Electronic Supplementary Material (ESI) for ChemComm.

A quantitative assay to study the lipid selectivity of membrane-associated systems using solution NMR

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Contents

Reagents

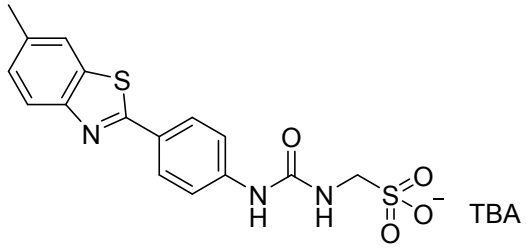
Lipids and Chemicals. The lipids used in this paper are referred to as: cardiolipin (CL), dimyristoylphosphatidylcholine (PC), palmitoyloleoylphosphatidylethanolamine (PE), soy phosphatidylinositol (PI), dipalmitoylphosphatidylserine (PS), egg phosphatidylglycerol (PG) and brain sphingomyelin (SM). All lipids were obtained from Avanti Polar Lipids (Alabaster, AL). Spectrophotometric-grade solvents, Asolectin from Soybean and all other chemicals were purchased from Sigma Aldrich. Glassware was used instead of plastic in each step of the procedure to avoid contaminants. Tetramethyl silane (TMS) and Trimethyl Phosphate (TMP) were used in this study as NMR reference standard and internal standard respectively, and used at a purity of at least 99%.

Solubilisation/hydrolysis of Styrene-Maleic Anhydride to produce Styrene-Maleic Acid (SMA). We followed the same protocol described in Townshend et al.[1] Commercially available styrene-maleic anhydride polymer, with a ratio of 2:1 styrene to maleic anhydride (SMA2000 polymer purchased from Cray Valley via Dr. Timothy Knowles), was first hydrolysed under reflux conditions. Styrene-maleic anhydride anhydride powder (25 g) was suspended in NaOH (250 mL, 1.0 M) with anti-bumping granules (0.1 g) and slowly heated to reflux (100 °C) with constant stirring. The solution was refluxed for ~4 hours, until all of the solid styrene-maleic anhydride dissolved. Once the solution cooled (20 ^{o}C) the SMA was precipitated by reducing the pH to below 5 with the addition of concentrated HCl and checked using pH paper. The precipitated polymer was centrifuged at 11,000 g for 15 minutes (4 ^{o}C), the supernatant was removed and the precipitate was washed with Milli Q water (3 x 250 mL). The polymer was then re-suspended in NaOH (0.6 M) and adjusted to pH 7.4 and left in an incubator overnight. Once completely solubilised the polymer was lyophilized and stored at room temperature.

CLIC1 expression and purification. The Human CLIC1 gene (clone HsCD00338210 from the Plasmid service at HMS) was cloned into a pASG vector (IBA) containing an N-terminal twin strep tag. CLIC1 was expressed recombinantly in the C43 *E.coli* strain (Lucigen). The cells were lysed by sonication, and the membrane and soluble fractions were separated by ultracentrifugation at 117734 g. The soluble fraction was purified in the absence of any detergent using affinity chromatography with a Strep-Tactin XT column and a subsequent step of gel filtration using a Superdex200 Increase column (GE) in 20 mM HEPES buffer with 20 mM NaCl at pH 7.4.

Chemical Synthesis

General remarks. A positive pressure of nitrogen and oven dried glassware were used for all reactions. All solvents and starting materials were purchased from known chemical suppliers or available stores and used without any further purification unless specifically stipulated. The NMR spectra were obtained using a Bruker AV2 400 MHz or AVNEO 400 MHz spectrometer. The data was processed using ACD Labs, MestReNova or Topspin software. NMR Chemical shift values are reported in parts per million (ppm) and calibrated to the centre of the residual solvent peak set (s = singlet, br = broad, d = doublet, t = triplet, q = quartet, m = multiplet).



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Compound 1: This compound was synthesised in line with our previously published methods. Proton NMR were found to match our previously published values. TBA = tetrabutylammonium.[2] ^{1}H NMR (400 MHz, DMSO-d_6): δ: 9.19 (s, 1H), 7.91 – 7.84 (m, 4H), 7.57 (d, *J* = 8.74 Hz, 2H), 7.32 – 7.29 (m, 1H), 6.80 (t, *J* = 5.88 Hz, 1H), 3.93 (d, *J* = 5.88 Hz, 2H), 3.15 – 3.12 (m, 8H), 2.43 (s, 3H), 1.59 – 1.51 (m, 8H), 1.34 – 1.25 (m, 8H), 0.92 (t, *J* = 7.32 Hz, 12H).

Lipid extraction

Lipid extraction from cells. Approximately 1.5 g of COLO-680N cells in buffer B (20 mM Hepes, 20 mM NaCl pH 7.6) were lysed by several freeze/thaw cycles. The membrane fraction was separated by ultracentrifugation at 206,000 g for 30 mins and mixed with 1 mL of buffer B by vortex mixer. Lipids from this cell line were extracted using the Folch method.[3] Briefly, the suspension was washed with a 2 : 1 (v/v) chloroform-methanol mixture to a final dilution 20-fold the initial volume of the membrane fraction. The mixture was shaken for 1 hour at room temperature and then phase separation was induced by addition of water to the mixture in the proportion 8 : 4 : 3 (v/v) chloroform:methanol:water. The suspension was centrifuged at 2,320 g for 10 mins and the lower (chloroform) phase was collected and dried.

CLIC1 Annular lipid isolation. Lipids were dissolved in buffer B and approximately 1 mg of soluble CLIC1 was added (at an approximate molar ratio of 5:1 total lipid:CLIC1). CLIC1 membrane insertion was induced by addition of $ZnCl_2$ (3 mM final concentration). The solution was gently shaken for 1 h at room temperature. After ultracentrifugation (206,000 g for 30 mins), the supernatant is discarded, removing all unbound Zn. The membrane pellet was resuspended in buffer B and 0.25 % (w/v) of SMAs were added. The solution was gently shaken at room temperature overnight and was then ultracentrifuged again (206,000 g for 30 mins) and the supernatant was transferred to a 7 KDa cut-off dialysis membrane. The discarded pellet contains all lipid material not associated with the SMA-nanodiscs. The solution was dialysed against buffer B overnight at room temperature to remove free SMAs. The sample was purified by size exclusion chromatography using a Superdex200 column (GE). Negative staining TEM was used to confirm the presence of SMA nanodics (Figure S17-S18). Finally, the lipids contained in the SMA-nanodiscs were extracted using the same Folch extraction method, as described above, and dissolved in 600 μL of solvent mixture A with 0.315 mM TMP and 0.05 % TMS.

SSA Annular lipid isolation. 5 mg of *E. Coli* lipid extract/DMPC at a 2:3 ratio was dissolved in buffer B and approximately 0.25 mg of SSA Compound 1 was added (at a molar ratio of 7:1 Total Lipid:Compound 1). The solution was gently shaken for 1 h at 37C. Then, 0.25 % (w/v) of SMAs were added. The solution was gently shaken at 37C overnight and was then ultracentrifuged again (206,000 g for 30 mins). The discarded pellet contains all lipid material not associated with the SMA-nanodiscs. The supernatant was purified by size exclusion chromatography using a Superdex200 column (GE). Finally, the lipids contained in the SMA-nanodiscs were extracted using the same Folch extraction method, as described above, and dissolved in 600 μL of solvent mixture A with 0.315 mM TMP and 0.05 % TMS.

NMR Methodology

NMR Experiments. ^{1}H-^{31}P HSQC spectra were acquired on a Bruker Avance III spectrometer at a proton frequency of 600MHz using a QCIP cryoprobe with a standard ^{31}P pre-amplifier without enhanced sensitivity from cryogenic cooling. Spectra were acquired using the pulse sequence na_hsqcetf3gpxy.[4] Time domain data of 1048 and 128 complex points were used in the direct and indirect domains respectively. The sweep widths used in the ^{1}H and ^{31}P dimensions were 14423 and 2915 Hz respectively. The inter scan delay was set to 1 s and 128 scans were acquired per increment with 4 dummy scans at the start of each experiment. Offsets of 7.470 and -1 ppm were used for the ^{1}H and ^{31}P channels. Decoupling was achieved using a GARP4 sequence and the CPMG sequence during the ^{1}H-^{31}P/ ^{31}P-^{1}H transfers used 256 delays of 200 μs. 1D spectra were processed and analysed using MestReNova. All 1D spectra were automatically phased, baseline corrected using a polynomial function and calibrated to the centre of the DSS peak. 2D spectra were processed with NMRPipe[5] and analysed using NMRView.[6] All 2D spectra were manually phased, zero filled and calibrated to the centre of the DSS and TMP peak. ^{31}P 1D skyline projection of the ^{1}H-^{31}P HSQC spectra was performed in Topspin 3.6.1.

