Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information For:

Sterol-modified PEG lipids: Alteration of bilayer anchoring moiety has an unexpected effect on liposome circulation

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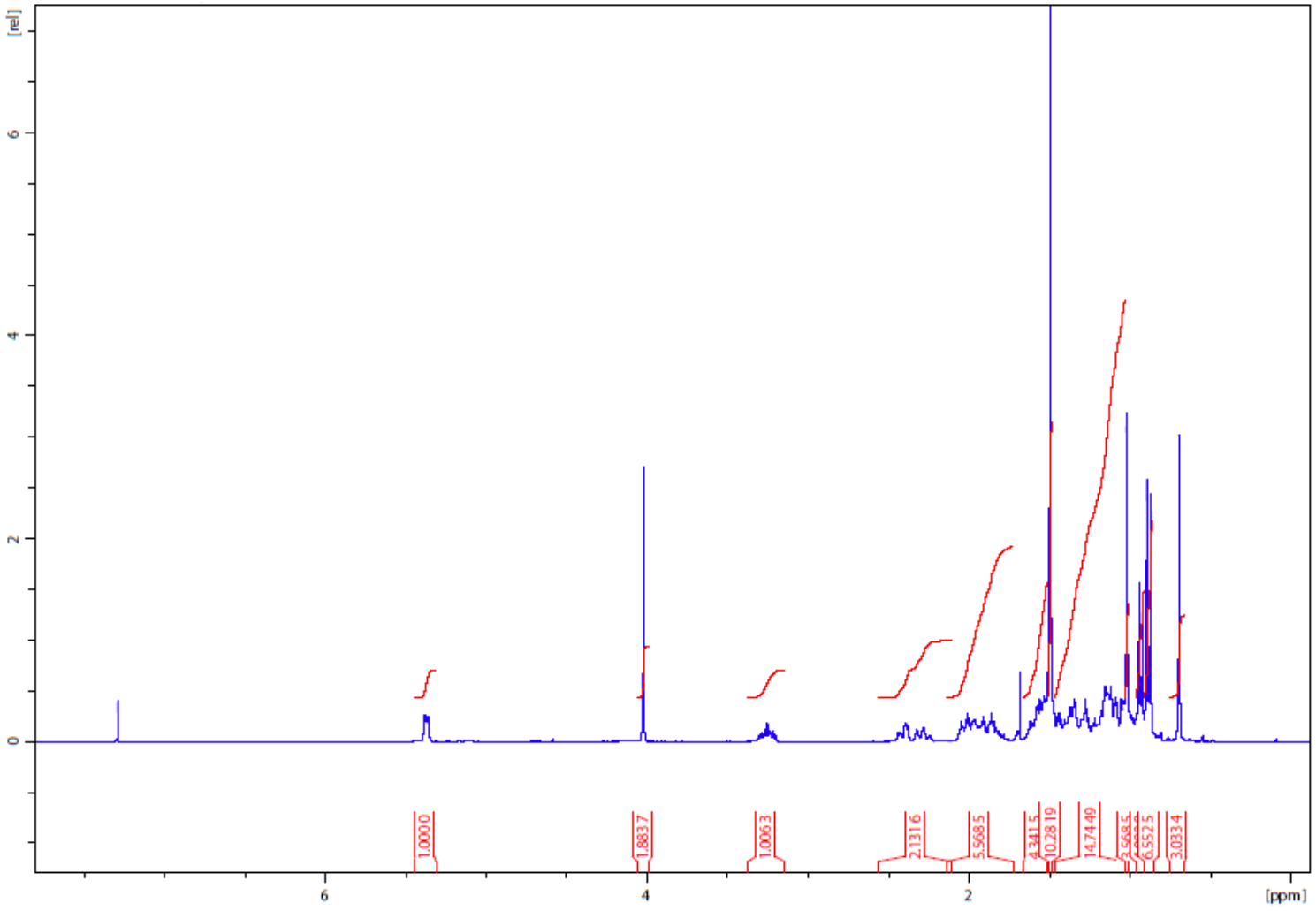
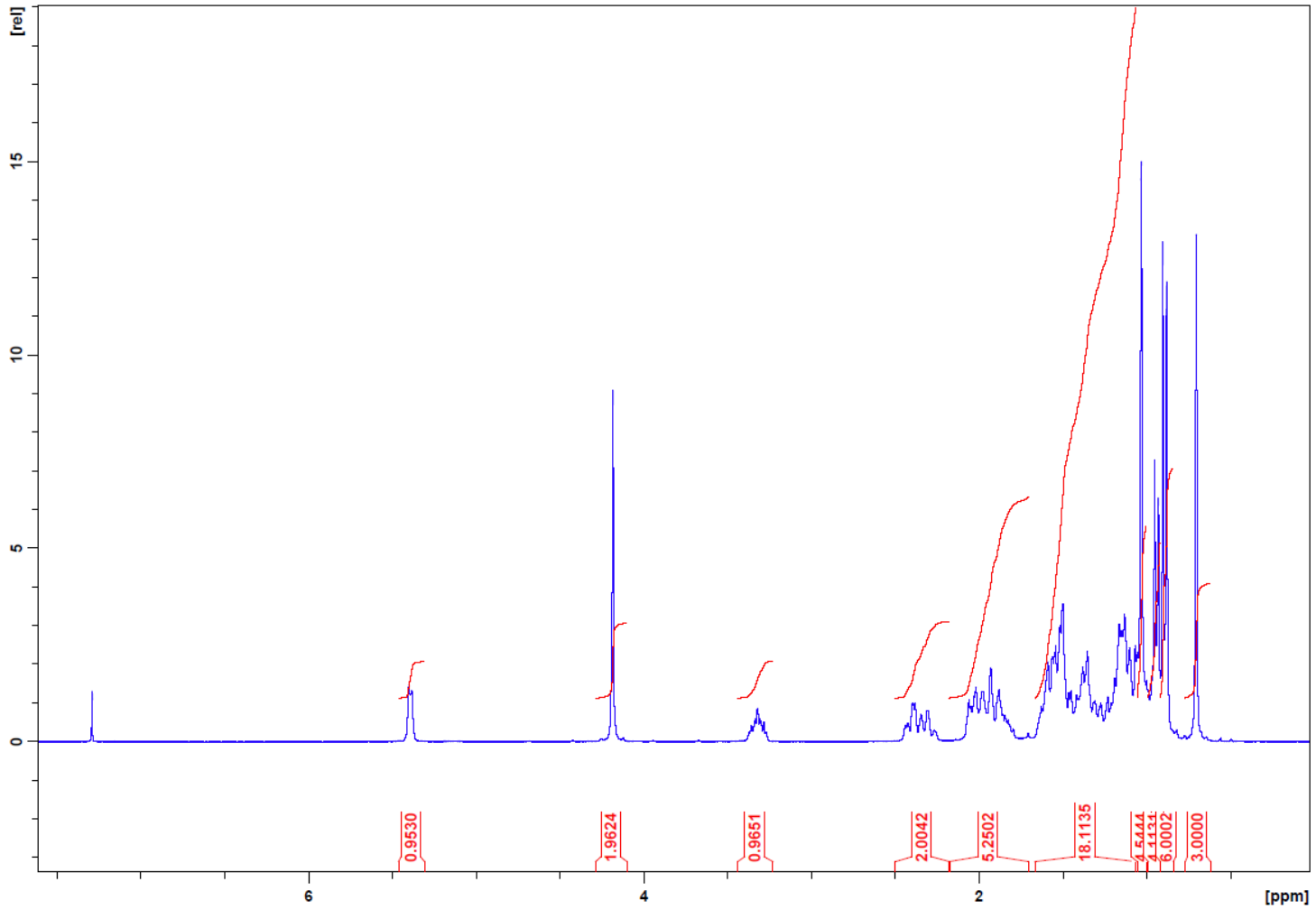
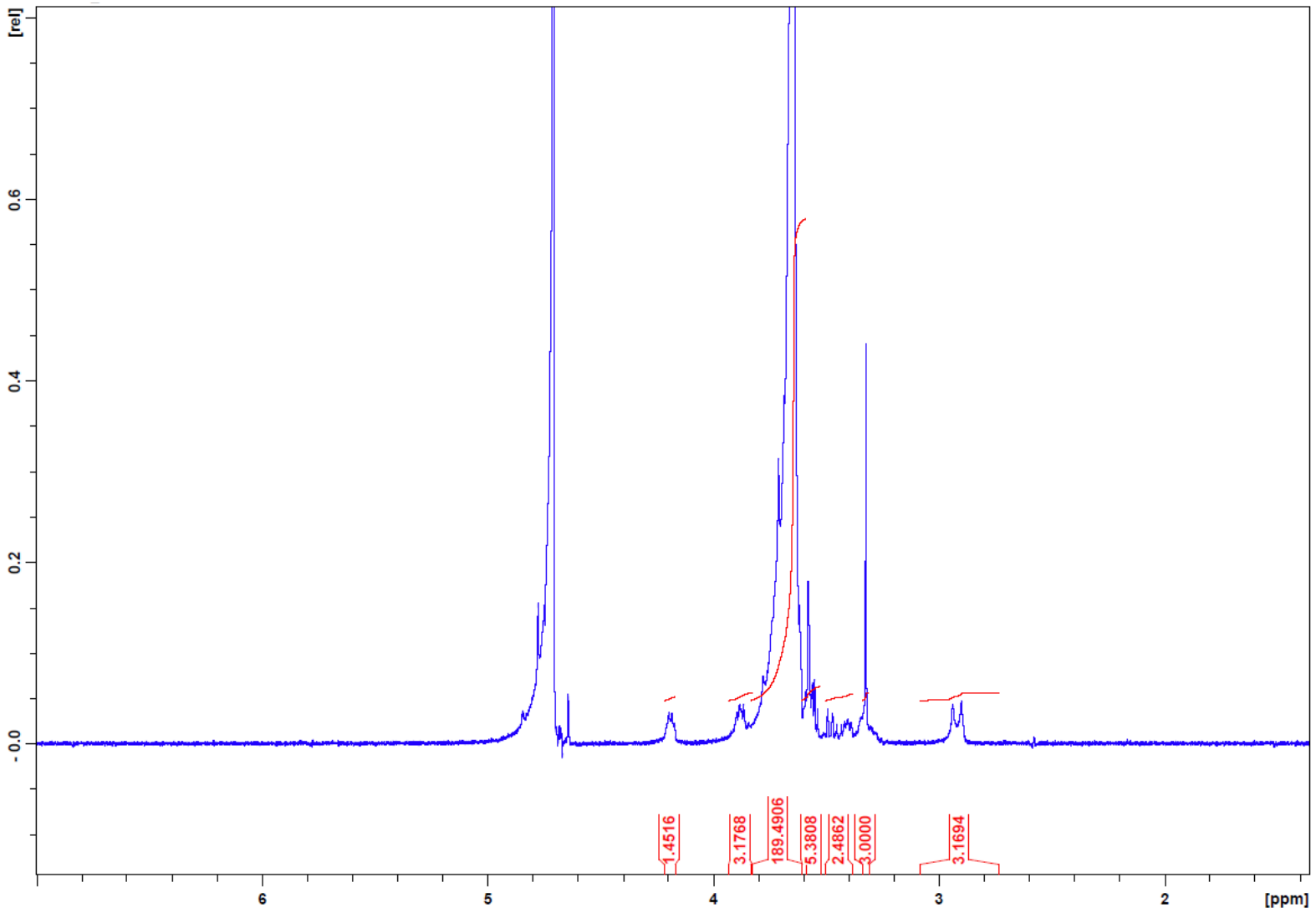
1.1 Figure S1: Compound 1 ^{1}H NMR



