

Electronic Supplementary Information (ESI)

Simple and Rapid Quantification of Phospholipids for Supramolecular
Membrane Transport Assays

Robert Hein, Can Berk Uzundal and Andreas Hennig*

Department of Life Sciences and Chemistry, Jacobs University Bremen, Campus Ring 1,
28759 Bremen (Germany), Email: a.hennig@jacobs-university.de

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Abbreviations

ANTS: 8-aminonaphthalene-1,3,6-trisulfonic acid; BE: berberine chloride; CB: cucurbituril; CB7: cucurbit[7]uril; CB8: cucurbit[8]uril; CF: 5(6)-carboxyfluorescein; CX4: *p*-sulfonatocalix[4]arene; DPPC: 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DPX: *p*-xylene-bis-pyridinium; EYPC: egg yolk phosphatidylcholine; HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPTS: 8-hydroxypyrene-1,3,6-trisulfonic acid; LCG: lucigenin; LOQ: limit of quantification; MDAP: *N,N'*-dimethyl-2,7-diazapyrenium; NMR: nuclear magnetic resonance; POPC: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPS: 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine; SEC: size exclusion chromatography; SNR: signal-to-noise ratio; TMSP: 3-(trimethylsilyl)propionic-2,2,3,3- d_4 acid; TRIS: tris(hydroxymethyl)-aminomethane.

Materials and Methods

Methanol- d_4 was from Deutero GmbH (Kastellaun, Germany). Lipids were from Avanti Polar Lipids, Inc. and received as stock solutions in chloroform or as solid (DPPC), except EYPC, which was from Lipoid AG (Steinhausen, Switzerland). BE and DPX were from Sigma-Aldrich, CF and HPTS were from Fluka, and ANTS was from Molecular Probes. All other chemicals were obtained in highest available purity from Sigma-Aldrich or Fluka and used as received. NAP-25 columns were purchased from GE Healthcare. CB7, CB8, CX4 and MDAP were synthesized according to the literature.¹⁻⁵

Absorption Spectroscopy. Absorption measurements were performed in quartz glass cuvettes with a Varian Cary 4000.

Dynamic Light Scattering. Dynamic light scattering experiments were carried out on a Malvern Instruments DTS Nano 2000 Zeta-Sizer. 5 μ L liposome solutions were diluted to 1 mL with the outside buffer. The analysis was performed in disposable plastic cuvettes under standard settings.

NMR Spectroscopy. ^1H NMR measurements were performed at ambient temperature on a Jeol ECX400 or ECS400 NMR spectrometer equipped with a 5 mm probehead operating at 400 MHz (9.4 T). The instrument's standard settings (45° pulse angle, 0.67 s acquisition time, 3s relaxation delay, 15 ppm spectral width) were used. Locking and shimming was performed on the signal of the exchangeable deuterium atoms of the $\text{D}_2\text{O}/\text{CD}_3\text{OD}$ mixtures. In total, 64 scans were performed leading to a total acquisition time of 8 min. Data processing was performed with MestReNova (Mestrelab Research, v9.0.1). After zerofilling to 64k data points, apodization (exponential and Gaussian functions (1 Hz)), Fourier transformation, phase- and baseline correction, the peak areas were determined by integration. TMSP was used as an internal standard ($\delta = 0$ ppm). For samples with increased amounts of water, presaturation of the water peak ($\delta = 4.79$ ppm) was used to ensure that the dynamic range of the instrument was not exceeded during the measurement. To verify full spin-lattice relaxation between individual pulses, randomly selected samples were also measured with 6 s relaxation delay, which showed no differences compared to the standard relaxation delay of 3 s.

Liposome Preparation^{6,7}

CF Liposomes (EYPC \rightarrow CF). A thin lipid film was formed by evaporating a solution of 25 mg EYPC in chloroform with a stream of nitrogen and drying overnight under vacuum. The thin lipid film was rehydrated with 1 mL of 10 mM HEPES, 50 mM CF, pH 7.5 and agitation at room temperature for 30 min. This was followed by 5 freeze-thaw cycles (5 min freezing in liquid N_2 and thawing in a water bath at 40 °C) and 15 times extrusion through a polycarbonate membrane (pore size 100 nm). Extravesicular components were removed by size exclusion chromatography (NAP-25 column) with

10 mM HEPES, 107 mM NaCl, pH 7.5. Final conditions; inside: 10 mM HEPES, 50 mM CF, pH 7.5; outside: 10 mM HEPES, 107 mM NaCl, pH 7.5.

ANTS/DPX Liposomes (EYPC \rightarrow ANTS/DPX). A thin lipid film was formed by evaporating a solution of 25 mg EYPC in chloroform with a stream of nitrogen and drying overnight under vacuum. The thin lipid film was rehydrated with 1 mL 10 mM HEPES, 20 mM NaCl, 12.5 mM ANTS, 45 mM DPX, pH 7.5 and agitation at room temperature for 30 min. This was followed by 5 freeze-thaw cycles (5 min freezing in liquid N₂ and thawing in a water bath at 40 °C) and 15 times extrusion through a polycarbonate membrane (pore size 100 nm). Extravesicular components were removed by size exclusion chromatography (NAP-25 column) with 10 mM HEPES, 107 mM NaCl, pH 7.5. Final conditions; inside: 10 mM HEPES, 20 mM NaCl, 12.5 mM ANTS, 45 mM DPX, pH 7.5; outside 10 mM HEPES, 107 mM NaCl, pH 7.5.

HPTS Liposomes (EYPC \rightarrow HPTS). A thin lipid film was formed by evaporating a solution of 25 mg EYPC in chloroform with a stream of nitrogen and drying overnight under vacuum. The thin lipid film was rehydrated with 1 mL of 10 mM HEPES, 100 mM NaCl, 1 mM HPTS, pH 7.0 and agitation at room temperature for 30 min. This was followed by 5 freeze-thaw cycles (5 min freezing in liquid N₂ and thawing in a water bath at 40 °C) and 15 times extrusion through a polycarbonate membrane (pore size 100 nm). Extravesicular components were removed by size-exclusion chromatography (NAP-25 column). Final conditions; inside: 10 mM HEPES, 100 mM NaCl, 1 mM HPTS, pH 7.0; outside 10 mM HEPES, 100 mM NaCl, pH 7.0.