Lipid quantification. Quantification of lipids was carried out using the ^{31}P 1D skyline projection of the ^{1}H-^{31}P HSQC spectra. The integrals are calculated for TMP and each identified phospholipid peak. The percentage of total phospholipid in each sample is calculated using the ratio of each phospholipid integral over the sum of the integrals of all phospholipids.

Sample Preparation. All samples for ^{1}H-^{31}P-NMR analysis were prepared in solvent mixture A (75 % $CDCl_3$, 25 % MeOD, 0.315 mM TMP and 0,05 % TMS). Samples containing 0.1-10 mg of lipid dissolved in 600 μL of solvent mixture A were used to obtain ^{1}H NMR fingerprint spectra for each lipid.

References

1 G. Townshend, G. S. Thompson, L. J. White, J. R. Hiscock and J.-L. J. L. O. Roldan, *Chem. Commun. (Camb).,2020, **56**, 4015-4018*

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4 B. Luy and J. P. Marino, *J. Am. Chem. Soc.*, 2001, **123**, 11306–11307.

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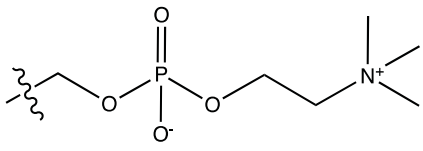
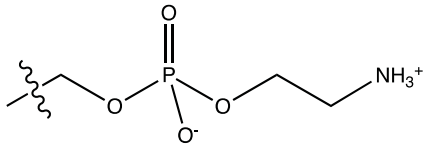
6 B. A. Johnson, *Methods Mol. Biol.*, 2004, **278**, 313–352.

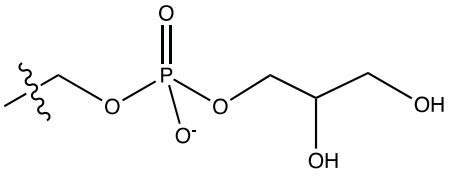
Phospholipid Headgroup Chemical Structures



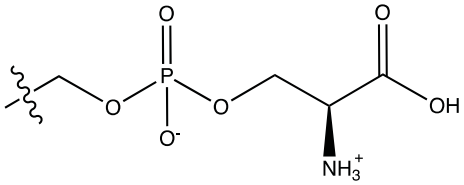
Phosphatidylcholine (PC)



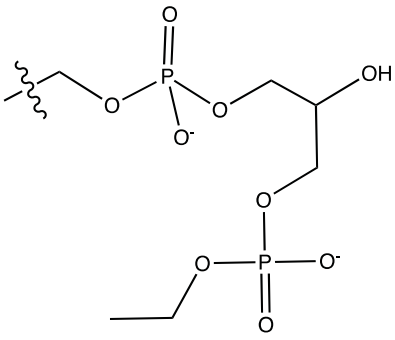
Phosphatidylethanolamine (PE)



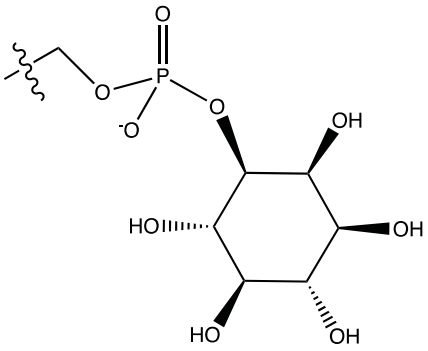
Phosphatidylglycerol (PG)



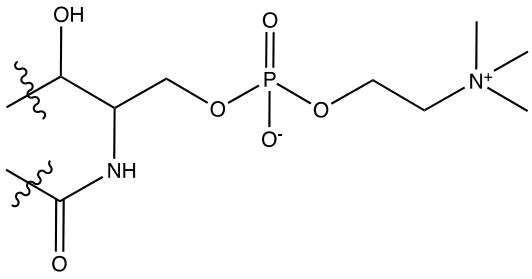
Phosphatidylserine (PS)



Cardiolipin (CL)



Phosphatidylinositol (PI)



Sphingomyelin (SM)

Figure S1: Chemical structures of the headgroups of the phospholipids assayed.

^{1}H -^{31}P NMR Lipid data

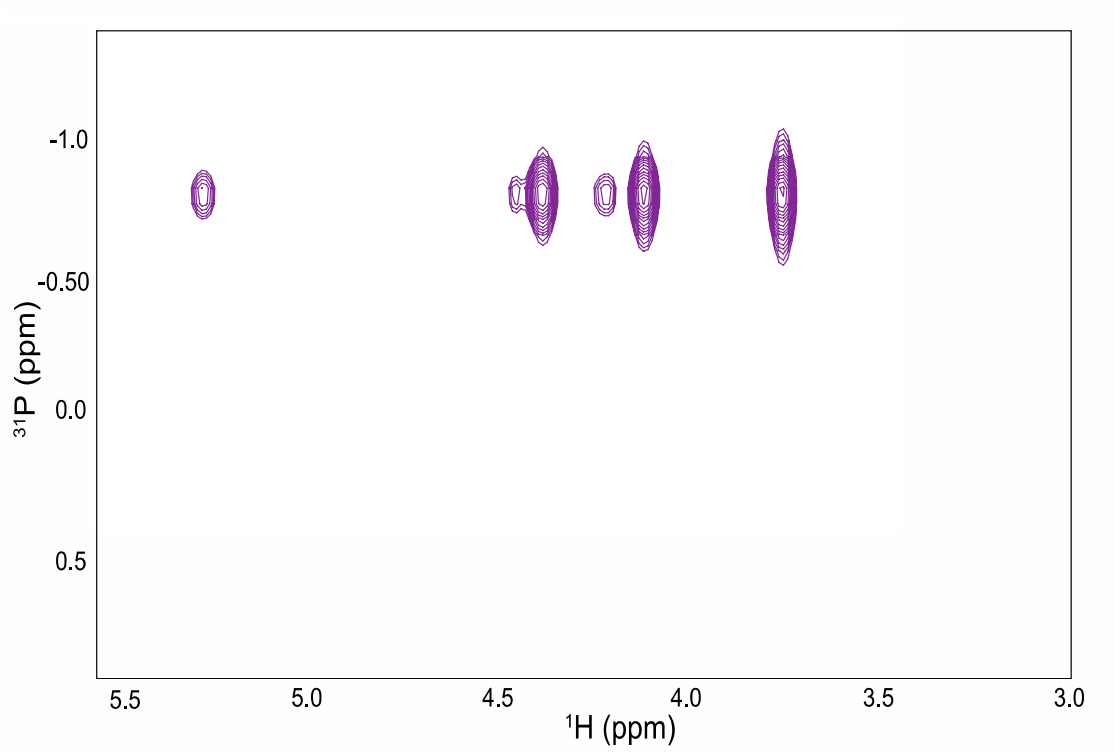


Figure S2 – ^{1}H-^{31}P HSQC NMR Spectrum of 17.4 mg/ml PC in 75 % $CDCl_3$, 25 % MeOD, and 0.05 % TMS.

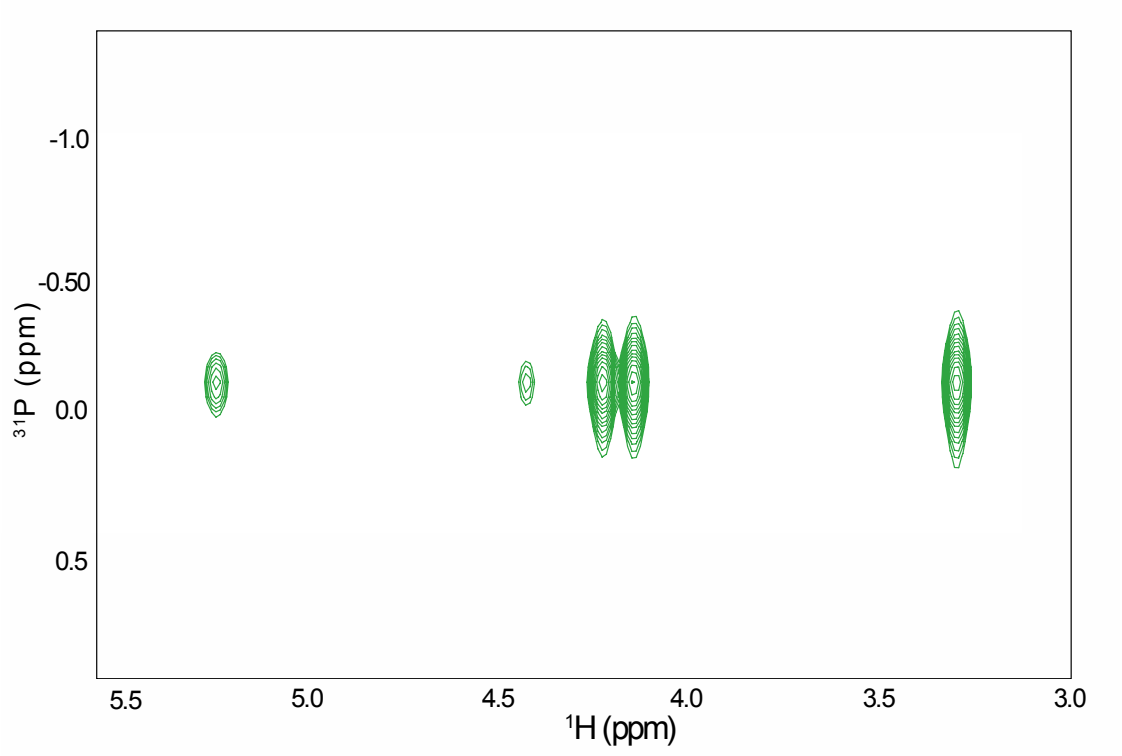


Figure S3 – ^{1}H-^{31}P HSQC NMR Spectrum of 14.1 mg/ml PE in 75 % $CDCl_3$, 25 % MeOD, and 0.05 % TMS.