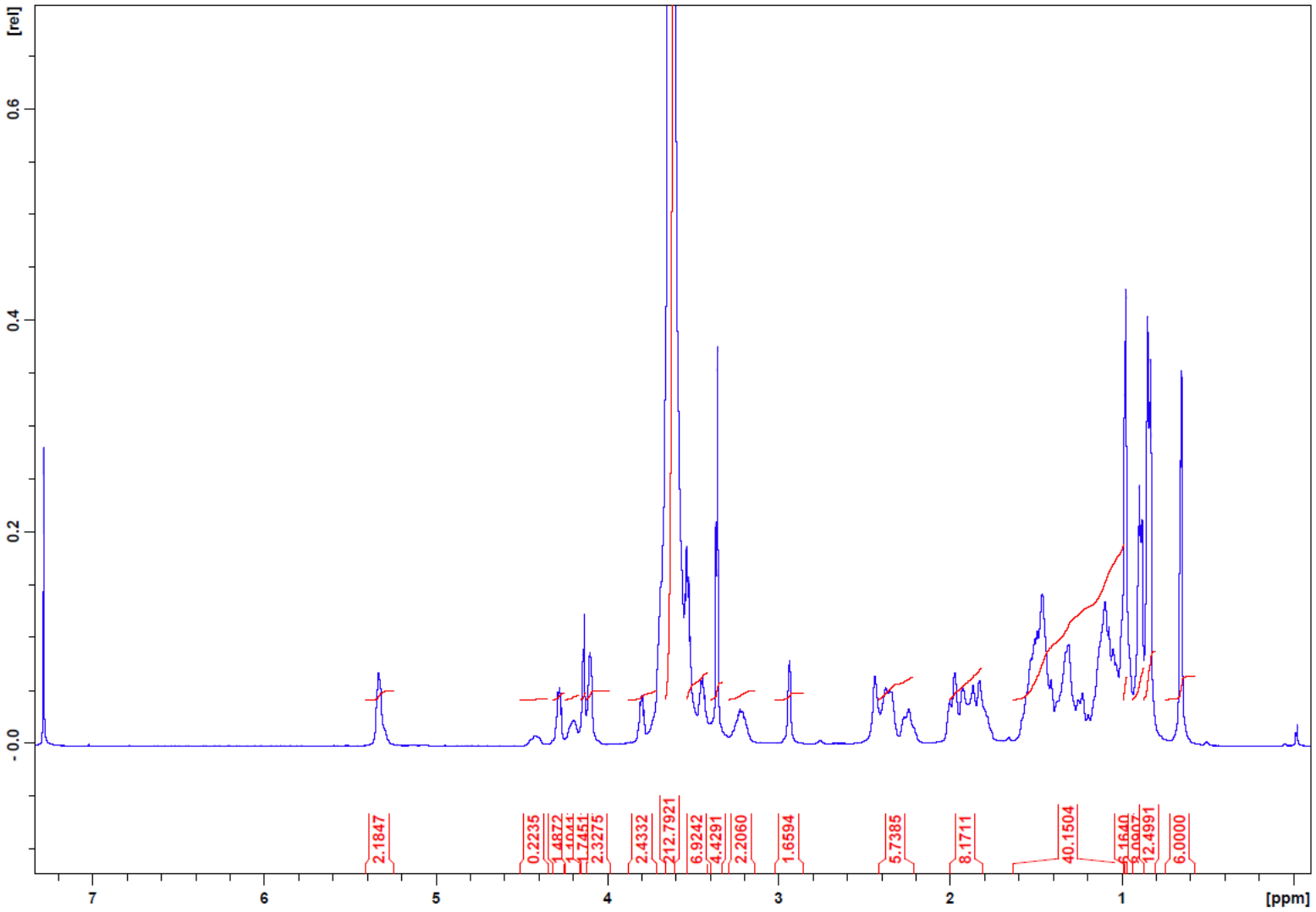
1.2 Figure S2: Compound 2 ^{1}H NMR



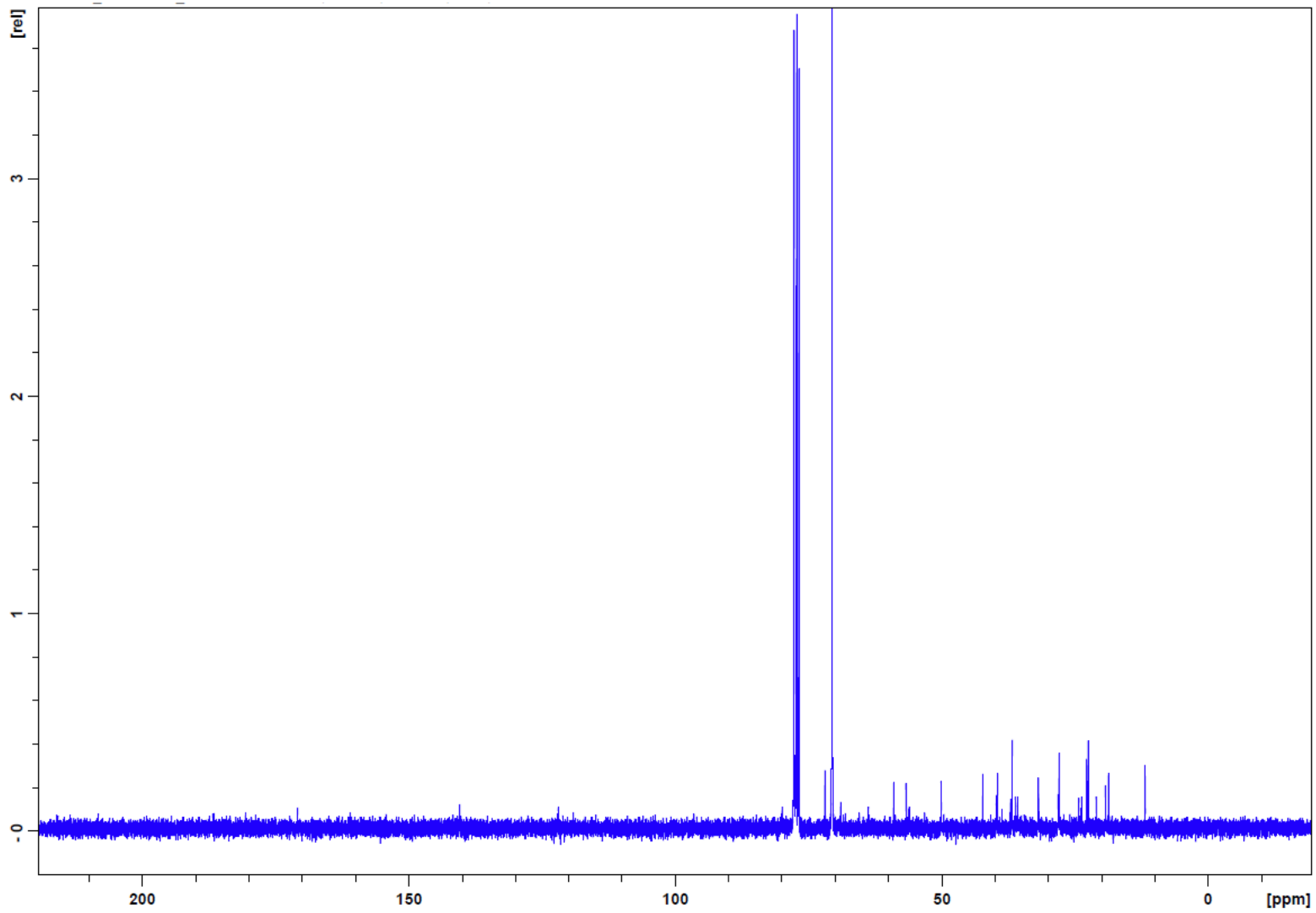
