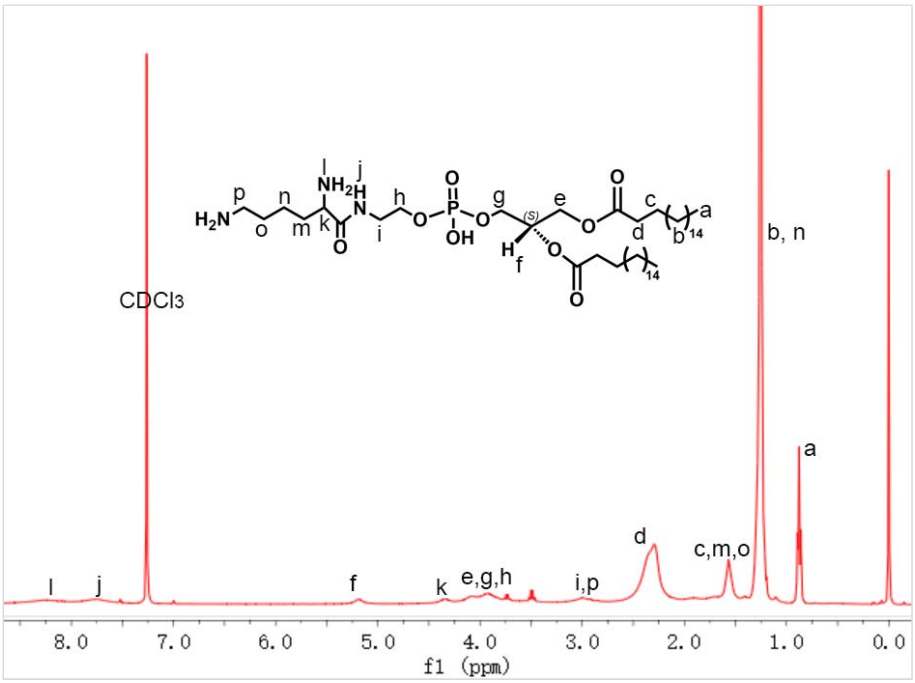

Figure S4. ^{1}H-NMR spectrum of DPL-1 in CDCl3.

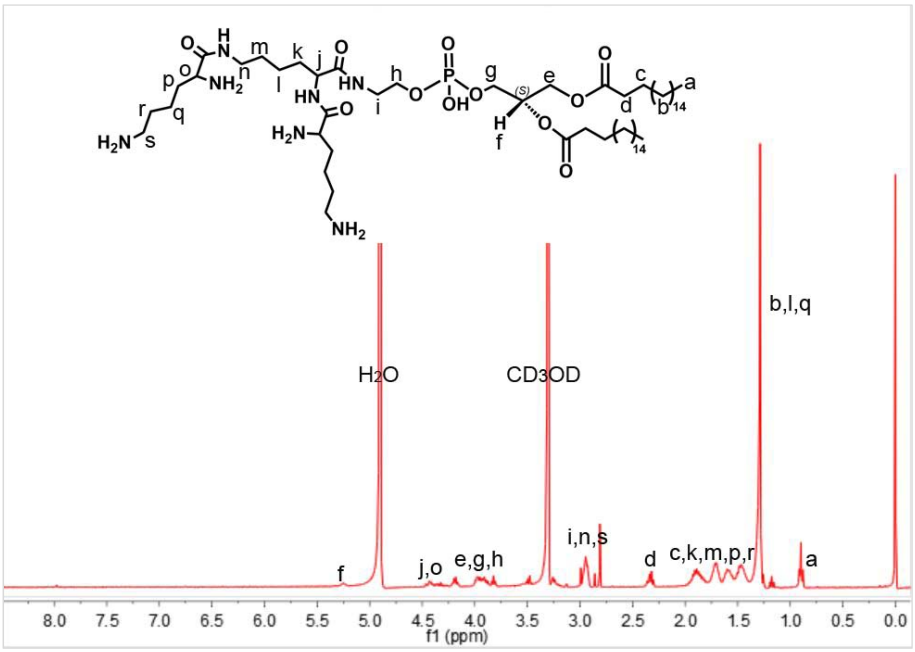

Figure S5. ^{1}H-NMR spectrum of DPL-2 in CH3OD.