CX4/LCG Liposomes (POPC \rightarrow CX4/LCG). A thin lipid film was formed by evaporating a solution of 2.5 mg of POPC in chloroform with a stream of nitrogen and drying overnight under vacuum. The thin lipid film was rehydrated with 1 mL 10 mM sodium phosphate, 700 μ M CX4 and 500 μ M LCG, pH 7.4 and agitation at room temperature for 30 min. This was followed by 15 freeze-thaw cycles (5 min freezing in liquid N₂ and thawing in a water bath at 40 °C). Extravesicular components were removed by size exclusion chromatography (NAP-25 column) with 10 mM sodium phosphate, pH 7.4. Final conditions; inside: 10 mM sodium phosphate, 700 μ M CX4, 500 μ M LCG, pH 7.4; outside: 10 mM sodium phosphate, pH 7.4.

LCG Liposomes (POPC \rightarrow LCG). A thin lipid film was formed by evaporating a solution of 25 mg of POPC in chloroform with a stream of nitrogen and drying overnight under vacuum. The thin lipid film was rehydrated with 1 mL 10 mM HEPES, 50 mM NaNO₃, 500 μ M LCG, pH 7.0 and agitation at room temperature for 30 min. This was followed by 15 freeze-thaw cycles (5 min freezing in liquid N₂ and thawing in a water bath at 40 °C). Extravesicular components were removed by size exclusion chromatography (NAP-25 column) with 10 mM HEPES, 50 mM NaNO₃, pH 7.0. Final conditions; inside: 10 mM HEPES, 50 mM NaNO₃, 500 μ M LCG, pH 7.0; outside: 10 mM HEPES, 50 mM NaNO₃, pH 7.0.

CB7/BE Liposomes (POPC/POPS \rightarrow CB7/BE). A thin lipid film was formed by evaporating a solution of 2.5 mg POPC and 0.33 mg POPS in chloroform with a stream of nitrogen and drying overnight under vacuum. The thin lipid film was rehydrated with 1 mL 10 mM HEPES pH 7.0, 300 μ M CB7, 300 μ M BE, pH 7.0 and agitation at room temperature for 30 min. This was followed by 15 freeze-thaw cycles (5 min freezing in liquid N₂ and thawing in a water bath at 40 °C). Extravesicular components were removed by size exclusion chromatography (NAP-25 column) with 10 mM HEPES, pH 7.0. Final conditions; inside: 10 mM HEPES, 300 μ M CB7, 300 μ M BE, pH 7.0; outside 10 mM HEPES, pH 7.0.

CB8/MDAP Liposomes (POPC/POPS \rightarrow CB8/MDAP). A thin lipid film was formed by evaporating a solution of 2.5 mg POPC and 0.33 mg POPS in chloroform with a stream of nitrogen and drying overnight under vacuum. The thin lipid film was rehydrated with 1 mL 10 mM HEPES pH 7.0, 550 μ M MDAP, 500 μ M CB8 and agitation at room temperature for 30 min. This was followed by 15 freeze-

thaw cycles (2 min freezing in liquid N₂ and 5 min thawing in a water bath at 40 °C). Extravesicular components were removed by size-exclusion chromatography (NAP-25 column) and 10 mM HEPES, pH 7.0. Final conditions; inside: 10 mM HEPES, 500 μM CB8, 550 μM MDAP, pH 7.0; outside 10 mM HEPES, pH 7.0.

POPC Liposomes. A thin lipid film was formed by evaporating a solution of 25 mg POPC in chloroform with a stream of nitrogen and drying overnight under vacuum. The thin lipid film was rehydrated with 1 mL of 10 mM TRIS, pH 7.5 and agitation at room temperature for 30 min. This was followed by 5 freeze-thaw cycles (5 min freezing in liquid N₂ and thawing in a water bath at 40 °C) and 15 times extrusion through a polycarbonate membrane (pore size 100 nm).

Assay Protocols

Stewart Assay. The Stewart assay was adapted from the original procedure with slight modifications.⁸ For calibration curves, solid DPPC was dissolved in chloroform (0.1 mg/mL) and taken as standard. DPPC is expected to give the same response as POPC, but is a non-hygroscopic solid and thus provides a more accurate standard. Varying amounts of this standard (0 to 2.4 mL) were filled up to 3 mL with chloroform in 15-mL-polypropylene tubes, 0.5 mL water and 2 mL 0.1 M FeCl₃, 0.4 M NH₄SCN in distilled water were added, and the mixture was vortexed for 20 s. After centrifugation at 1000 rpm for 5 min, 2 mL of the lower chloroform layer was carefully transferred into a quartz glass cuvette with the aid of a pipette and the absorbance was measured at 485 nm.

For analysis of liposome solutions, an aliquot of the aqueous liposome solution (2 to 50 μL) was filled up to 0.5 mL with water in a 15-mL-polypropylene tube, 3 mL chloroform and 2 mL 0.1 M FeCl₃, 0.4 M NH₄SCN in distilled water were added, and the mixture was vortexed for 20 s. After centrifugation at 1000 rpm for 5 min, 2 mL of the lower chloroform layer was carefully transferred into a quartz glass cuvette with a pipette and the absorbance was measured at 485 nm. Unknown liposome solutions were analyzed at least in triplicates.