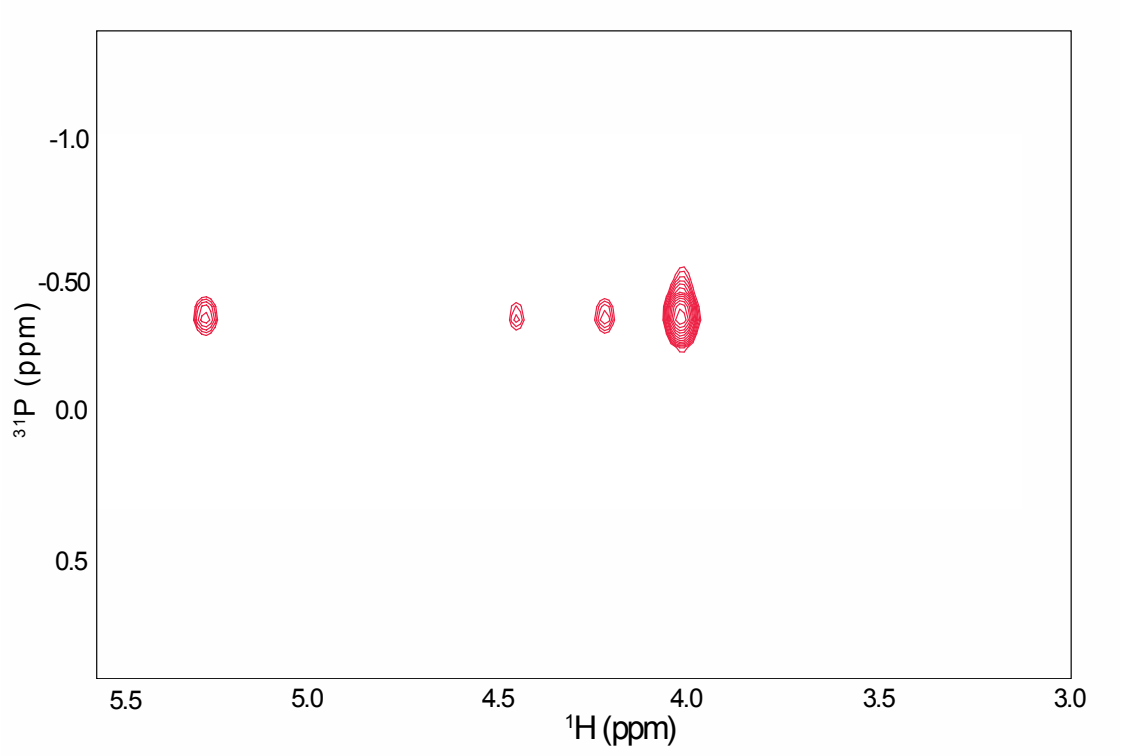


Figure S4 – ^{1}H-^{31}P HSQC NMR Spectrum of 10 mg/ml PS in 75 % $CDCl_3$, 25 % MeOD, and 0.05 % TMS.

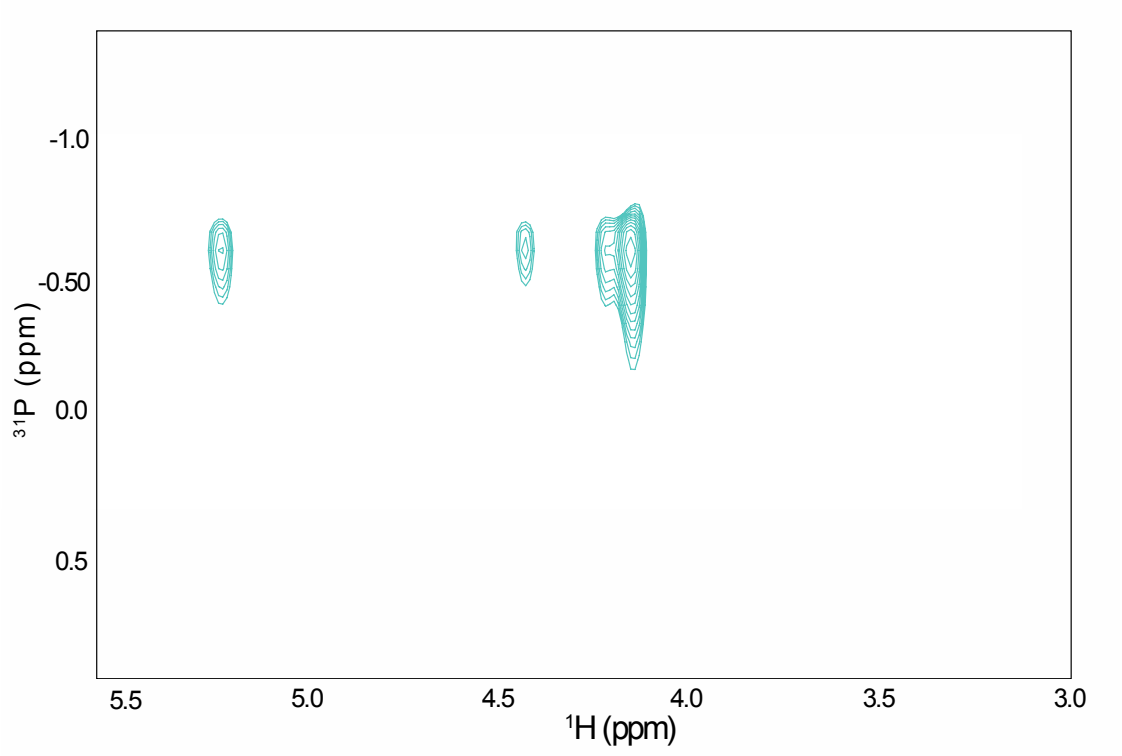


Figure S5 – ^{1}H-^{31}P HSQC NMR Spectrum of 10 mg/ml brain PI in 75 % $CDCl_3$, 25 % MeOD, and 0.05 % TMS.

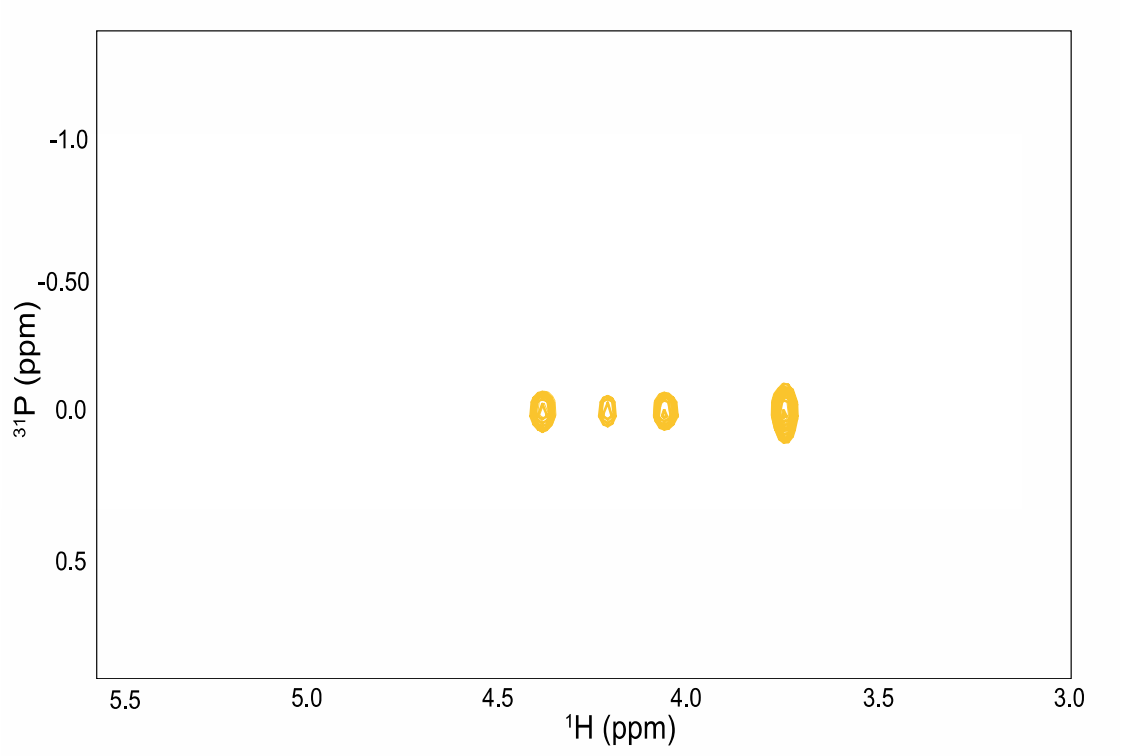


Figure S6 – ^{1}H-^{31}P HSQC NMR Spectrum of 15.3 mg/ml SM in 75% $CDCl_3$, 25% MeOD, and 0.05% TMS.

