Supporting Information

Disterolphospholipids: Non-exchangeable Lipids and Their Application to Liposomal Drug Delivery

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Contents:

Synthesis of disterol-modified phospholipids

*1,2-Dicholesterylcarbonoyl-sn-glycero-3-phosphocholine (**dSML1**):*

Glycerophosphocholine (0.514 g, 2 mmol) and sodium tetraphenylborate (0.719 g, 1.05 equiv.) were dissolved in 15 mL methanol. Solvent was evaporated, and the residue was azeotropically dried with toluene twice. The dried solid was then dissolved in anhydrous pyridine (60 mL), followed by the addition of 4,4-dimethylaminopyridine (0.732 g, 6 mmol). Cholesteryl chloroformate (2.70, 6 mmol) was added into the reaction mixture in portion at r.t. with vigorous stirring. The reaction flask was then purged with nitrogen and kept under dark for 3 days. Volatiles were removed by rotary evaporation under reduced pressure. The crude product was dissolved in chloroform-methanol (2:1, 150 mL), and washed with 50 mL distilled water. The organic layer was dried by anhydrous sodium sulfate, filtered and evaporated. The residue was loaded on a Flash40+M column (Biotage, Charlottesville, VA), and purified on a Biotage high performance flash column (HPFC) system (Horizon™). Chloroform was used as the elution solvent A, and a mixture of methanol and water (25/4, v/v) was used as the more polar elution solvent B. The flash column was equilibrated with 240 mL chloroform, then eluted by solvents of increasing strength ranging from 0% to 25% B in A. Detailed parameters and methods of HPFC are listed as follows:

Flow rate: 40 mL/min; fraction volume: 12 mL/tube; column equilibration: 240 mL chloroform (diverted to waste bottle); column elution (collected in glass tubes): segment 1) 15% - 25% B in A, 480 mL, segment 2): 25% - 25% B in A, 600 mL.

Fractions #67-84 were pooled, evaporated, and dried under high vacuum to afford the pure product with a yield of 0.38 g (17.7%). TLC: R_f = 0.4 ($CHCl_3$-MeOH-H_2O 65/25/4).

^{1}H NMR ($CDCl_3$), δ 0.68 (s, 6H); 0.85-1.65 (m, 66H); 1.81-2.06 (m, 10H); 2.37 (m, 4H); 3.42 (s, 9H); 3.89 (m, 2H); 4.06 (m, 2H), 4.26 (m, 2H); 4.36 (m, 2H); 4.44 (m, 2H); 5.04 (m, 1H); 5.38 (d, J = 4.4, 2H). MALDI-MS calcd for $C_{64}H_{109}NO_{10}P^+$ $[M + H]^+$ 1082.79, found 1082.73.

*1,2-Dicholesterylhemisuccinoyl-sn-glycero-3-phosphocholine (**dSML2a**):*

Glycerophosphocholine (1.03 g, 4 mmol) and sodium tetraphenylborate (1.33 g, 1 equiv.) were dissolved in 30 mL methanol. Solvent was evaporated, and the residue was azeotropically dried with toluene twice. The dried solid was then dissolved in anhydrous pyridine (120 mL), followed by the addition of 4,4-dimethylaminopyridine (0.9 g) and cholesteryl hemisuccinate (4.86 g, 10 mmol). The mixture was gently warmed up to dissolve the solid completely. Dicyclohexylcarbodiimide (2.32 g, 11 mmol) was added to the reaction mixture after it was cooled to the room temperature. The mixture was stirred under nitrogen at room temperature for 3 days. Volatiles were evaporated, and the residue was dissolved in chloroform-methanol (2:1, 300 mL), washed with 80 mL distilled water. The organic layer was dried over anhydrous sodium sulfate, filtered and evaporated. The crude product was purified by HPFC with a method similar to that of dSML **1**. Yield, 2.3 g (48%). TLC: R_f = 0.42 ($CHCl_3$-MeOH-H_2O 65/25/4). ^{1}H NMR ($CDCl_3$), δ 0.68 (s, 6H); 0.85-1.65 (m, 66H); 1.81-2.06 (m, 10H); 2.30 (m, 4H); 2.60 (m, 8H); 3.39 (s, 9H); 3.85 (m, 2H); 3.99 (m, 2H), 4.23 (m, 2H); 4.35 (m, 2H); 4.58 (m, 2H); 5.22 (m, 1H); 5.37 (d, J = 4.4, 2H). MALDI-MS calcd for $C_{70}H_{116}NO_{12}P^+$ $[M + H]^+$ 1194.84, found 1194.79.