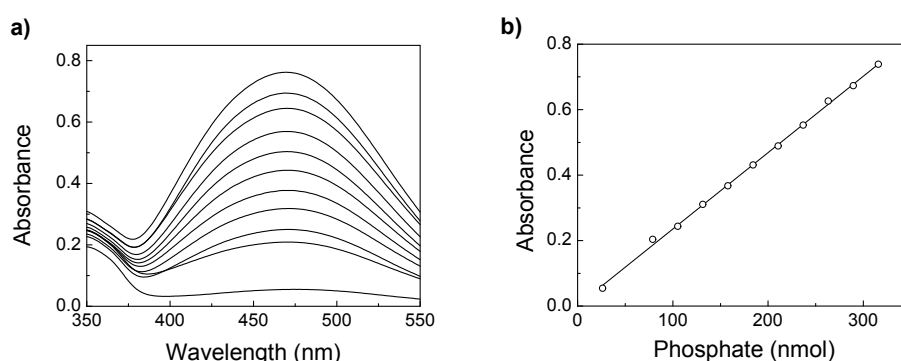


Figure S1. Representative calibration curve with DPPC for the Stewart assay. a) Obtained absorption spectra, and b) the resulting calibration curve ($\lambda_{\text{obs}} = 485 \text{ nm}$).

Rouser Assay. The assay was adapted from the original procedure with slight modifications.⁹ Calibration curves were obtained by taking increasing amounts (0 to 70 μL) of an aqueous sodium dihydrogen phosphate standard (3.226 mM) and filling up to 3.3 mL with water in 10 mL glass vials. 650 μL perchloric acid (70 %), 0.5 mL ammonium molybdate solution (0.025 mg/mL) and 0.5 mL freshly prepared ascorbic acid solution (0.1 mg/mL) were then added. The vials were then incubated in a hot water bath (100 °C) for 10 min. After cooling to room temperature, the absorbance was measured at 800 nm.

Liposome solutions were analyzed by incubating <50 μL liposome solution with 650 μL perchloric acid (70 %) at 180 °C for 50 min (closed vials under constant stirring). After cooling to r.t. the vials were filled up to 3.95 mL with water. The vials were then treated in the same manner as described above for the calibration curve.

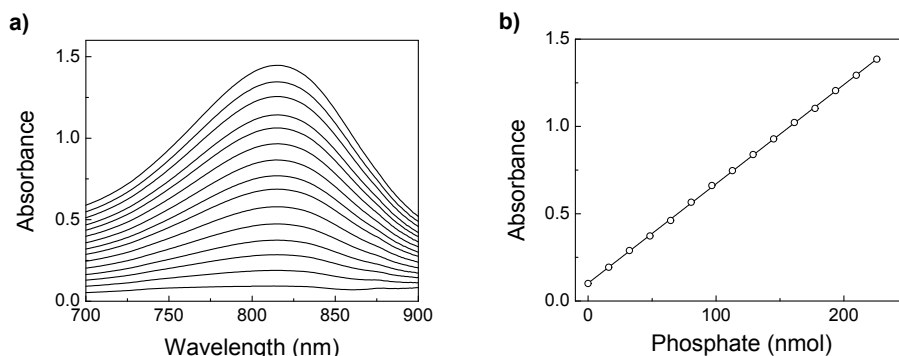


Figure S2. Representative calibration curve with the Rouser assay. a) Obtained absorption spectra, and b) the resulting calibration curve ($\lambda_{\text{obs}} = 800 \text{ nm}$).

¹H NMR. *Method A (for concentrated liposomes, e.g. with ca. 25 mg/ml lipids):* Up to 50 μL of liposome stock solution were directly mixed with 430 μL CD_3OD and 20 μL 50 mM TMSP in D_2O in standard economy NMR tubes and vortexed for 10 s. The NMR spectrum was measured as described above. In all cases a clear, one-phase system was obtained. *Method B (for less concentrated liposomes requiring larger amounts of liposome stock solution, e.g. with ca. 2.5 mg/ml lipids):* Up to 175 μL of liposome stock solution were directly mixed with 430 μL CD_3OD , 100 μL CDCl_3 , and 20 μL 5 mM TMSP in D_2O in standard economy NMR tubes and vortexed for 10 s. The NMR spectrum was measured as described above including presaturation to suppress the water signal. In all cases a clear, one-phase system was obtained.

Since a phospholipid molecule has two fatty acid sidechains with one terminal methyl group each, the signal around 0.88 ppm refers to six hydrogen atoms. By comparing the integrated peak area of this signal with that of the signal from TMSP at 0 ppm, the phospholipid concentration c_{PL} can easily be obtained according to equation S1, where c_{St} is the concentration of the TMSP standard in the stock solution, V_{PL} and V_{St} are the volumes of the liposome solution and the standard, and I_{PL} and I_{St} are the integrated peak areas of the phospholipid and standard.

$$c_{\text{PL}} = \frac{3c_{\text{St}}V_{\text{St}}I_{\text{PL}}}{2V_{\text{PL}}I_{\text{St}}} \quad (\text{eq S1})$$

Table S1. Comparison of phospholipid concentrations used during lipid film rehydration and final concentrations after size exclusion chromatography (SEC) determined by the ¹H NMR method.

Liposome	[Lipid] / mM	
	For lipid rehydration	After SEC
EYPC \supset CF	32.5	16.1 \pm 1.1
EYPC \supset ANTS/DPX	32.5	16.4 \pm 1.3
EYPC \supset HPTS	32.5	17.1 \pm 0.5
POPC \supset CX4/LCG	3.3	1.50 \pm 0.20
POPC/POPS \supset CB8/MDAP	4.4	2.80 \pm 0.04
POPC/POPS \supset CB7/BE	4.4	3.00 \pm 0.01

Sensitivity¹⁰

The sensitivity was expressed as the limits of quantification (LOQ) and detection (LOD). For the spectrophotometric response of the Stewart and Rouser assay, these values were determined according to equations S2 and S3 from the slope of the calibration curve s and the standard deviation of the blank σ ($n \geq 3$) or the standard deviation of the y -intercept of the calibration curve σ (with $n \geq 7$).

$$LOQ = \frac{10 \sigma}{s} \quad (\text{Eq. S2})$$

$$LOD = \frac{3.3 \sigma}{s} \quad (\text{Eq. S3})$$

For the ¹H NMR assay, the signal-to-noise ratio (SNR) of the peak around 0.88 ppm assigned to the terminal methyl groups of the fatty acid side chains of the phospholipids was determined from unprocessed spectra (i.e. after zero-filling and Fourier transformation, but omitting apodization or other windowing functions) with the MestReNova software (v9.0.1). Also here, the LOQ is reached at $SNR \leq 10$, which corresponds to 65 nmol (determined with standard method/settings: 100 μ L liposome stock solution, 20 μ L 5 mM TMSP, 430 μ L CD₃OD, 100 μ L CHCl₃, 64 scans, water presaturation). The LOD is reached at $SNR \leq 3.3$.