1.3 Figure S3: Compound 3 ^{1}H NMR



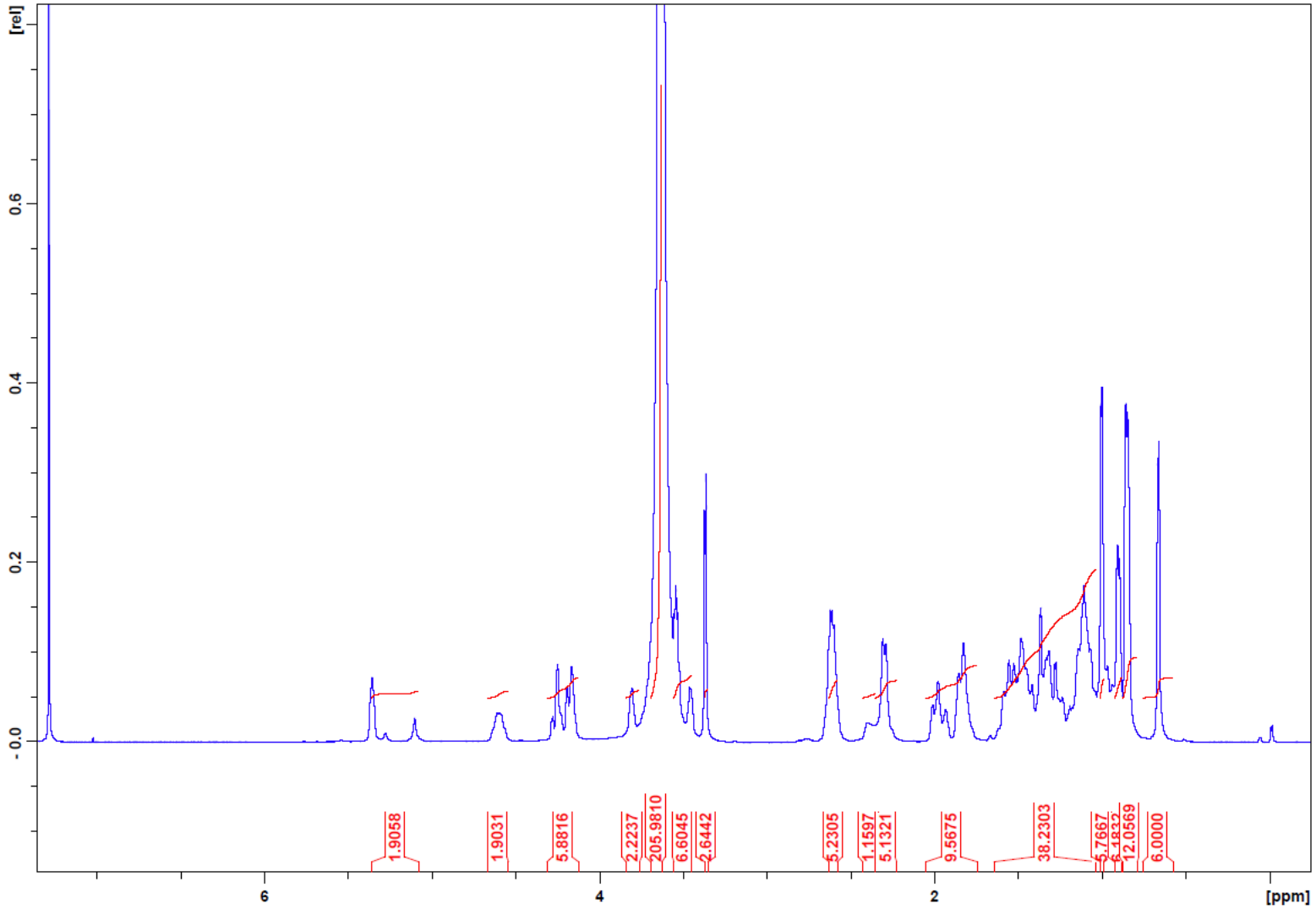
1.4 Figure S4: Compound 4-DiCHOL-PEG ^{1}H NMR