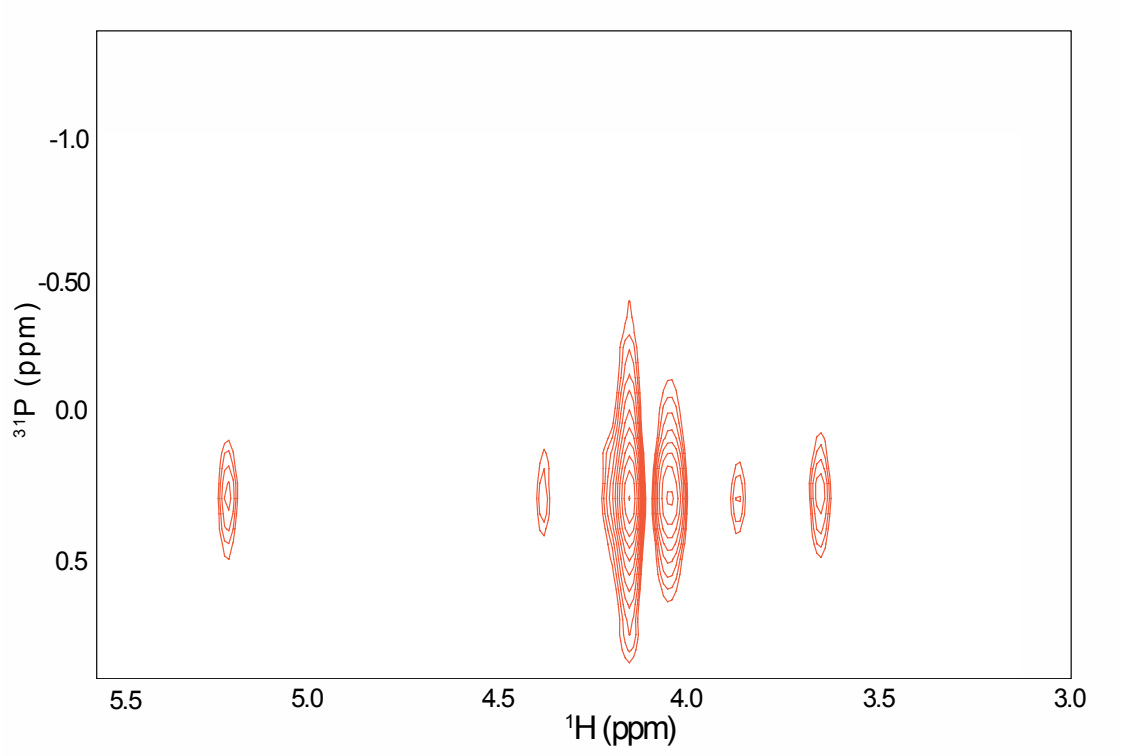


Figure S7 – ^{1}H-^{31}P HSQC NMR Spectrum of 12.3 mg/ml PG in 75% $CDCl_3$, 25% MeOD, and 0.05% TMS.

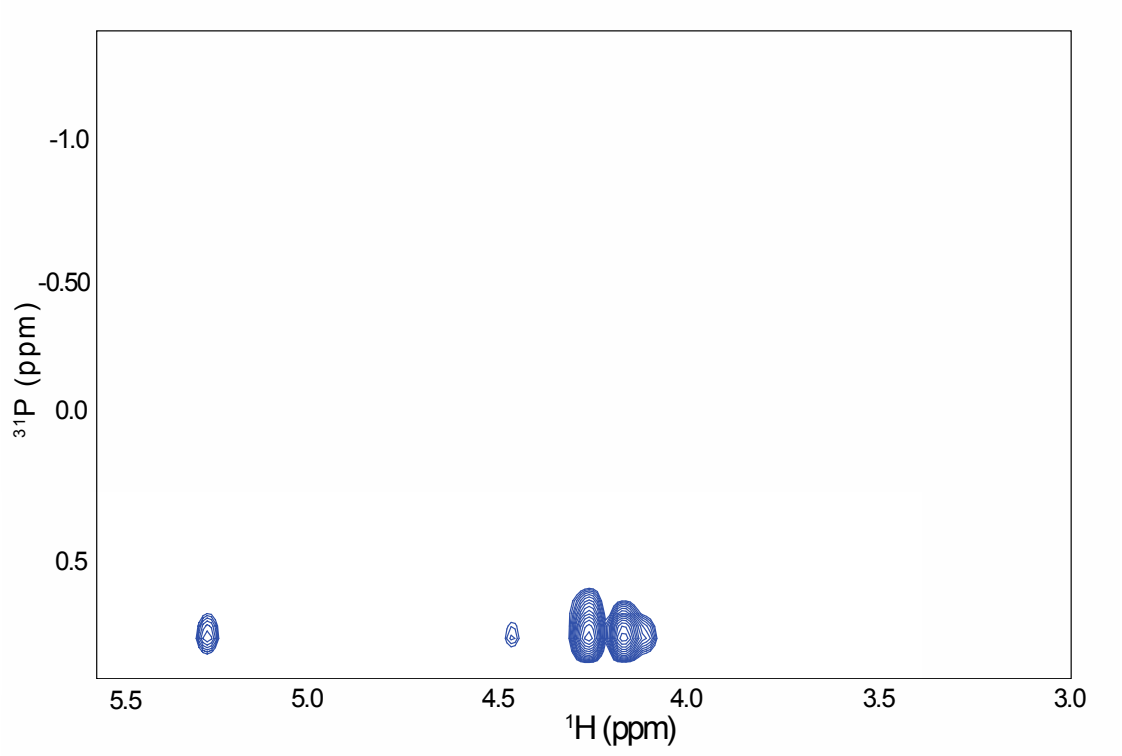


Figure S8 – ^{1}H-^{31}P HSQC NMR Spectrum of 2 mg/mL CL in 75% $CDCl_3$, 25% MeOD, and 0.05% TMS.

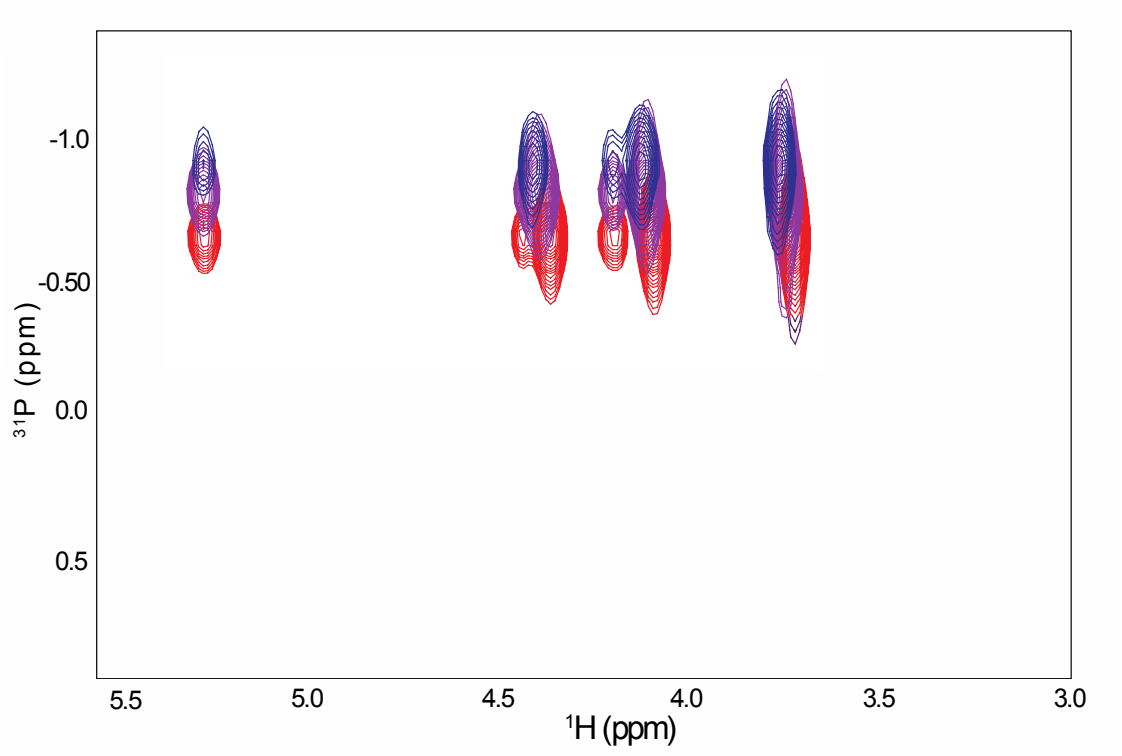


Figure S9 – ^{1}H-^{31}P HSQC NMR Spectra of PC at 20.3 mg/mL (red), 17.4 mg/mL (purple) and 15.2 mg/mL (blue) in 75 % $CDCl_3$, 25 % MeOD, and 0.05 % TMS.