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Supporting Figures

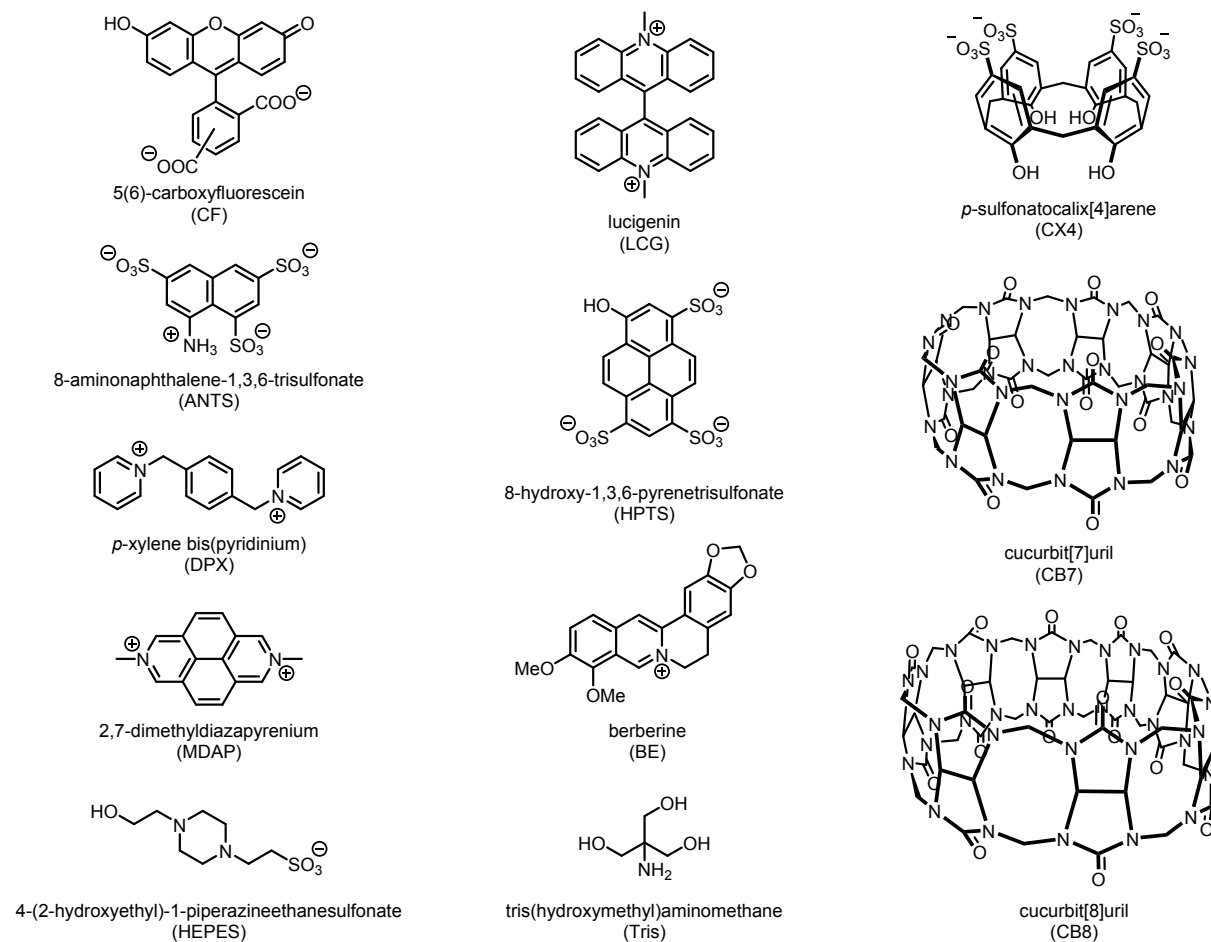


Chart S1. Common substances for supramolecular transport assays.