1,2-Distigmasterylhemisuccinoyl-*sn*-glycero-3-phosphocholine (***dSML2b***): This compound was synthesized according to the same procedure of ***dSML2a***. TLC: Rf = 0.36 ($CHCl_3$-MeOH-H_2O 65/25/4). ^{1}H NMR ($CDCl_3$), δ 0.81 (m, 20H); 1.11 (m, 30H); 1.56

(m, 20H); 1.96 (m, 10H); 2.30 (m, 4H); 2.58 (m, 8H); 3.30 (s, 9H); 3.34 (m, 2H), 3.76 (m, 2H); 3.96 (m, 2H); 4.28 (m, 2H); 4.57 (d, J = 6.0, 2H); 5.08 (m, 5H); 5.37 (d, J = 4.2, 2H). MALDI-MS calcd for $C_{74}H_{121}NO_{12}P^+$ $[M + H]^+$ 1246.87, found 1246.98.

Freeze-Fracture Electron Microscopy (FF-EM)

The FF-EM images were taken on a JEOL 100 CX electron microscope by Dr. Brigitte Papahadjopoulos-Sternberg (Nanoanalytical Laboratory, San Francisco, CA). Sonicated liposome sample (DPPC/dSML2a, 2/1, 20 mM) was quenched using sandwich technique and liquid nitrogen-cooled propane. Using this technique a cooling rate of 10,000 Kelvin per second is reached avoiding ice crystal formation and artifacts possibly caused by the cryo-fixation process. The fracturing process was carried out in JEOL JED-9000 freeze etching equipment and the freshly exposed fracture planes were shadowed with Pt for 30 seconds in an angle of 25-35 degree and with carbon for 35 seconds (2 kV/60-70 mA, 1×10^{-5} Torr). The replicas produced this way were cleaned with concentrated, fuming HNO_3 for 24 hours followed by repeating agitation with fresh chloroform/methanol (1/1 by volume) at least 5 times. The cleaned replicas were then examined by the electron microscope.

Differential Scanning Calorimetry. Differential scanning calorimetric (DSC) measurements were carried out by using a high-temperature MC-DSC 4100 calorimeter (Calorimetry Sciences Corp., Lindon, UT) with three reusable Hastelloy sample ampoules and a reference ampoule. Data was collected typically over a range of 5 ºC–85 °C at 0.5 °C/min with Milli-Q® water as the reference. The CpCalc 2.1 software package from Calorimetry Sciences Corp was used to convert the raw data into molar heat capacity (MHC). Data were then imported into Origin 6.0 (Microcal, Northampton,

MA) for further processing and calculation. Liposomes used for DSC measurement were prepared by hydrating the lipid film (10 μmol) in Milli-Q® water (200 μL) at 65 °C under argon for 15 min with intermittent vortex. Samples were then cooled to room temperature, degassed, and loaded into the sample ampoule using gas-tight Hamilton® syringe (100 μL per sample). Samples were scanned through a heating-cooling-heating cycle and the second heating scan data was used for analysis.

