Electronic Supplementary Information

Zwitterionic Sulfobetaine Lipids that Form Vesicles with Salt-Dependent Thermotropic Properties

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Materials and Methods

DPPC, DSPC, and DSPG were purchased from Avanti Polar Lipid. DiD (D-307) was purchased from Molecular Probes (Eugene, OR). Solvents were purchased from VWR Scientific. All other chemicals were purchased from Sigma Aldrich. All buffers were made with MilliQ water and passed through a filtration system. NMR measurements were taken on a Bruker 300 MHz Avance system and analyzed with Topspin software. Chemical shifts are expressed as parts per million with tetramethylsilane as internal standard. HPFC column purifications were performed on a Reveleris Flash System (Grace Division Biosciences) with pre-packed GraceResolv silica cartridges (67 Å, 40.5 μm). All sonication was performed in a G112SP1 Special Ultrasonic Cleaner from Laboratory Supplies Co., Inc (Hicksville, NY).

Synthesis and Characterization

The SBL library was synthesized from a 3-(dimethylamino)-1,2-propanediol core via a two step synthesis without the need for column purification for the saturated chain versions. After the alkylation with 1,3-propanesultone in the presence of DIPEA, the reaction was washed with 1 M HCl and 1M Na_2CO_3 ,and then precipitated from acetone, hexanes, and acetonitrile sequentially, to afford pure SBL products in moderate yields. Five SBLs were made varying only at the alkyl chains (lauric acid = DLSB (C_{12}), myristic acid = DMSB (C_{14}), palmitic acid = DPSB (C_{16}), stearic acid = DSSB (C_{18}), and oleic acid = DOSB ($C_{18:1}$)). This straightforward synthesis requires relatively inexpensive materials and a purification that could make it suitable for cost-effective scale-up and result in a less expensive alternative to PCLs for some applications.

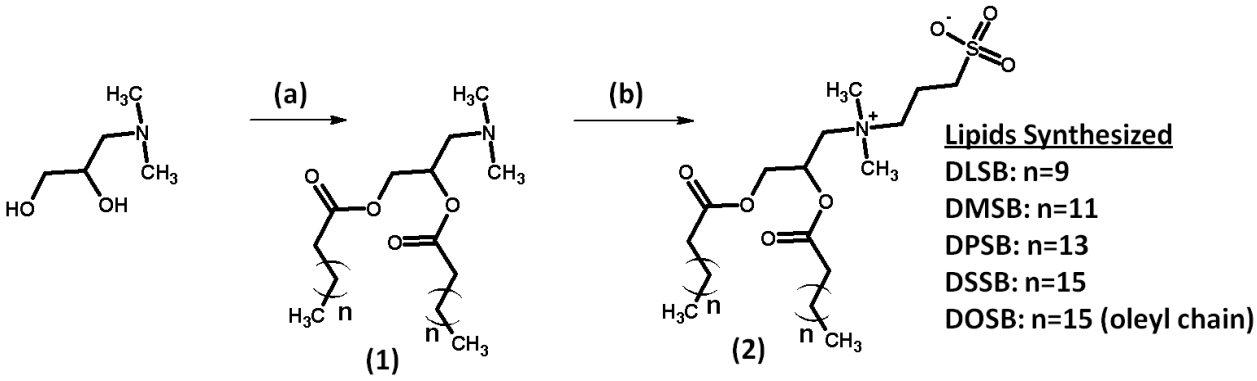

Fig. S1: Chemical Synthesis (a) Cn+3 alkyl acid, DCC, DMAP, CH2Cl2, r.t. 4 hrs. (b) 1,3-Propanesultone, DIPEA, CH2Cl2, MeOH (4:1), 40 °C, 18 hrs.

Synthesis of (1): 1g of N,N-dimethylamino-1,2-propanediol (8.3 mmoles) and 2.2 molar equivalent (18.3 mmoles) of alkyl acid chain (i.e. 4.7 g palmitic acid (MW 256) for DPSB) were dissolved in methylene chloride while stirring at room temperature. Then, 0.1 g 4-dimethylaminopyridine (DMAP) and 2.2 molar equivalent (18.3 mmoles) of *N,N'*-Dicyclohexylcarbodiimide (DCC) (3.78 g) were added and the solution was stirred for 3-4 hours or until complete by TLC. Additional small portions of DCC were added if necessary to drive the reaction to completion. The solution was then filtered to remove the precipitated dicyclohexyuracil and washed 2X with 1M HCl. The organic layer was collected and dried with

sodium sulfate and solvent was removed by rotary evaporation. The di-substituted dimethylaminoglycerol product (1) was moved forward with no further purification.