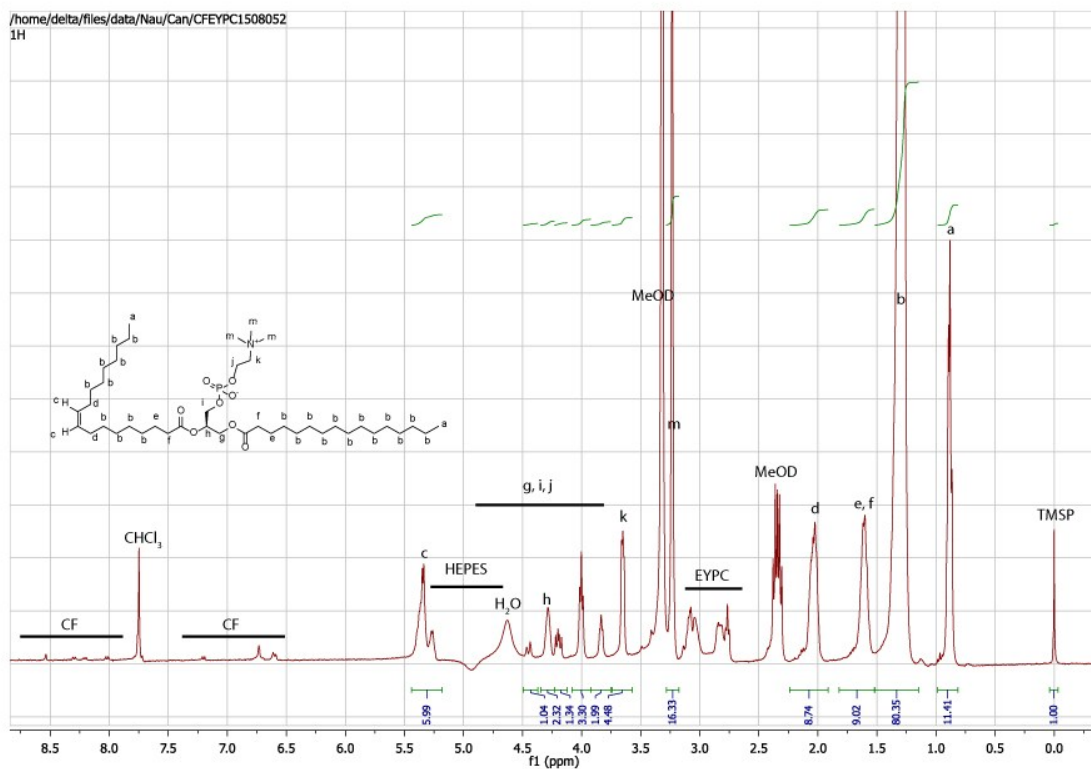


Figure S3. Phospholipid quantification by ^1H NMR of CF liposomes. 100 μL EYPC \supset CF liposomes (in 10 mM HEPES, 107 mM NaCl, pH 7.5) were dissolved in 430 μL CD_3OD , 100 μL CDCl_3 , and 20 μL 5 mM TMSP in D_2O . The proton signal from water was suppressed by presaturation.

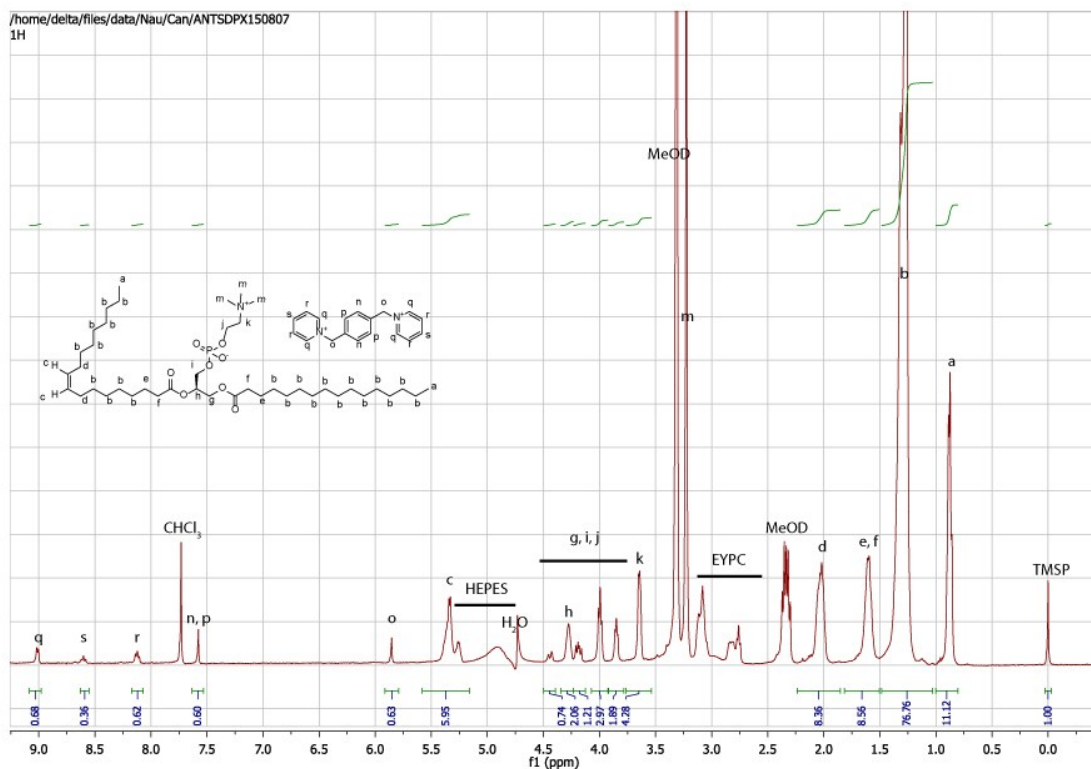


Figure S4. Phospholipid quantification by ^1H NMR of ANTS/DPX liposomes. 100 μL EYPC \supset ANTS/DPX liposomes (in 10 mM HEPES, 107 mM NaCl, pH 7.5) were dissolved in 430 μL CD_3OD , 100 μL CDCl_3 , and 20 μL 5 mM TMSP in D_2O . The proton signal from water was suppressed by presaturation.