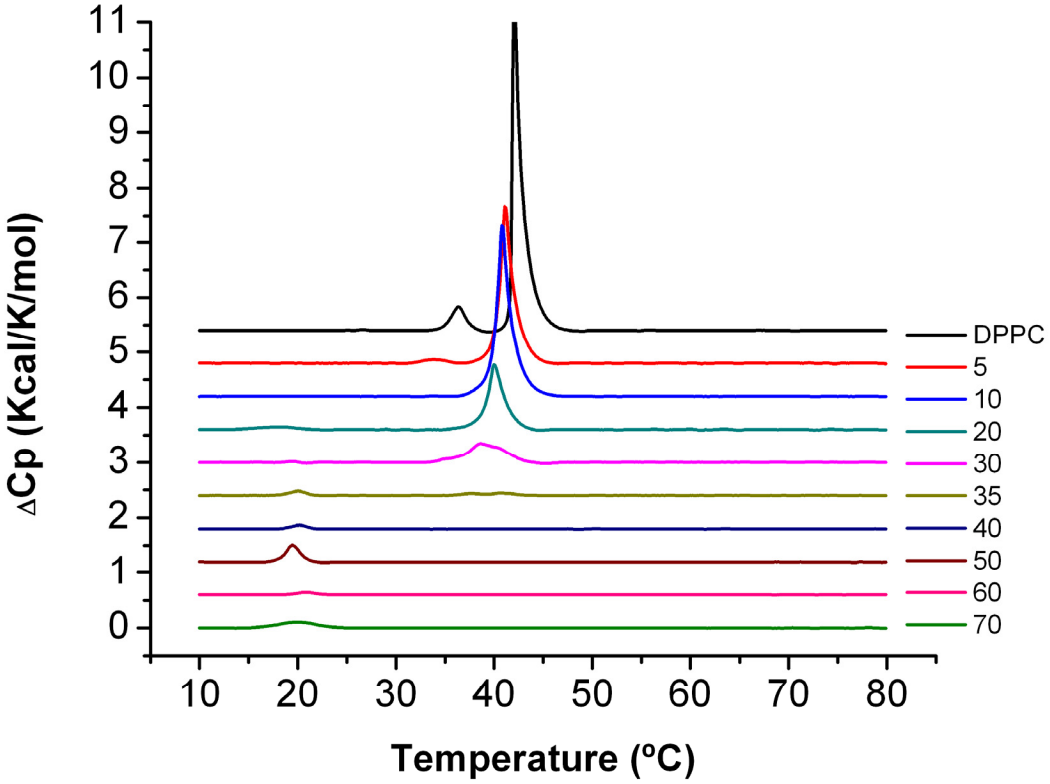

Figure 1. DSC thermogram of dSML **2b**/DPPC. Label indicates the percentage of cholesterol in the liposome formulation.

Cholesterol Exchange Experiment. Unilamellar liposomes were prepared by the extrusion method. The donor liposomes consisted of 40% cholesterol (or equivalent from dSML), 10% negatively charged corresponding phosphatidylglycerol (PG), and 50% 1,2-diacyl-*sn*-glycero-3-phosphocholine (PC) (or the equivalent from dSML). Specifically,

the two donor liposomes were formulated at the following molar ratios: 1) Chol/DPPC/DPPG(4/5/1), 2) dSML/DPPC/DPPG(5/13/2). A ten-fold molar excess of neutral POPC liposome was used as the acceptor liposome. For the exchange experiments, 1 mL donor liposomes (10 mM) and 1 mL acceptor liposomes (100 mM, 10 fold) were warmed at 37 °C first, then mixed and incubated at 37 °C. An aliquot (250 μL) of mixture was sampled at given time point and applied to a small (ca. 2 cm in length) anion exchange column (Q-Sepharose XL). The column was pretreated with 0.1 mL 10 mM POPC before the loading of the exchange sample to reduce the nonspecific binding of the neutral liposome. The column was eluted with 1 mL pH 7.4 10 mM NaCl, 10 mM HEPES buffer. The eluate was lyophilized and analyzed by the cholesterol assay[1] to quantify the amount of cholesterol exchanged. Briefly, the lyophilized lipid powder was suspended in 100 μL distilled water and 50 μL was transferred to screw-capped glass tube (13mm ×100mm). After the addition of 5 mL cholesterol assay reagent (339 mg ferric perchlorate hexahydrate in 300 mL ethyl acetate mixed with 200 mL concentrated sulfuric acid at 4 °C), the mixture was heated at 100 °C for 90 seconds, immersed immediately into ice-water. The absorbance at 610 nm was measured and the amount of cholesterol was calculated according to the standard curve.

Leakage in 30% Fetal Bovine Serum. Carboxyfluorescein (CF) was purified to remove hydrophobic fluorescent impurities, and encapsulated in liposomes as previously described.[2] Briefly, the dry lipid film (10 μmol) of given formulation was hydrated in 1 mL CF solution at 60 °C under argon for 15 min with intermittent vortex. Then liposomes were extruded through 200 nm polycarbonate membrane, and the free CF was removed by eluting liposomes loaded on a PD-10 column (GE Healthcare, Piscataway,

NJ) with HEPES buffer saline (pH 7.4, 20 mM HEPES, 130 mM NaCl) as the isomotic eluent. An aliquot of liposome sample (10 μL) was diluted in 96-well plate by FBS to a total volume of 200 μL that containing 0.02% sodium azide. The plate was then sealed with transparent plastic film and incubated at 37 °C. Samples were lysed with 10 μL 15% C12E10 detergent to release all encapsulated contents so that total fluorescence can be determined. Fluorescence intensities of samples were monitored at different time points on a Fluorostar fluorescence plate reader (BMG LABTECH Inc, Durham, NC), and the percentage of CF released from liposome was determined by the following formula.