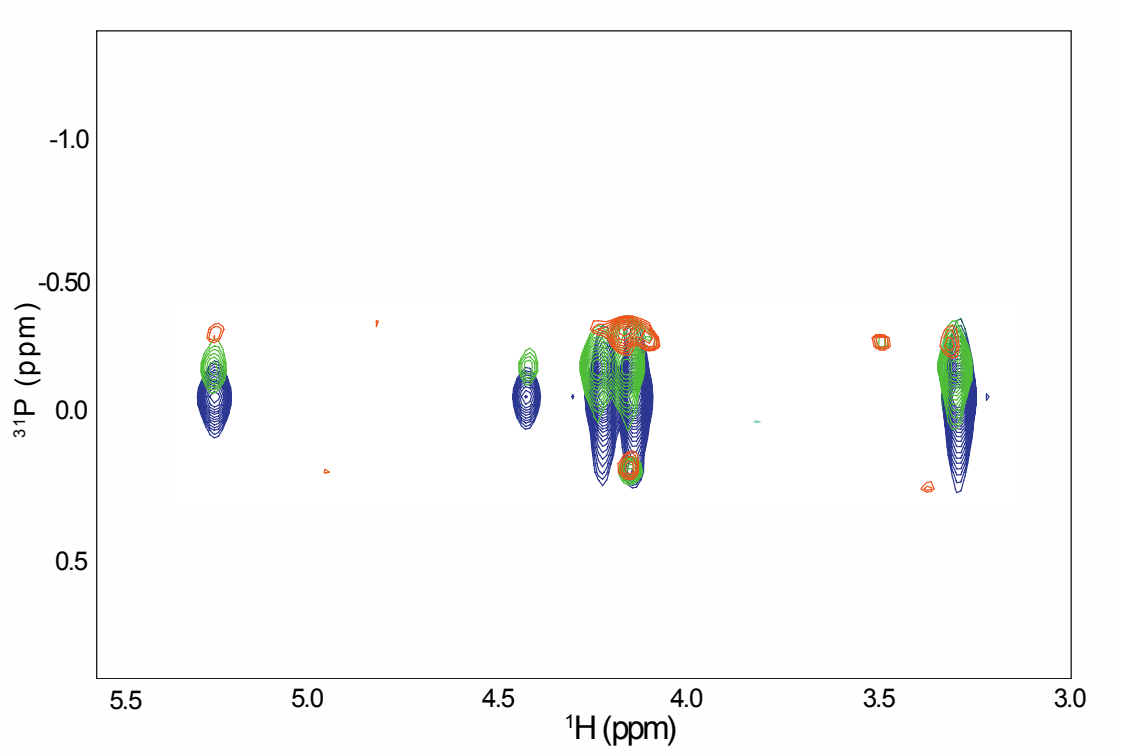


Figure S10 – ^{1}H-^{31}P HSQC NMR Spectra of PE at 16.5 mg/mL (purple), 14.1 mg/mL (green) and 12.4 mg/mL (orange) in 75 % $CDCl_3$, 25 % MeOD, and 0.05 % TMS.

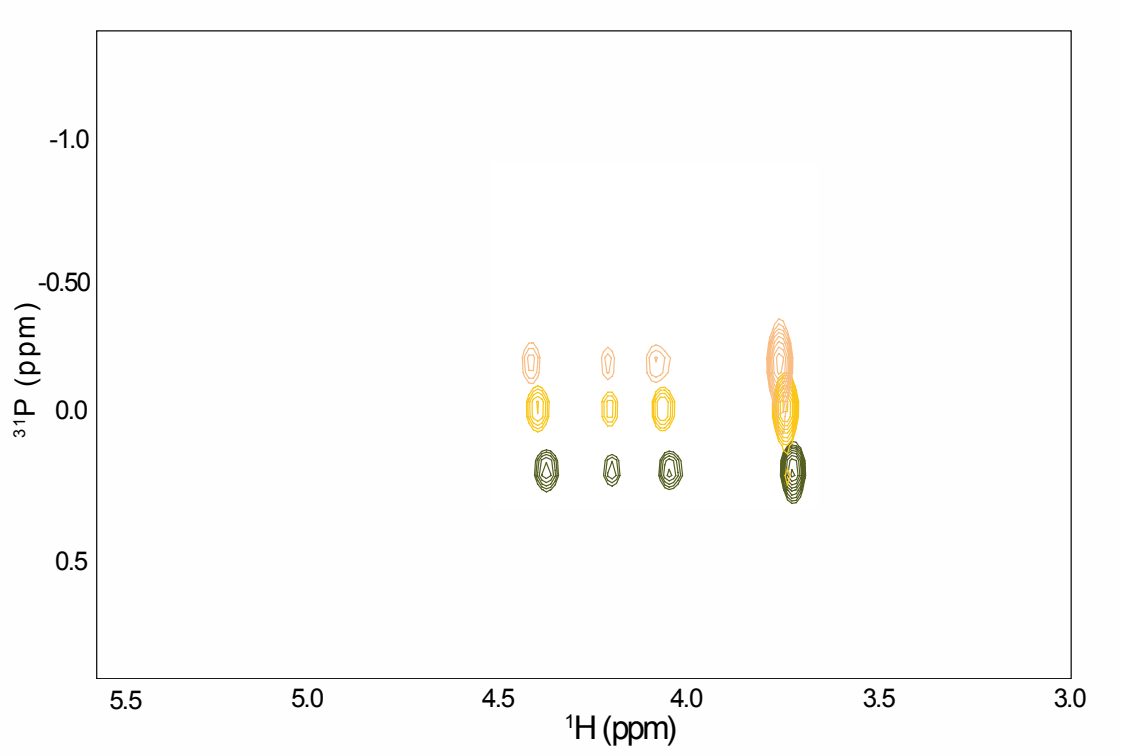


Figure S11 – ^{1}H-^{31}P HSQC NMR Spectra of SM at 17.8 mg/mL (gray), 15.3 mg/mL (yellow) and 13.3 mg/mL (dark yellow)in 75 % $CDCl_3$, 25 % MeOD, and 0.05 % TMS.