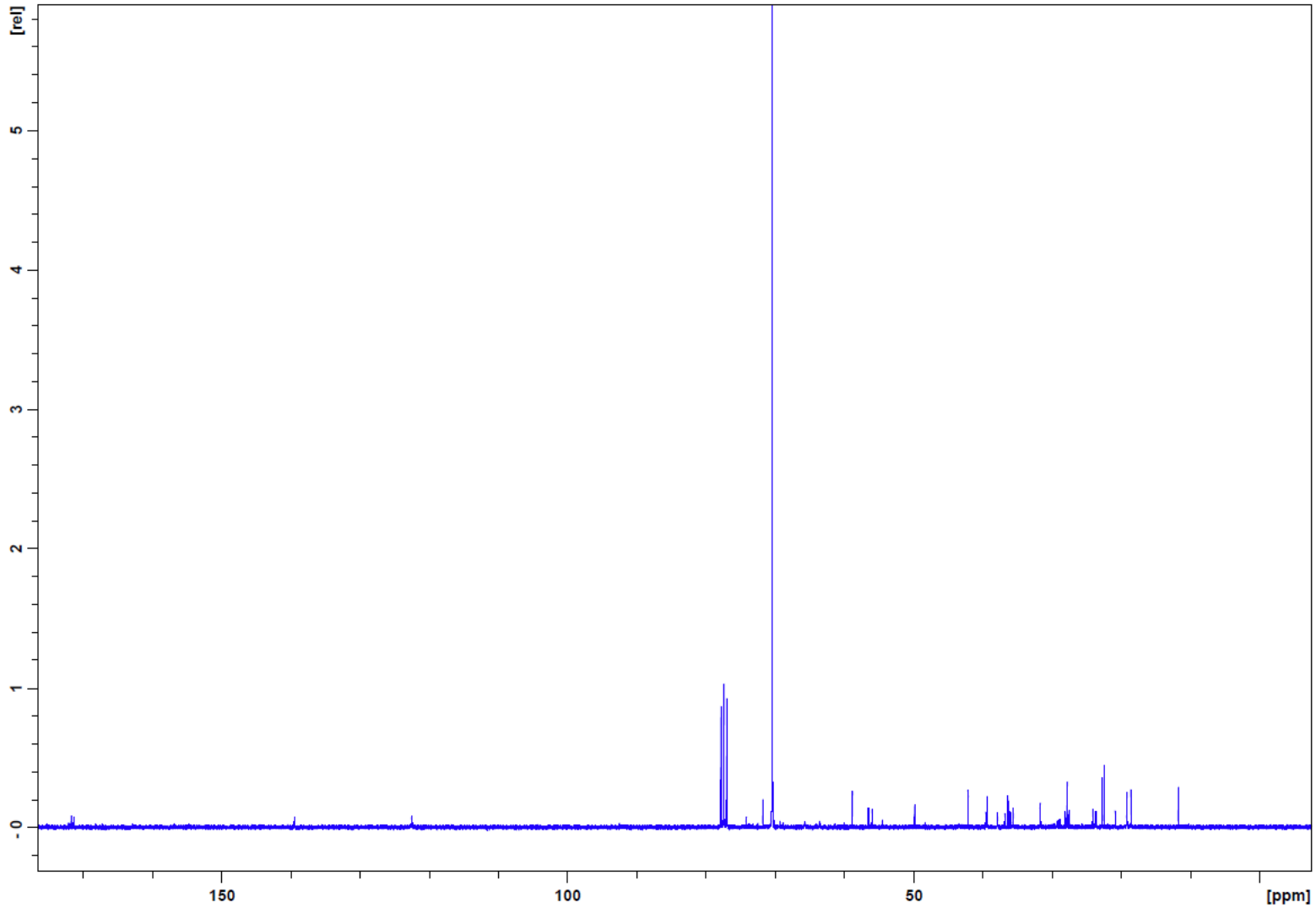
1.5 Figure S5: Compound 4-DiCHOL-PEG ^{13}C NMR



1.6 Figure S6: Compound 6-DiCHEMS-PEG ^{1}H NMR



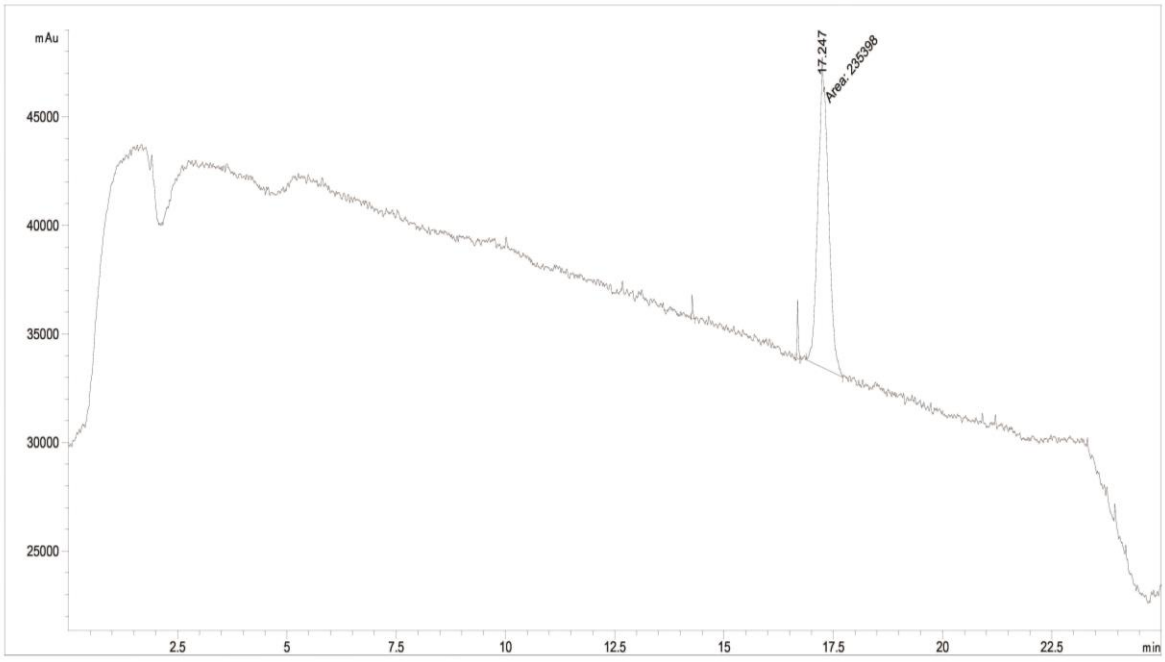
1.7 Figure S7: Compound 6-DiCHEMS-PEG ^{13}C NMR