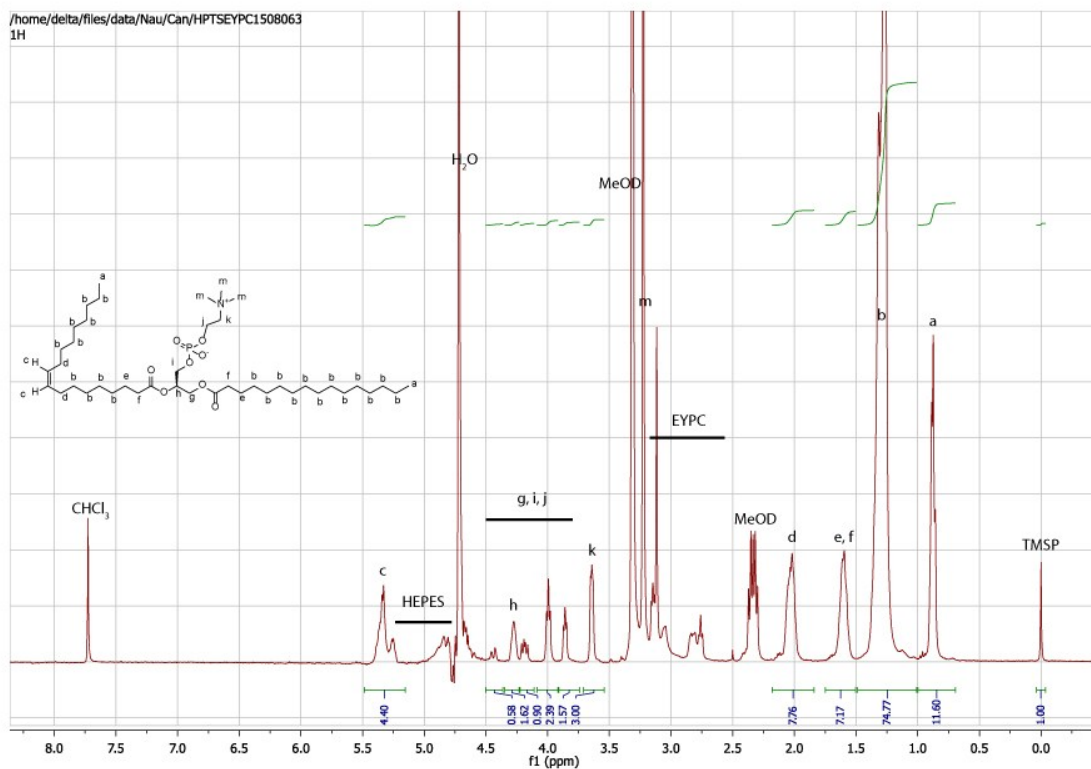


Figure S5. Phospholipid quantification by ^1H NMR of HPTS liposomes. 100 μL EYPC \supset HPTS liposomes (in 10 mM HEPES, 100 mM NaCl, pH 7.0) were dissolved in 430 μL CD_3OD , 100 μL CDCl_3 , and 20 μL 5 mM TMSP in D_2O . The proton signal from water was suppressed by presaturation.

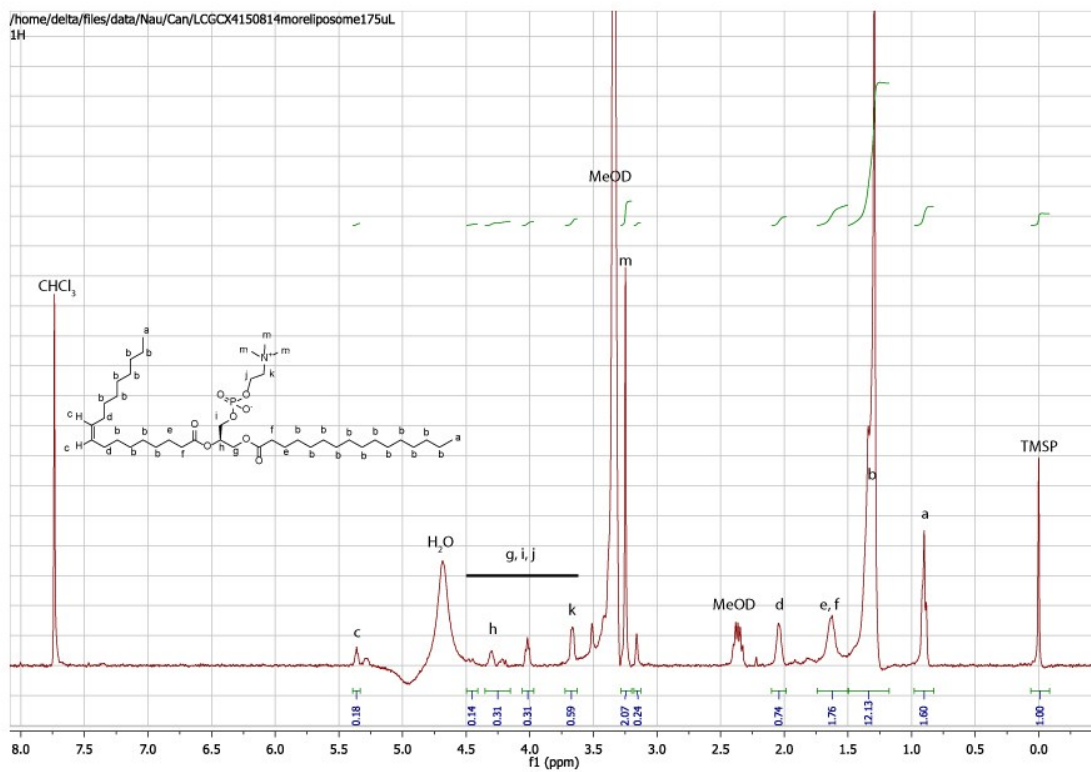


Figure S6. Phospholipid quantification by ^1H NMR of CX4/LCG liposomes. 175 μL POPC \supset CX4/LCG liposomes (in 10 mM sodium phosphate, pH 7.4) were dissolved in 430 μL CD_3OD , 100 μL CDCl_3 , and 20 μL 5 mM TMSP in D_2O . The proton signal from water was suppressed by presaturation.