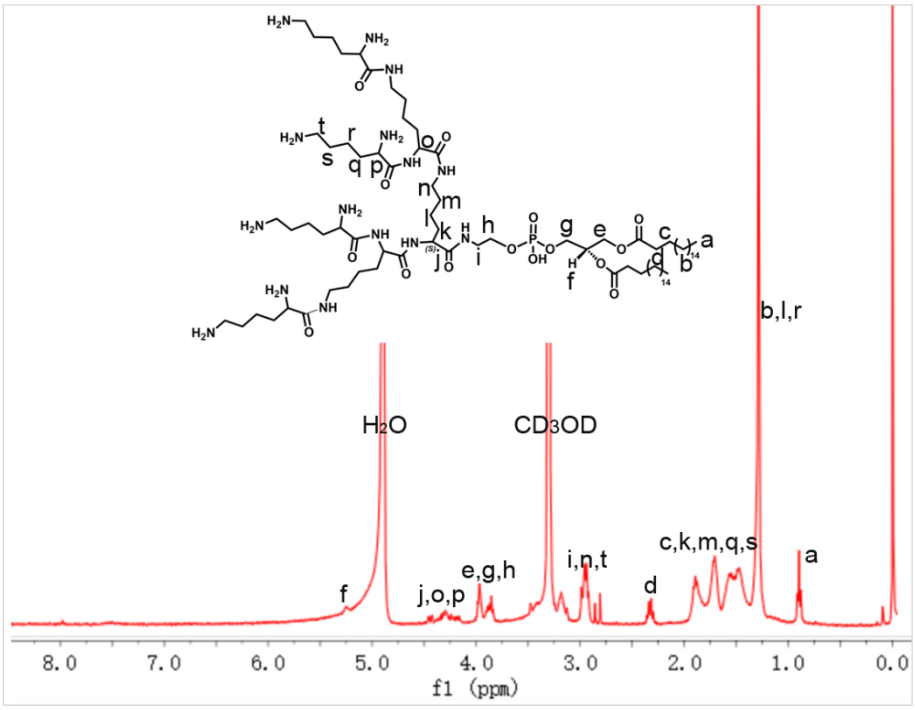

Figure S6. ^{1}H-NMR spectrum of DPL-3 in CH3OD.

Cellular uptake

Flow cytometer was used to quantitatively analyze the cellular uptake of two DPNs. In 4T1 cells, the fluorescence intensity of DOX-loaded DG3-DPNs group is 3.0 fold higher than that of DOX-loaded DG2-DPNs group (**Fig. 7**). As showed in **Fig. 8**, in BGC823 cells, the fluorescence intensity of DOX-loaded DG3-DPNs group is 1.8 fold higher that of DOX-loaded DG2-DPNs group.

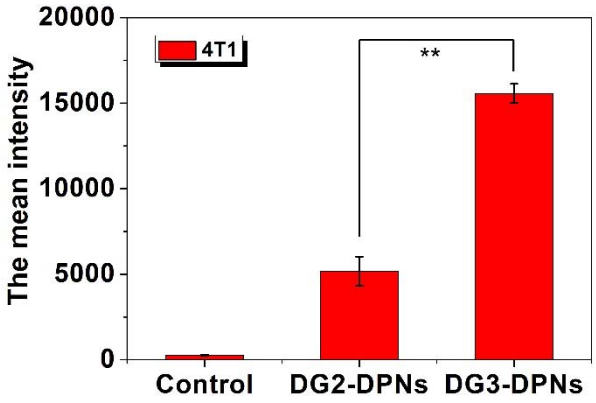

Figure S7 The mean intensity of DOX in 4T1 cells for DOX-loaded DPNs measured by flow cytometry. (P<0.01)**

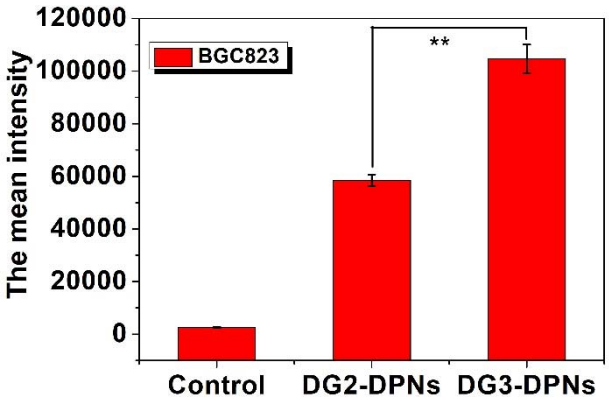

Figure S8 The mean intensity of DOX in BGC823 cells for DOX-loaded DPNs measured by flow cytometry. (P<0.01)**