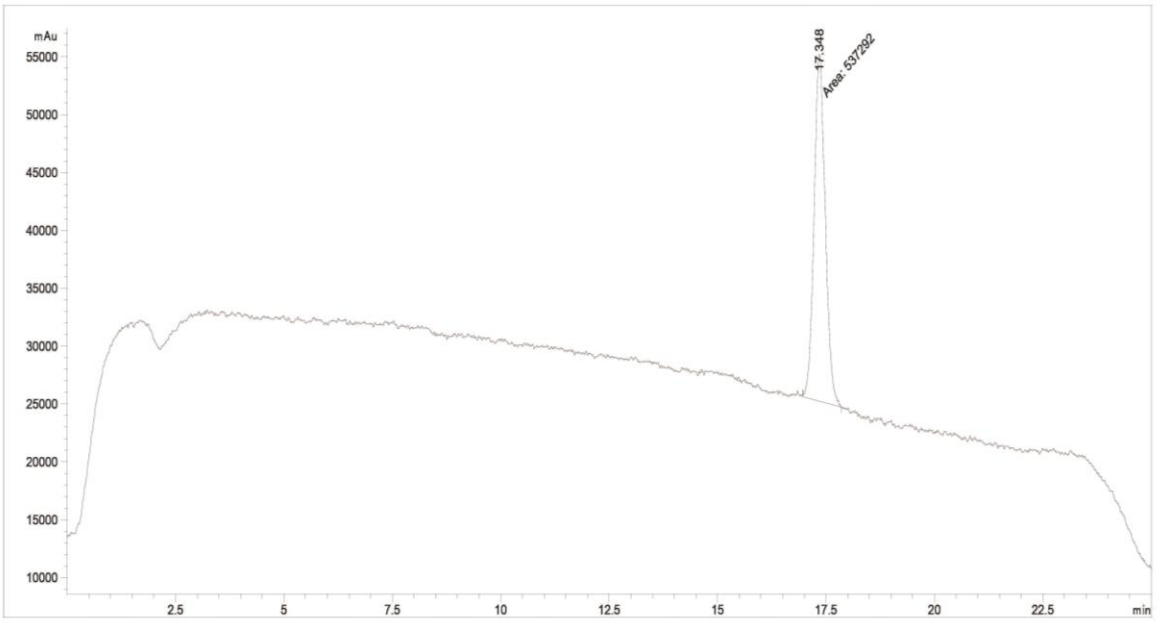
1.8 Figure S8: Compound 4,6 Thin-layer chromatography (TLC)

Left: Compound 6; Right: Compound 4
10 % MeOH in $CHCl_3$, Cerium molybdate stain

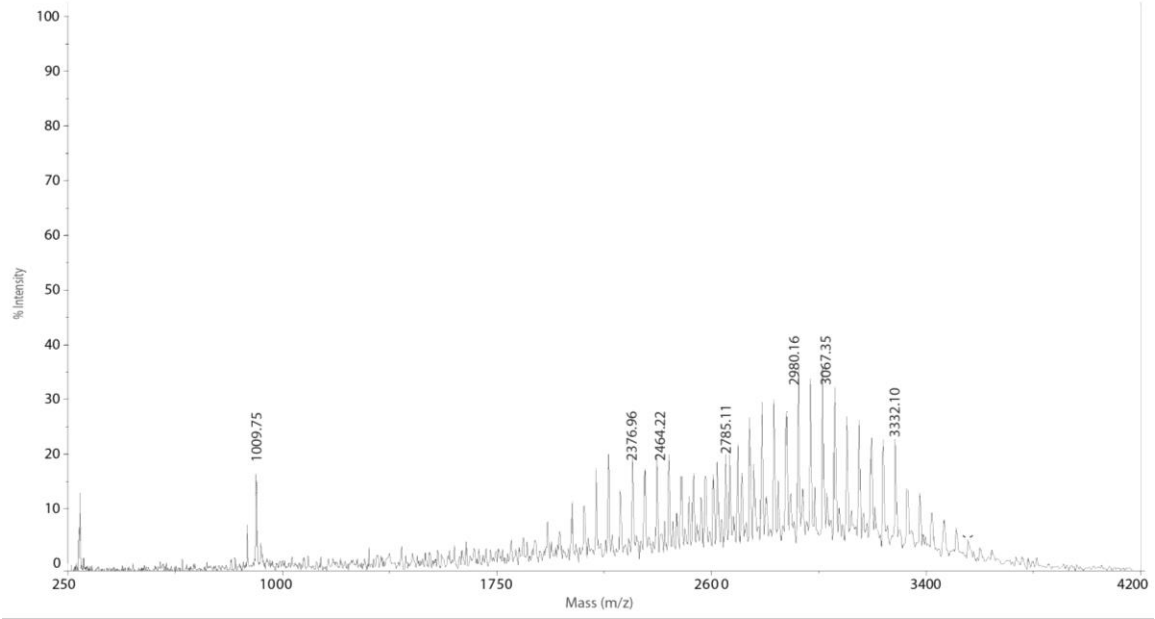
1.9 Figure S9: Compound 4 HPLC



1.10 Figure S10: Compound 6 HPLC

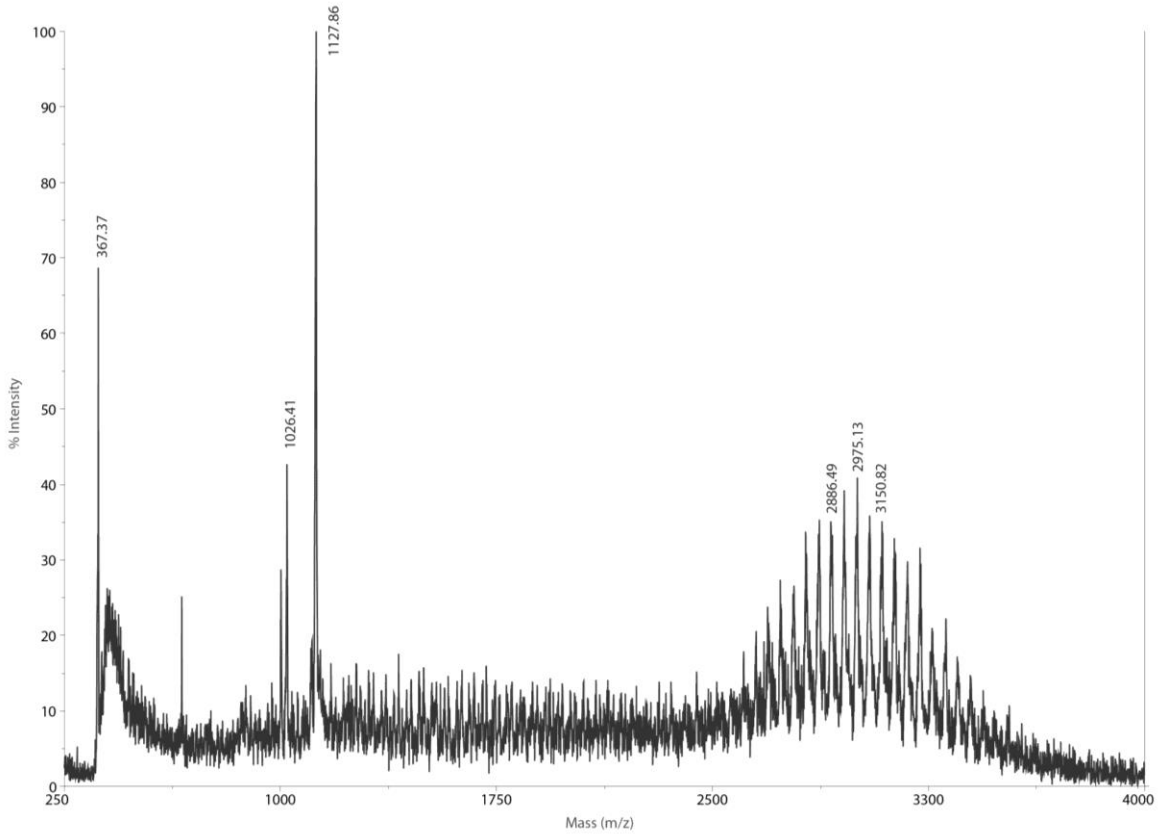


1.11 Figure S11: Compound 4 MALDI



MALDI Fragmentation by Bruker Daltonics microflex using para-nitroaniline as a matrix: Loss of single cholesterol (2376.96, 2464.22), loss of PEG (1009.75). Sample is a single spot as indicated by TLC using 9:1 $CHCl_3$: MeOH and stained by Cerium molybdate and I_2.