$$CF\% = (Ft - F0)/(Fa - F0)*100\%$$

Wherein

F0 = background fluorescence signal

Fa = total fluorescence signal

Ft = fluorescence signal at the time of measurement

Encapsulation of doxorubicin in dSML liposomes. Doxorubicin was encapsulated in dSML liposomes by the remote loading method. [3] A dried lipid film of given formulation was hydrated in ammonium sulfate solution (250 mM) at 60 °C under argon, sonicated for 5 min, exposed to three cycles of freeze-thaw (ethanol-dry ice bath for 2 min, then 60 °C bath for 1 min), then extruded through polycarbonate membranes of 200 nm, 100 nm, and 50 nm, sequentially. Free ammonium sulfate was removed by dialysis liposomes against 5% glucose 4 times in 24 hour. Liposomes with ammonium sulfate encapsulated were then incubated with doxorubicin solution in 5% glucose at 65 °C for 60 min, cooled to room temperature, mixed with Dowex resin, rotated for 60 min, and run through PD-10 column. The pH of purified dSML-Dox was adjusted to 6.5 by adding

a 10 mM histidine buffer. The diameter of dSML1-Dox was 113.5 nm, and 118.2 nm for dSML2a-Dox-F1, 188.3 nm for dSML2a-Dox-F2 according to the dynamic light scattering measurements (Zetasizer 3000, Malvern). The commercially available Doxil purchased from the UCSF hospital pharmacy has a diameter of 93.7 nm.

C26 Murine Adenocarcinoma Model.[4] All animal experiments were performed in compliance with the NIH guidelines for animal research under a protocol approved by the Committee on Animal Research at the University of California, San Francisco. For all chemotherapy experiments, on day 0, BALB/c mice were given subcutaneous injections of C-26 tumor cells (4×10^5 cells per mouse) in the right flank and were then randomized with 5 mice per group and numbered. On day 8 post-tumoring mice received 0.2 mL via a single tail vein injection of either phosphate buffered saline, doxorubicin at 10 mg/kg, Doxil™ at 15 mg/kg or doxorubicin at 15 mg/kg encapsulated in dSML/DSPC/DSPE-PEG2000/α-tocopherol (33.0/61.8/5.0/0.2, mole ratio) or in dSML/DSPE-PEG2000/α-tocopherol (94.8/5.0/0.2). Mice were weighed and tumor sizes were monitored daily during the experimental period. The tumor volume was estimated by measuring three orthogonal diameters (*a*, *b*, and *c*) with calipers; the volume was calculated as ($a \times b \times c$) $\times$ 0.5 cm^3. Tumors that were just palpable were defined as 1 mm $\times$ 1 mm $\times$ 1 mm. In each experiment the mice were monitored for up to 60 days post-inoculation or until one of the following conditions for euthanasia was met: 1) their body weight dropped below 15% of their initial mass; 2) their tumor was greater than 2.0 cm across in any dimension; 3) they became lethargic or sick and unable to feed; or 4) they were found dead. On day 60, all surviving mice were euthanized. The average tumor volume of each group was plotted over the time of observation (Figure 2).