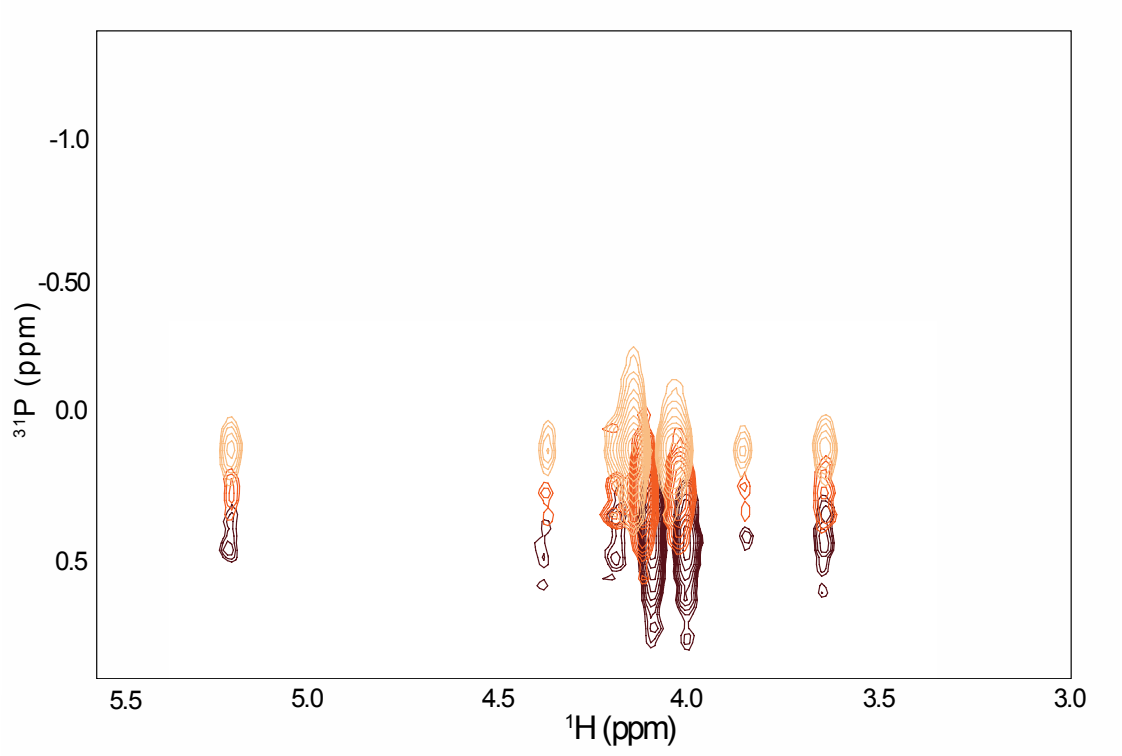


Figure S12 – ^{1}H-^{31}P HSQC NMR Spectra of PG at 16.4 mg/mL (dark red), 14.1 mg/mL (orange) and 12.3 mg/mL (yellow) in 75% $CDCl_3$, 25% MeOD, and 0.05% TMS.

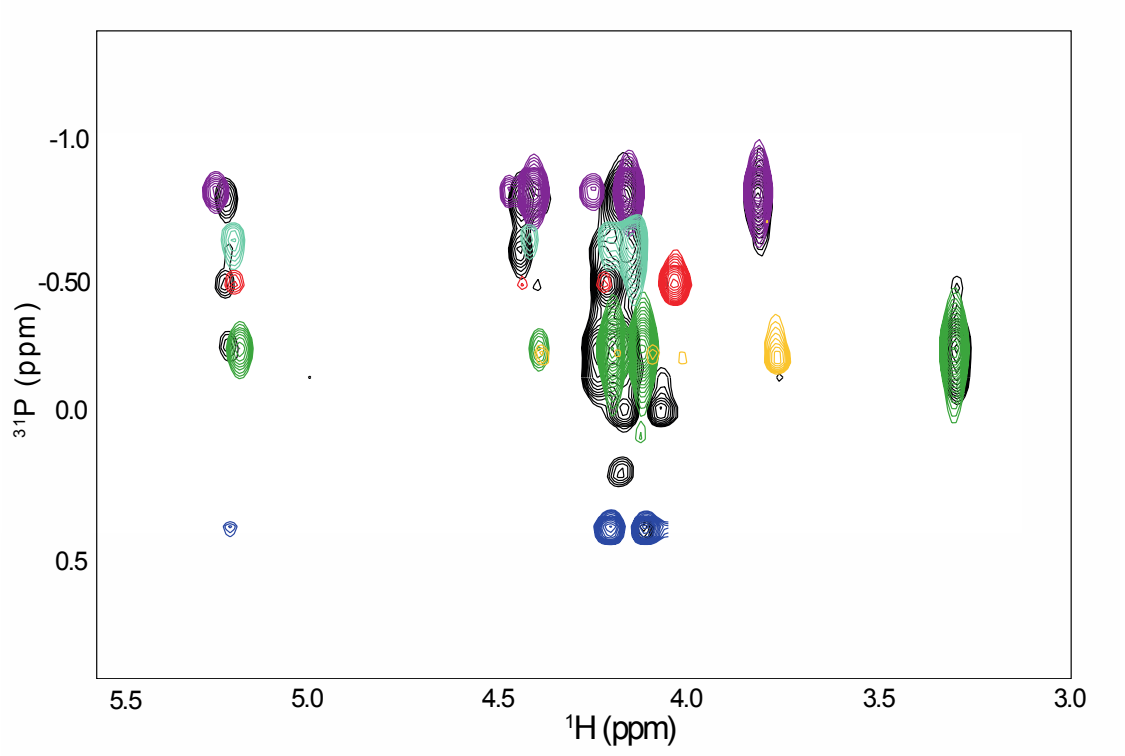


Figure S13 – Overlay of the ^{1}H-^{31}P HSQC NMR spectrum of VERO cells lipid extract (black) with the spectra of PC (purple), PE (green), PI (cyan), PS (red), PG (orange) and CL (blue) in 75% $CDCl_3$, 25% MeOD, and 0.05% TMS.

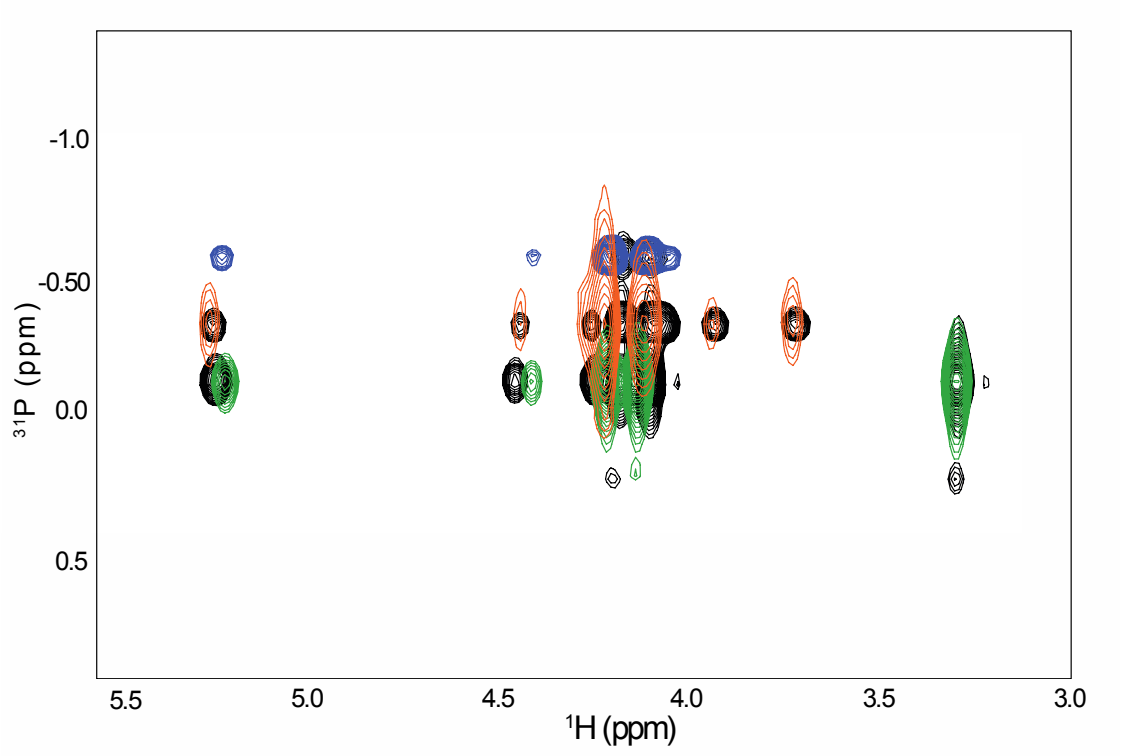


Figure S14 – Overlay of the ^{1}H-^{31}P HSQC NMR spectrum of E. coli total lipid extract (black) with the spectra of PE (green), PG (orange) and CL (blue) in 75% $CDCl_3$, 25% MeOD, and 0.05% TMS.