1.12 Figure S12: Compound 6 MALDI



MALDI Fragmentation by Bruker Daltonics microTOF using para-nitroaniline as a matrix: Single PEG (1127.86), loss of PEG (1026.41), cholesterol (367.37). Sample is a single spot as indicated by TLC using 9:1 $CHCl_3$: MeOH and stained by Cerium molybdate and I_2.

1.13 Figure S13: Liposome fluorescence anisotropy

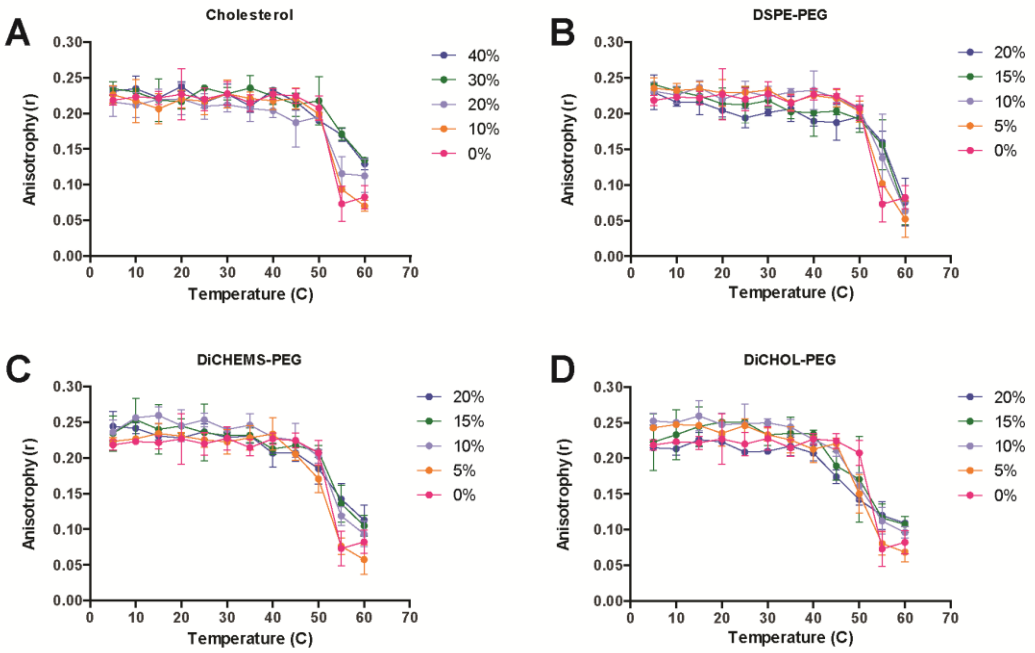


Fig S13 DPH fluorescence anisotropy of DSPC vesicles with increasing amounts of additives. A) Cholesterol, B) DSPE-PEG, C) DiCHEMS-PEG, and D) DiCHOL-PEG. DSPC liposomes containing sterol-anchored PEG exhibit similar anisotropy traces to liposomes with cholesterol. The mole percent of unmodified cholesterol is twice that of the mole percent of DiCHEMS-PEG or DiCHOL-PEG to have an equivalent amount of cholesterol in the bilayer. Errors bars represent mean ± SD.

1.14 Figure S14: Liposome plasma binding

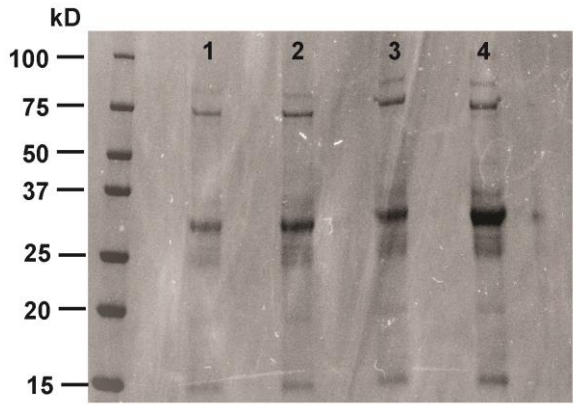


Fig S14 Liposome plasma binding. 1, DSPC:Cholesterol:DSPE-PEG (85:10:05); 2, DSPC:DiCHOL-PEG (95:05); 3, DSPC:Cholesterol:DSPE-PEG (55:40:05); 4, DSPC:DiCHEMS-PEG (95:05). Normalized binding of liposomes to plasma proteins show only minor differences in plasma adhesion with the DSPC:Cholesterol:DSPE-PEG (85:10:05) displaying the least plasma adhesion. Protein content normalized to liposome concentration.

1.15 Figure S15: ApoE3 cellular uptake

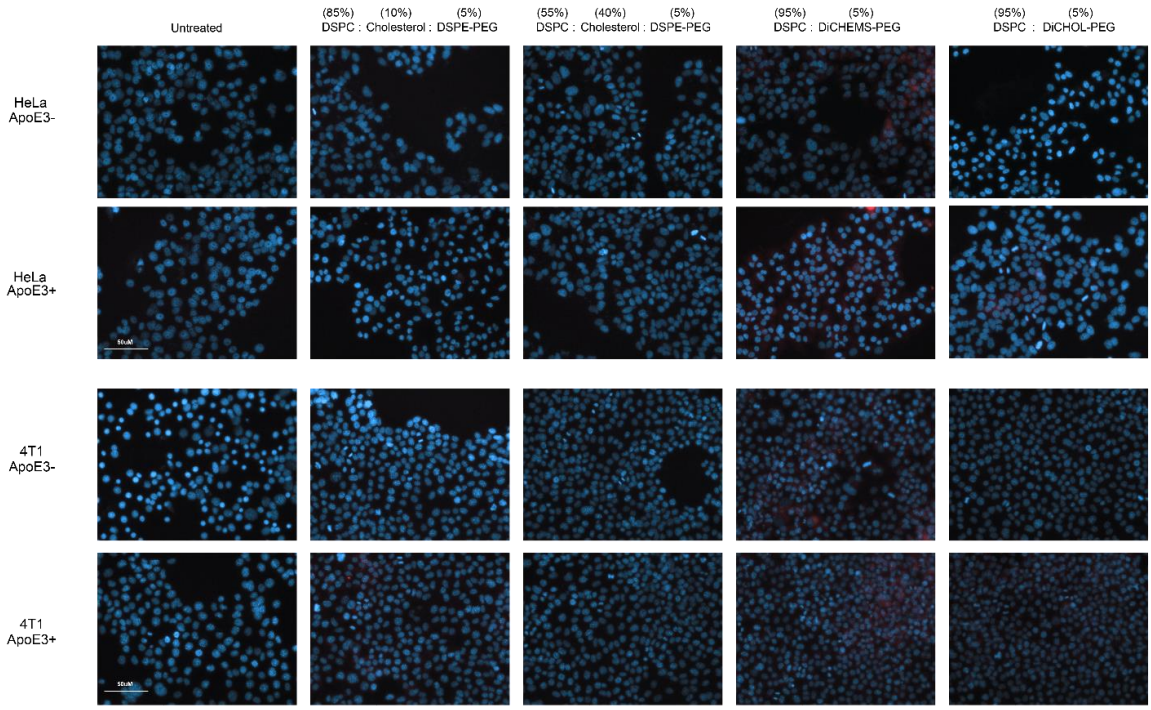


Fig S15 ApoE3 cellular uptake. Liposomal uptake in HeLa (Top) and 4T1 (Bottom). Liposomes containing DiCHEMS-PEG displayed the greatest levels of cellular uptake. Cell nuclei stained in DAPI (blue); liposomes containing DiR (red).