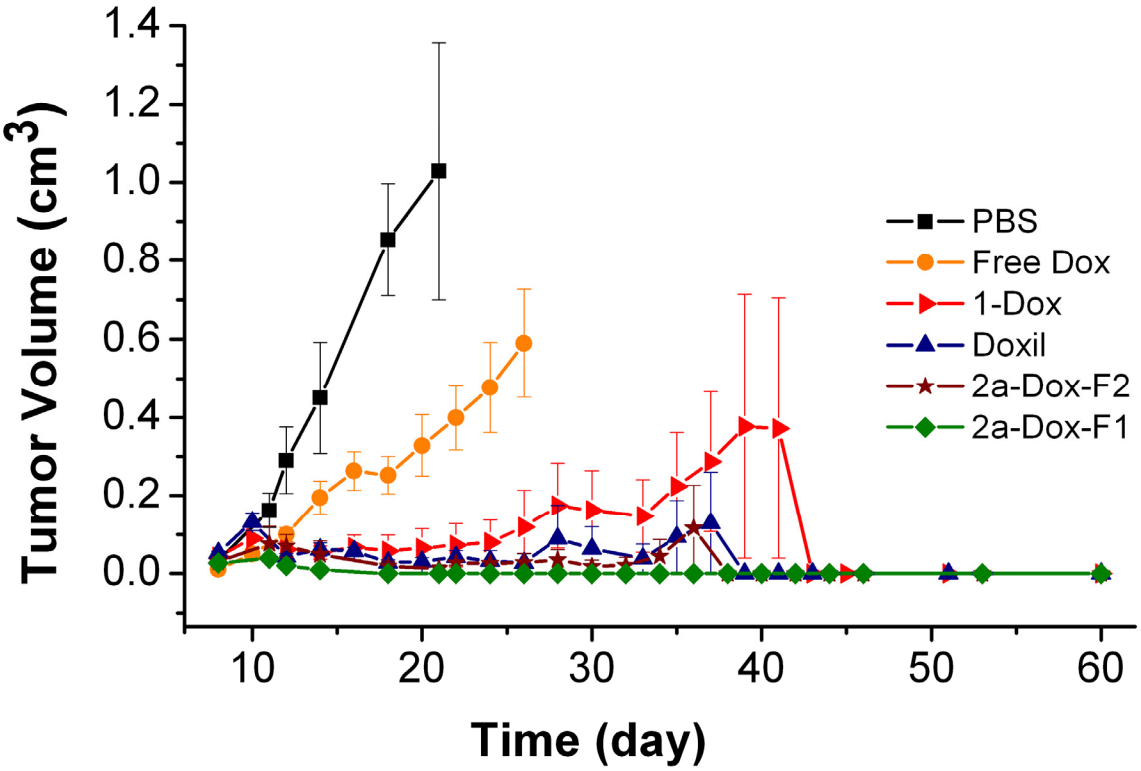

Figure 2. Tumor growth curves. 1-Dox: dSML1/DSPC/DSPE-PEG2000/α-tocopherol (33.0/61.8/5.0/0.2); 2a-Dox-F1:dSML2a /DSPC/DSPE-PEG2000/α-tocopherol (33.0/61.8/5.0/0.2); 2a-Dox-F2: dSML2a/DSPE-PEG2000/α-tocopherol (94.8/5.0/0.2)

Biodistribution Study of the Encapsulated Doxorubicin in Tumored Mice

Eight days after tumor inoculation, when the tumors were approximately 5 mm wide, mice (3 per group) were injected via the tail vain with either 15 mg/kg of doxorubicin as Doxil™ or encapsulated in dSML/DSPC/DSPE-PEG2000/α-tocopherol (33.0/61.8/5.0/0.2, mole ratio) in ca. 200 μL of PBS. Two control mice received 200 μL of PBS. Blood was collected from the mice via the orbital sinus 6, and 24, after dosing; at 48 h, the group was sacrificed for tissue collection. Prior to euthanasia, mice were anesthetized with a ketamine-xylazine-acepromazine cocktail by intraperitoneal injection. For all mice, blood was collected by heart puncture, and the whole tumors were dissected

and their weights recorded. Up to 300 mg of each tumor was placed in a 2 mL tube containing 1 mL of acidified alcohol (90% isopropanol / 0.075 M HCl) and zirconia beads. The tumor was homogenized by bead beating (Bead Beater, Biospec, Bartlesville, OK) at 5,000 rpm. The blood was allowed to coagulate at 4 ºC and then centrifuged for 10 min at 14,000 rpm. The serum (upper phase) was collected and its volume recorded. Up to 400 μL of serum was transferred to a 2 mL tube containing 1 mL of acidified alcohol. The homogenized tumor samples and the sera were stored at 4 °C overnight to extract the drug. The samples were then centrifuged at 14,000 rpm for 5 min at 4 ºC. An aliquot (400 μL) of each supernatant was transferred to tubes containing 1.6 mL of acidified alcohol. Fluorescence emission from the drug was measured (Ex: 490 nm, Em: 590 nm) using a Spex Fluorolog photon counting instrument (Model FL1/2, Jobin Yvon, Edison, NJ) equipped with a 150 W xenon light source. A calibration curve was prepared for doxorubicin using concentrations from 0.04 to 5 μg/mL in acidified alcohol. To subtract the background fluorescence from each tissue type, a calibration curve was prepared using serial dilutions of the tumor and sera extracts of the control mice.

References:

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