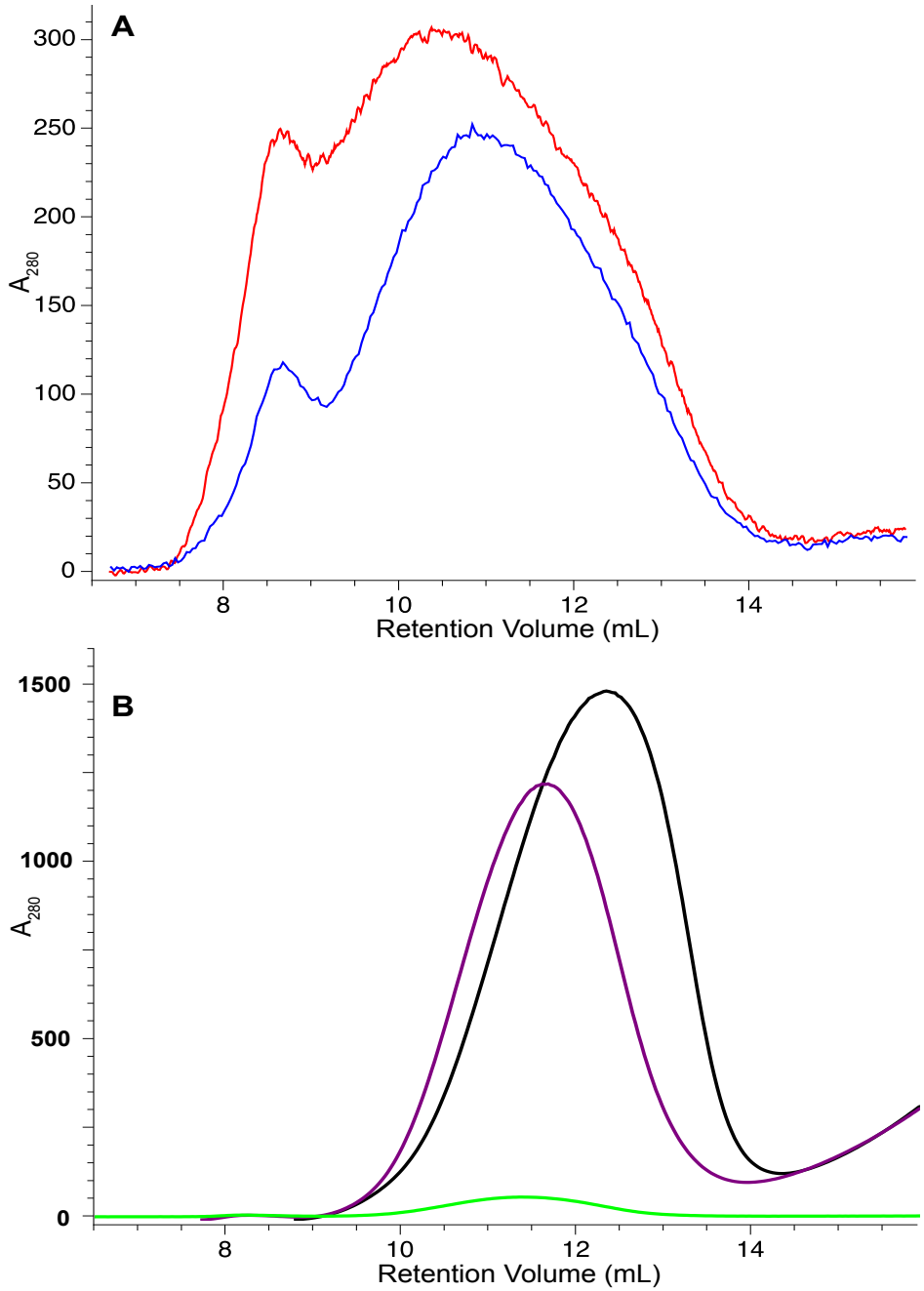


Figure S15 – A. Size exclusion chromatography traces of CLIC1-containing (red) and empty (blue) SMA nanodiscs. Both samples were injected on a Superdex200 column and the absorbance of the SMA moiety and tryptophan residues on CLIC1 were monitored at 280 nm. The left most peak corresponds to aggregates and high-molecular weight species eluting at the void volume of the column. The second major peak on the right corresponds to the SMA nanodiscs. B. Size exclusion chromatography traces of SSA compound 1-containing (purple and green) and empty (black) SMA nanodiscs. Both samples were injected on a Superdex200 column and the absorbance of the SMA moiety was monitored at 260 nm (purple) and the absorption of the SSA compound 2 was monitored at 335 nm (green).

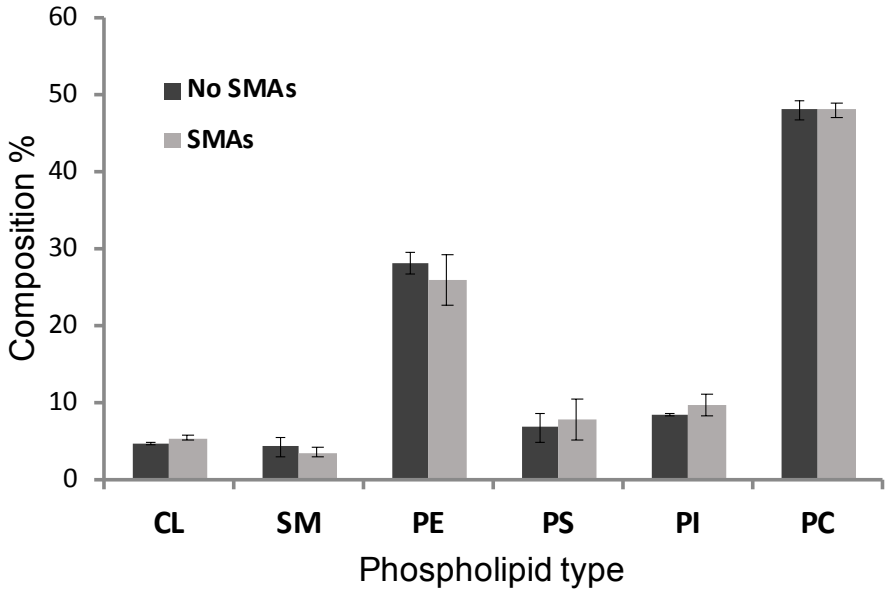


Figure S16 – Comparative analysis of lipids distribution in eukaryotic samples in the presence and the absence of SMA-nanodiscs (n=2).

TEM nanodisc characterisation data

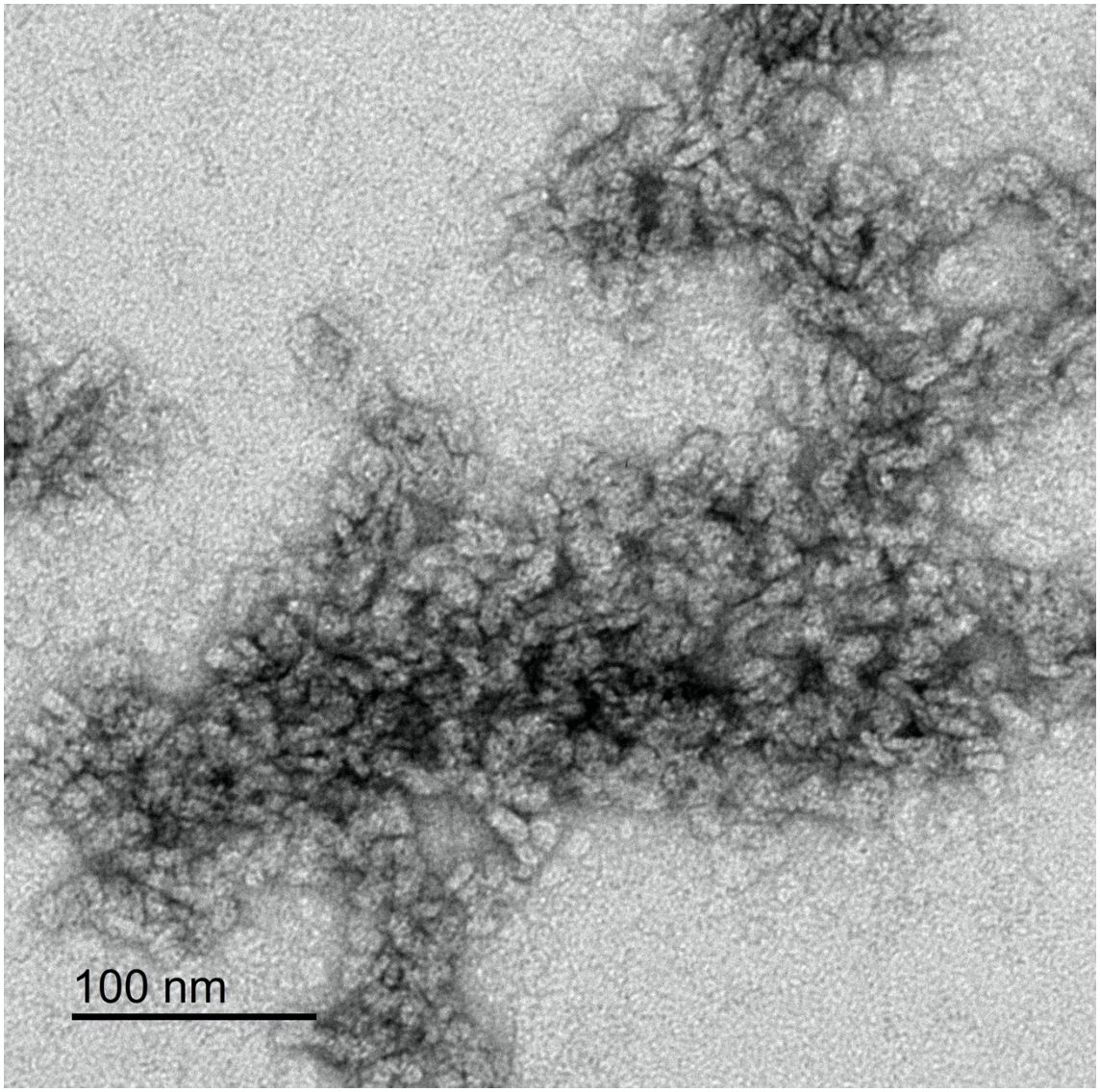


Figure S17 – TEM image of dehydrated Clic1 SMA nanodisc formed in COLO-680N cells lipid extract.