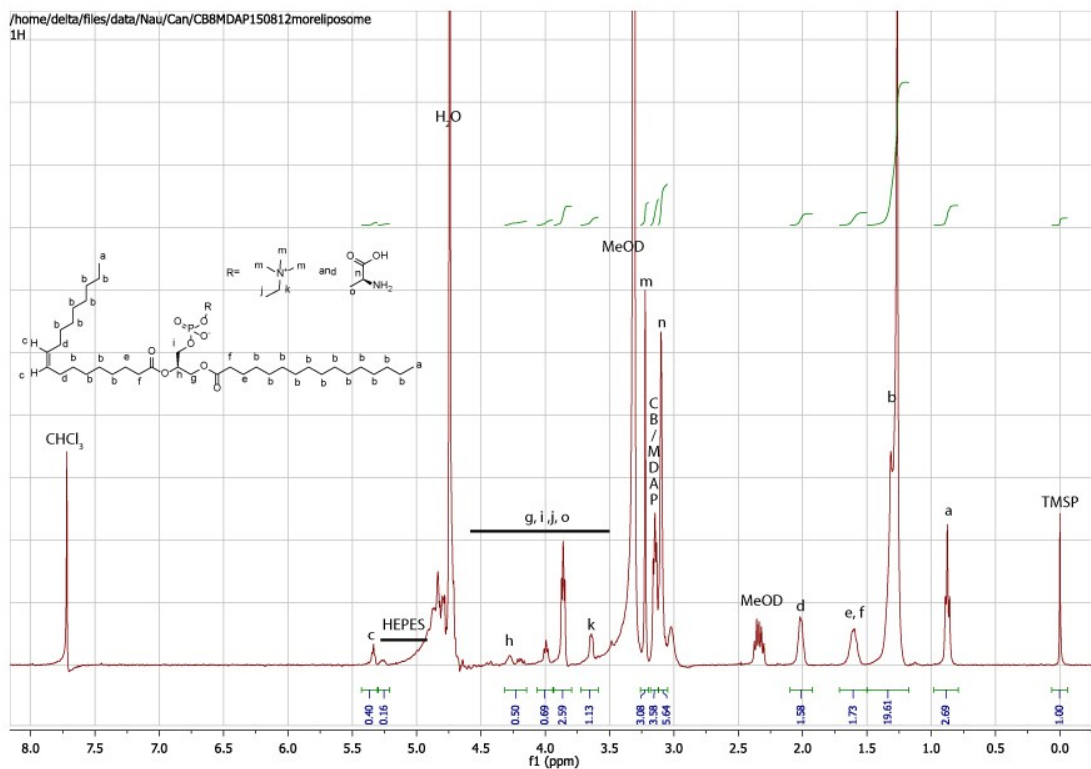


Figure S7. Phospholipid quantification by ¹H NMR of CB8/MDAP liposomes. 175 μ L POPC/POPS \rightarrow CB8/MDAP liposomes (in 10 mM HEPES, pH 7.0) were dissolved in 430 μ L CD₃OD, 100 μ L CDCl₃, and 20 μ L 5 mM TMSP in D₂O. The proton signal from water was suppressed by presaturation.

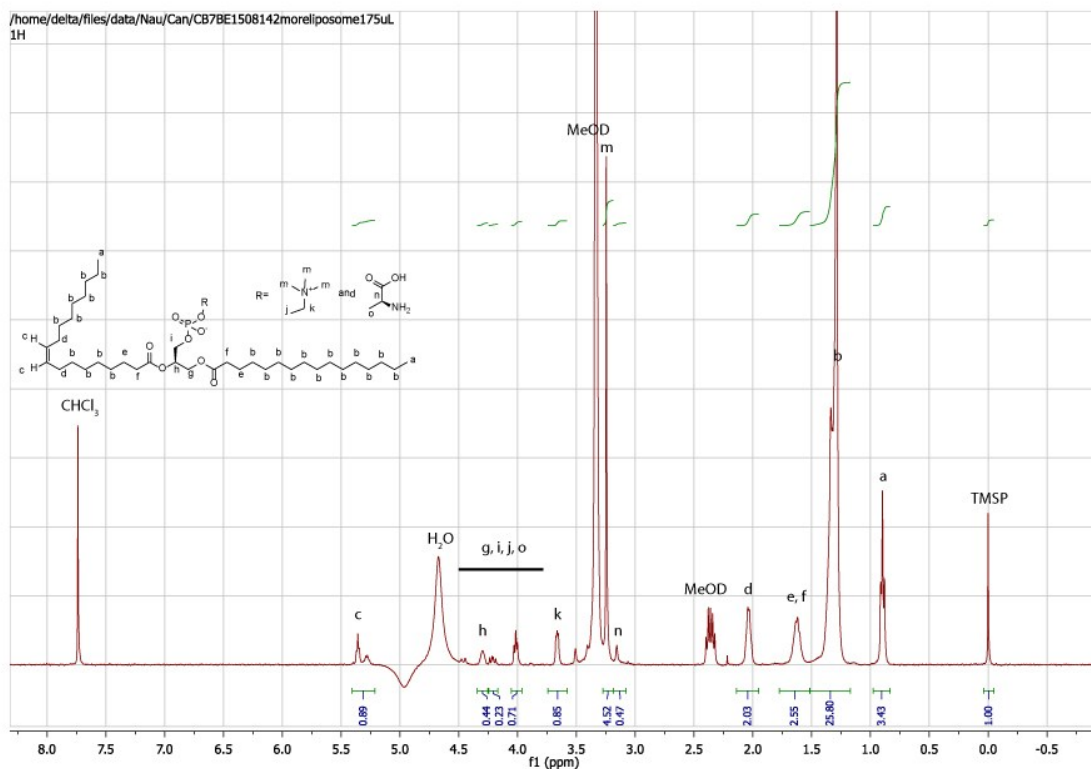


Figure S8. Phospholipid quantification by ¹H NMR of CB7/BE liposomes. 175 μ L POPC/POPS \rightarrow CB7/BE liposomes (in 10 mM sodium phosphate, pH 7.4) were dissolved in 430 μ L CD₃OD, 100 μ L CDCl₃, and 20 μ L 5 mM TMSP in D₂O. The proton signal from water was suppressed by presaturation.