2 Materials and methods

2.1 Instrumentation

NMR measurements were performed on a Bruker (Billerica, MA) 300MHz Advance system and analyzed using TopSpin software. MALDI-TOF measurements were performed on a Bruker Daltonics MicroFlex LT system (Billerica, MA). Elemental analysis was performed by the Microanalytical Laboratory at the University of California Berkeley using an ICP Optima 7000 DV instrument. Particle size and zeta measurements were carried out using a Nano-ZS Dynamic Light Scattering Instrument from Malvern (Westborough, MA). Fluorescence spectroscopy was measured on a FluoroLog-3 spectrofluorimeter (Horiba Jobin Yvon) equipped with a temperature- controlled stage (LFI-3751) or a SpectraMax M5 Micoplate Reader (Sunnyvale, CA) and data was collected through FluorEssence or SoftMax pro software, respectively. Cell imaging was completed on a Zeiss widefield microscope with images processed in the accompanying Zen image analysis program.

2.2 Materials

1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N- [methoxy(polyethylene glycol)-2000] (DSPE-PEG) were obtained from Avanti Polar Lipids (Alabaster, AL). tert-Butylbromoacetate and cell culture materials were purchased from Fisher Scientific (Hanover Park, IL). [Methoxy(polyethylene glycol)-2000]- Nitrophenyl Carbonate (mPEG-NPC) was purchased from Laysan Bio Inc (Arab, AL). 1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindotricarbocyanine iodide (DiR) was purchase from Biotium (Freemont, CA)

PD-10 sephadex columns were obtained from GE Healthcare (San Francisco, CA). Heat-inactivated fetal calf serum was purchased from General Electric (Logan, UT). Mouse plasma was purchased from Pel Freez Biologicals (Rogers, Arkansas). All other reagents were purchased from Sigma–Aldrich (St. Louis, MO).

2.3 Synthesis

Disterol-PEG derivatives were synthesized from acylation of an amino-propanediol backbone previously reported[1] and as outlined in Scheme 1.

2.3.1 Synthesis of compound 1

Compound **1** and **2** were synthesized as done previously.[2] Cholesterol (7 mmol), dissolved in anhydrous THF (12 mL) was treated with 60 % NaH dispersion in mineral oil (15 mmol) and tert-butylbromoacetate (18 mmol) was added dropwise at 0 °C. The reaction was quenched by addition of methanol (3.0 mL) at 0 °C after 24 h under inert atmosphere and stirred for 30 min before concentration by rotary evaporation. The resulting solid was dissolved in chloroform and washed three times with water. Organic phases were combined, dried over anhydrous sodium sulfate and filtered. The filtrate was purified by silica column chromatography with 10 % ethyl acetate in n-hexane.

2.3.2 Synthesis of compound 2

Compound 2 (2 mmol) was first dissolved in chloroform (2 mL) with formic acid slowly added while stirring (3.0 mL). After stirring overnight at room temperature, the reaction mixture was concentrated by rotary evaporation. 15 mL of chloroform was added 3X and concentrated to remove residual formic acid which yielded a pure product.

2.3.3 **Synthesis of compound 3**

3-methylamino-1,2-propanediol (0.8 mmol) was dissolved in dimethylformamide (DMF) (1 mL) and stirred under inert atmosphere. mPEG-NPC (0.4 mmol) was dissolved in DMF (3 mL) and added dropwise. The reaction was allowed to stir for 30 m before the dropwise addition of DIPEA (0.5 mmol). After overnight stirring the mixture was dialyzed against water for 24 h with four changes in a 2K MWCO tube. The resulting sample was lyophilized and recrystallized with diethylether to yield a pure product.

2.3.4 **Synthesis of compound 3**

Sterol addition to **3** was completed as done previously.[3] **3** (0.5 mmol) was solubilized in 5 mL chloroform. **2** (1.5 mmol) was added followed by dimethylaminopyridine (DMAP) (0.2 mmol) and 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (4 mmol). The reaction proceeded overnight at 40 ºC then dialyzed in a 2K MWCO tube in chloroform:methanol (50:50) with four solvent changes into 100 % methanol over 24 h. Sample was concentrated by rotary evaporation, dissolved in water and dialyzed over 24 h at 4 ºC. Lyophilization produced an off-white solid that was purified by silica column chromatography in 10 % ethylacetate in n-hexane with the product eluted in 5 % methanol in chloroform.

2.3.5 **Synthesis of compound 5**

Compound **5** was synthesized in the same manner as **3** using 2-methyl-2-amino-1,3-propanediol as the template.

2.3.6 **Synthesis of compound 6**

Sterol addition to **5** was completed in the same manner as **3** using commercial available cholesteryl hemisuccinate. Following lyophilization the compound did not require further purification.

2.4 Chemical Characterization

Compound 1. Yield: 30%. ^{1}H NMR ($CDCl_3$): δ 0.70 (s, 3H), 0.87 (d, 6H), 0.93 (d, 3H), 1.02 (s, 3H), 1.02-1.63 (m, 21H), 1.49 (s, 9H), 1.94 (m, 5H), 2.35 (m, 2H), 3.21 (m, 1H), 4.03 (s, 2H), 5.37 (d, 1H)

Compound 2. Yield: Quantitative. ^{1}H NMR ($CDCl_3$): δ 0.70 (s, 3H), 0.87 (d, 6H), 0.93 (d, 3H), 1.02 (s, 3H), 1.02-1.63 (m, 21H), 1.94 (m, 5H), 2.35 (m, 2H), 3.31 (m, 1H), 4.18 (s, 2H), 5.37 (d, 1H)

Compound 4. Yield 70%. ^{1}H NMR (D_2O): δ 2.92 (s,2H), 3.32 (s, 3H), 3.46 (m, 2H), 3.58 (m, 6H), 3.60 (s, 190H), 3.89 (t, 3H), 4.19 (m, 1H)

Compound 5: Yield: 50%. ^{1}H NMR ($CDCl_3$): δ 0.68 (s, 6H), 0.85 (d, 12H), .86 (d, 6H), .98 (s, 6H), 1.01-1.6 (m, 40H), 1.89 (m, 6H), 2.37 (m, 4H), 2.96 (s, 3H), 3.23 (m, 2H), 3.38 (s, 3H), 3.45 (m, 6H), 3.63 (s, 210H), 3.81 (t, 3H), 4.06 (s, 4H), 4.13 (m, 2H), 4.19 (m,1H), 4.26 (m, 2H), 4.42 (m,1 H), 5.34 (s, 2H). ^{13}C NMR ($CDCl_3$): 11.9, 18.7, 19.3, 21.4, 22.6, 24.1, 28.1, 31.9, 35.6, 36.6, 39.6, 42.2, 50.0, 56.5, 58.9, 70.9, 72.1, 122.1, 140.5, 170.6. MALDI-TOF, MW_{AVG}= 3.0 kDa, PDI=1.03. Elemental analysis for [C154H281NO53]: C, 61.8; H, 9.5; N, 0.5; Found: C, 60.3; H, 9.1; N, 0.3. Note: Carbon values are believed to be lower due to hydroscopic nature of PEG.