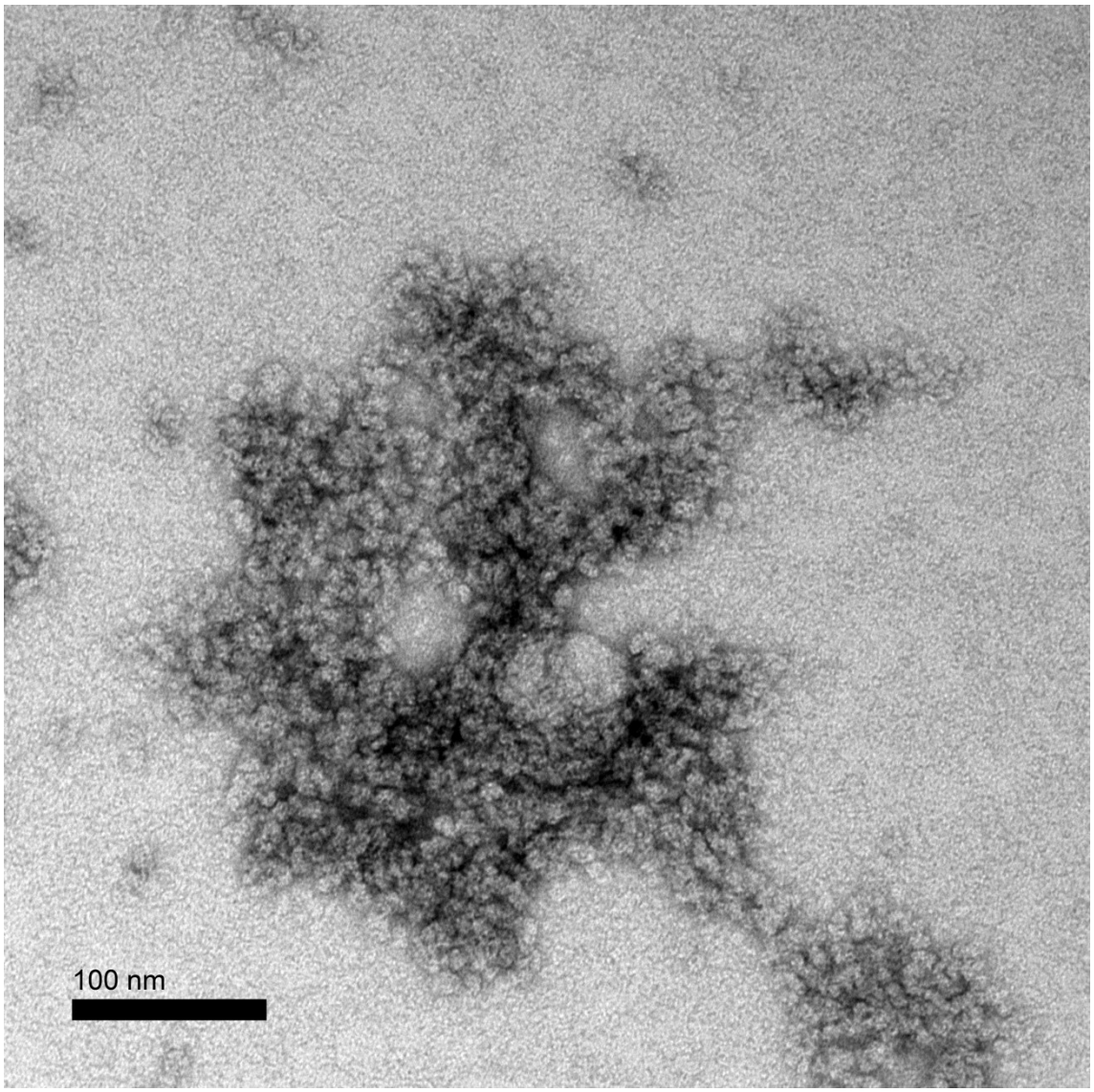


Figure S18 – TEM image of dehydrated SSA compound 1 loaded SMA nanodisc formed in DMPC - *E. coli* lipid extract mixture.

Compound characterisation NMR

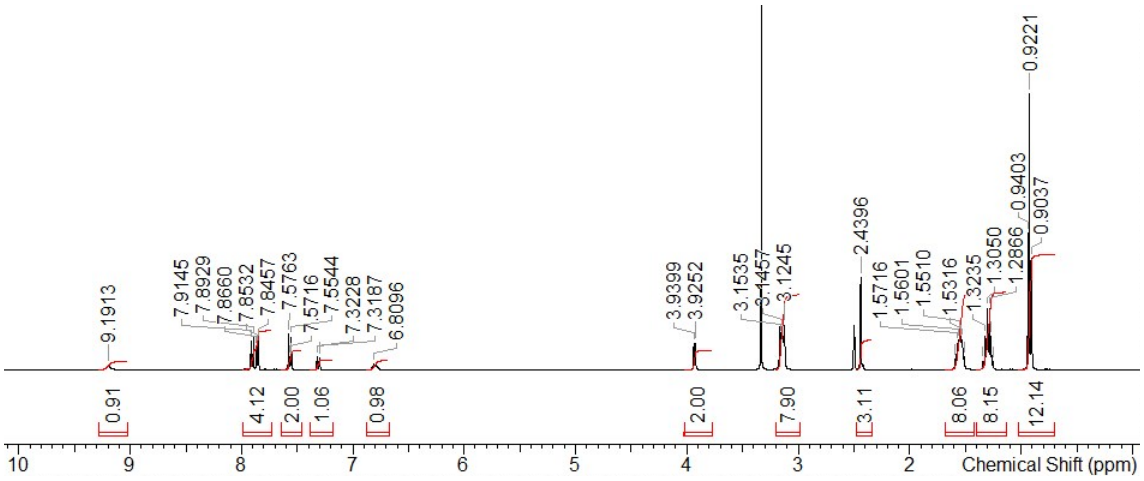


Figure S19 - ^{1}H NMR spectrum of SSA (compound **1)** in DMSO-d_6 conducted at 298.15 K.

Linear fitting of Experimental and Calculated data

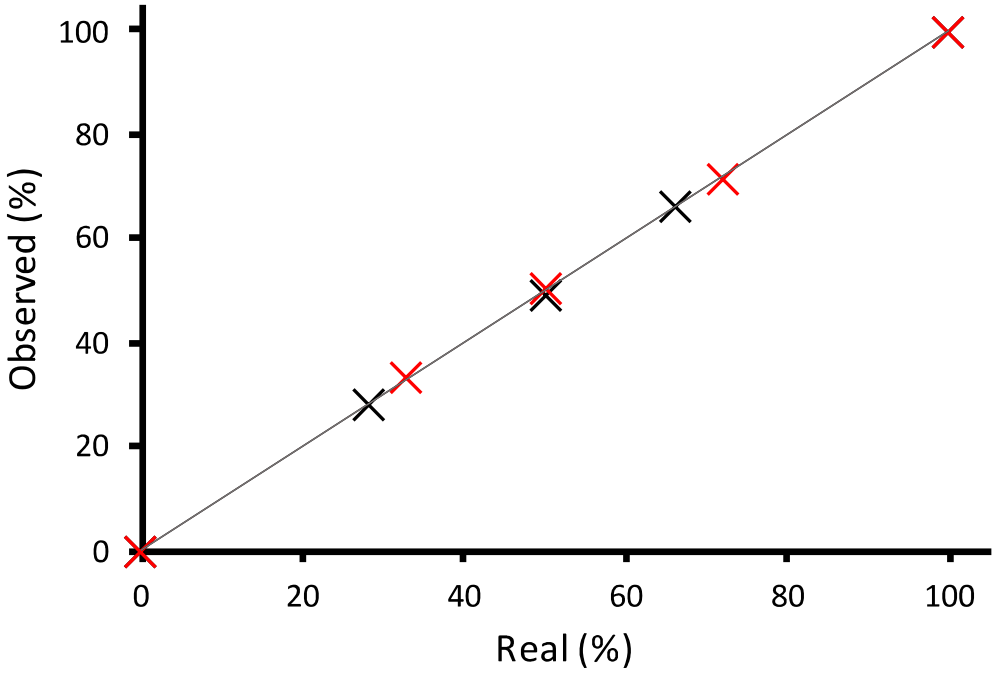


Figure S20 - *Linear fitting of real vs calculated concentrations of PE (black) and PC (red) lipids.*