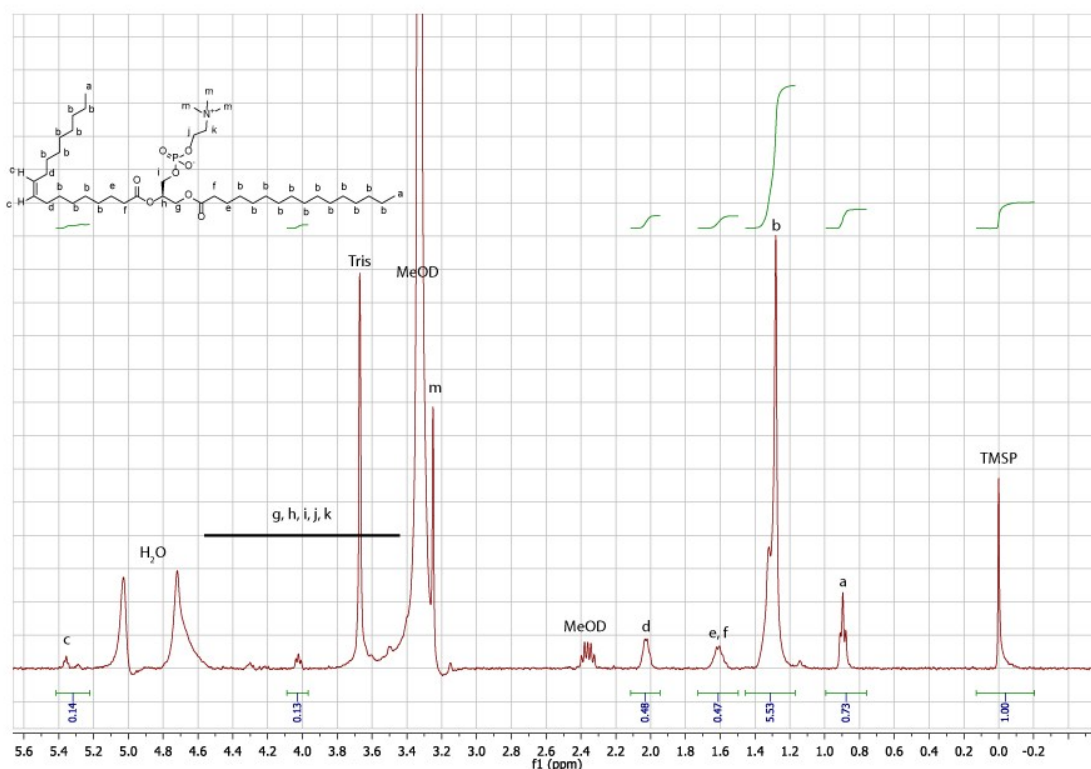


Figure S9. Phospholipid quantification by ^1H NMR of POPC liposomes. 50 μL POPC liposomes (in 10 mM TRIS, pH 7.5) were dissolved in 430 μL MeOD and 20 μL 50 mM TMSP in D_2O . The proton signal from water was suppressed by presaturation.

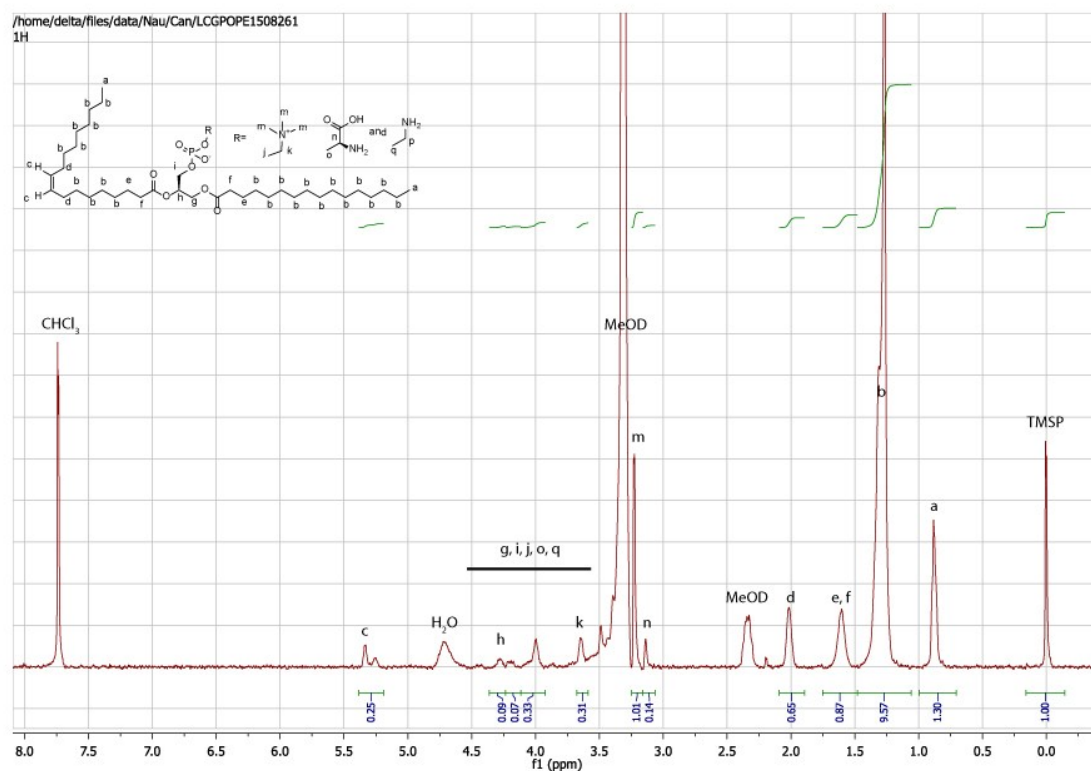


Figure S10. Phospholipid quantification by ^1H NMR of CX4/LCG liposomes. 100 μL POPE/POPC/POPS \rightarrow CX4/LCG liposomes (in 10 mM sodium phosphate, pH 7.2) were dissolved in 430 μL CD_3OD , 100 μL CDCl_3 , and 20 μL 5 mM TMSP in D_2O . The proton signal from water was suppressed by presaturation.