Compound 7: Yield: 70%. ^{1}H NMR ($CDCl_3$): δ 0.67 (s, 6H), 0.85 (d, 12H), 0.90 (d, 6H), 1.0 (d, 6H), 1.03-1.65 (m, 40H), 1.61-1.93 (m, 10H), 2.31 (d, 4H), 2.39 (m, 1H), 2.62 (m,

4H), 3.24 (s, 3H), 3.50 (m, 6H), 3.64 (s, 205H), 3.81 (t, 3H), 4.20 (m, 6H), 4.60 (m, 2H), 5.23 (m, 2H). ^{13}C NMR ($CDCl_3$): 11.7, 18.8, 19.2, 21.0, 22.7, 23.8, 27.9, 29.2, 31.7, 35.9, 36.4, 38.2, 39.3, 42.0, 50.0, 56.5, 58.8, 70.4, 71.9, 74.1,122.6, 139.7, 171.6. MALDI-TOF, MW_{AVG}= 3.1 kDa, PDI= 1.01. Elemental analysis for [C158H289NO54]: C, 61.9; H, 9.5; N, 0.5; Found: C, 61.6; H, 9.4; N, 0.5

2.5 Elemental Analysis

A total of 5–10 mg of dry lipid was submitted to Microanalytical Laboratory at the University of California Berkeley for elemental analysis determinations using an ICP Optima 7000 DV instrument.

2.6 HPLC Analysis

High-performance liquid chromatography (HPLC) was performed on an 1100 HPLC from Agilent (Santa Clara, CA) with an PL-ELS 2100 evaporative light scattering detector (ELSD) from Polymer Laboratories, and a Phenomenex Kinetex 5 µm, C8 (4.6 × 150 mm, i.d., 5 µm particle size) column equipped with Phenomenex C-8, AJO-4290 guard column. Samples (20 µL) were detected using a mobile phase consisted of 0.1% TFA in water (A) and 0.1 % TFA in methanol (B) using a linear gradient of 70% B to 100% B over 15 min and flow rate of 1.0 mL/min, a column temperature of 30 °C over 25 min. Nitrogen was used as nebulization gas at a rate of 1.6 L/min with a nebulizer temperature of 50 °C and an evaporation temperature of 90 °C.

2.7 Carboxyfluorescein (CF) release

The carboxyfluorescein encapsulating protocol was completed as done previously.[3] A lipid film (5 µmol) was rehydrated in 10 mM Tris, 100 mM

carboxyfluorescein (CF), pH 7.4 to a final concentration of 10 mM lipid. Each sample was heated at 60 ºC for 1 h and subsequently sonicated at 60 ºC for 10 min. Liposomes were then extruded 11-13 times through a 100 nm polycarbonate membrane. Free CF was removed by size exclusion chromatography with a PD-10 sephadex column. Liposomes were incubated in 200 µL of 105 mM NaCl, 10 mM HEPES pH 7.4 containing 30 % (v/v) fetal bovine serum, 0.02 % sodium azide at 37 ºC for one week. Leakage was measured on a SpectraMax M5 microplate reader with excitation at 485 nm and emission at 518 nm. Percent leakage values were obtained by normalization to the fluorescence of the samples after lysis of liposomes using 0.1 % Triton. Leakage measurements were run in triplicate.

2.8 Fluorescent anisotropy

Anisotropy was completed as done previously.[4] Lipids were dissolved in chloroform, dried to form a thin film, and placed under high vacuum overnight. The films were hydrated with 140 mM NaCl, 10 mM HEPES at 60 ºC and vortexed to obtain a lipid concentration of 5 mM. Each sample was heated at 60 ºC for 1 h and subsequently sonicated at 60 ºC for 10 min. Liposomes were then extruded 11-13 times through a 100 nm polycarbonate membrane. Liposomes were diluted 8-fold with PBS and 6 µl of 1,6-diphenyl-1,3,5-hexatriene in tetrahydrofuran (0.15 mg/ml) was added to the sample. The mixture was incubated at 65 °C for 1 h to allow DPH to integrate into the bilayer. Liposomes were heated or cooled in 5 °C increments from 5–65 °C, with a 3–10 m equilibration between transitions and anisotropy was detected by DPH fluorescence (Ex. 350 nm, Em. 430 nm).

2.9 Liposomes size and charge

Zeta and size measurements were obtained with 200 µM liposomes in 140 mM NaCl, 10 mM HEPES using a Malvern Nanosizer. All samples were run in triplicate.

2.10 Transmission electron microscopy.

TEM was performed as done previously.[1] Liposomes were buffer exchanged into 140 mM ammonium carbonate, 10 mM HEPES, pH 8. A 2.0 µL drop of liposomes were adsorbed for 60 s on a glow-discharged carbon-coated copper grid (Ted Pella, Redding, CA) and water was wicked off. Then, 2 µL of a 1 % uranyl acetate negative stain solution were added and left to stain for 60 s and wicked off. The grid was then washed with deionized water three times and the water was removed by wicking. Grids were imaged with a FEI Tecnai T12 TEM (FEI company, Hillsboro, OR) at 120kV. Data were acquired with a 4 x 4 Gatan UltraScan CCD camera (Gatan, Pleasanton, CA).

2.11 Albumin labeling

A 10 mg/mL solution of bovine serum albumin with 0.4 mg/mL fluorescein isocyanate in 100 mM carbonate buffer, pH 8 was allowed to stir for 3 h at room temperature. Albumin was separated from unreacted fluorescein by size-exclusion chromatography on a PD-10 column to yield labeled albumin. The resulting albumin had 5-10 moles fluorescein per mole albumin.

2.12 Protein binding

Liposomes were mixed at 5 mM with sterile-filtered fluorescently labeled albumin (10 mg/mL) or mouse plasma in 140 mM NaCl, 10 mM HEPES, 0.5 mM EDTA, 1 mM DTT for 5 h at 37 °C. Samples were centrifuged at 120,000 x*g* in a Beckman TL-100 ultracentrifuge to pellet the liposomes and any bound protein. After the supernatant was

removed, liposomes were resuspended and analyzed for associated proteins. Samples were run in triplicate and normalized based on liposome concentration.

2.13 Cell uptake

Cell uptake was performed as done previously.[5] HeLa or 4T1 cells were plated onto 12 well plates and grown to 70-80 % confluency. Media was removed and cells were washed 3X with PBS before being incubated in reduced serum media containing 100 µmol liposomes and 1 ug/mL ApoE3 for 5 h at 37 ºC with gentle rocking. Following the incubation, the media was removed and the cells were washed 3X with PBS then lysed using RIP-A at 4 ºC and spun down at 12,000 x*g* for 20 m. The supernatant was removed and uptake was determined by liposome fluorescence compared to the total initial concentration added initially. For imaging, following the 5 h incubation, cells were washed 3X with PBS then fixed in 2 % PFA for 10 m. Cells were washed 3X with PBS, permeabilized with 0.2 % triton-X100 for 5 m and washed again 3X with PBS. Cell nuclei were stained following a 5 m incubation with DAPI then washed prior to imaging on a Zeiss widefield microscope.

2.13.1 Pharmacokinetic studies

Mouse pharmacokinetics was conducted at Murigenics (California) in male CD-1 mice of 20-30 g following standard procedures. Mice were injected with 60 µmol/kg of lipid via tail vein with serum collected 24 h later. Plasma was shipped under dry ice and analyzed for fluorescence compared to a standard curve of liposomes with spiked amounts of plasma. Each liposome sample was injected into groups of three mice.

3 References

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