To all of (1) was added 2 molar equivalents 1,3-propanesultone (2 g, MW 123) with 1.5 mL DIPEA (8.3 mmoles, MW 129) in minimal methylene chloride/methanol (4:1). The reactions were stirred while heating at 40 °C for 18 hours. The solutions were diluted with the reaction solvent mixture and washed with 1M HCl followed by 1 M Na_2CO_3. The organic layer was collected and concentrated under rotary evaporation. DLSB, DMSB, DPSB, DSSB were purified through a series of precipitation from methylene chloride in acetone, acetonitrile, and hexanes. Small amounts of product were lost in each precipitation step and overall yields for the entire synthesis were 20%, 66%, 32%, and 38% for the DLSB, DMSB, DPSB, and DSSB respectively. DOSB could not be purified by precipitation and was instead purified by HPFC, and was eluted with 30% methanol in methylene chloride with a yield of 7.6%. Percent yields were calculated according to: Mass collected following purification / expected mass (starting moles of N,N-dimethylamino-1,2-propanediol X Product Molecular Weight). In the DOSB synthesis, a significant amount of side product with a molecular weight corresponding to two additions of the 1,3-propanesultone was observed on MALDI and it is possible that the 1,3-propanesultone was added across the alkene in one of the oleyl chains.

DLSB: ^{1}H NMR ($CDCl_3$/CD_3OD ~10:3): δ 0.87 (t, 6H); δ 1.26 (m, 32H); δ 1.60 (m, 4H); δ 2.23 (m, 2H); δ 2.32 (m, 4H); δ 2.90 (t, 2H); δ 3.14 (d, 6H); δ 3.66-3.78 (m, 4H); δ 4.05-4.09 (m, 1H); δ 4.43-4.48 (m, 1H); δ 5.60 (m, 1H). MALDI-MS calc'd mass 606.9, found 607.87. Elemental Analysis: 63.4 %C expected, 62.54 % C observed, 10.5 % H expected, 10.72 % H observed, 2.31 % N expected, 2.34 % observed

DMSB: ^{1}H NMR ($CDCl_3$/CD_3OD ~10:3): δ 0.89 (t, 6H); δ 1.27 (m, 40H); δ 1.62 (m, 4H); δ 2.22 (m, 2H); δ 2.38 (m, 4H); δ 2.87 (t, 2H); δ 3.14 (d, 6H); δ 3.59 (m, 2H); δ 3.71 (m, 2H); δ 4.04-4.08 (m, 1H); δ 4.46-4.47 (m, 1H); δ 5.62 (m, 1H). MALDI-MS calc'd mass 663.02, found 664.62. Elemental Analysis: 65.3%C expected, 64.92 % C observed, 10.8 % H expected, 10.54 % H observed, 2.12 % N expected, 2.13 % observed.

DPSB: ^{1}H NMR ($CDCl_3$/CD_3OD ~10:3): δ 0.88 (t, 6H); δ 1.26 (m, 48H); δ 1.60 (m, 4H); δ 2.21 (m, 2H); δ 2.33 (m, 4H); δ 2.88 (t, 2H); δ 3.14 (d, 6H); δ 3.60-3.70 (m, 4H); δ 4.02-4.08 (m, 1H); δ 4.43-4.48 (m, 1H); δ 5.60 (m, 1H). MALDI-MS calc'd mass 719.12, found 721.49. Elemental Analysis: 66.9 %C expected, 66.62 % C observed, 11.09 % H expected, 11.31 % H observed, 1.95 % N expected, 1.99 % observed.

DSSB: ^{1}H NMR ($CDCl_3$/CD_3OD ~10:3): δ 0.83 (t, 6H); δ 1.20 (m, 56H); δ 1.56 (m, 4H); δ 2.15 (m, 2H); δ 2.29 (m, 4H); δ 2.84 (t, 2H); δ 3.07 (d, 6H); δ 3.6 (m, 4H); δ 3.96-4.02 (m, 1H); δ 4.38-4.42 (m, 1H); δ 5.50 (m, 1H). MALDI-MS calc'd mass 775.23, found 776.51. Elemental

Analysis: 68.3 %C expected, 68.08 % C observed, 11.3 % H expected, 11.46 % H observed, 1.81 % N expected, 1.84 % observed.
DOSB: ^{1}H NMR ($CDCl_3$/CD_3OD ~10:3): δ 0.90 (t, 6H); δ 1.30 (m, 40H); δ 1.60 (m, 4H); δ 2.03 (m, 8H); δ 2.33 (m, 6H); δ 2.90 (m, 2H); δ 3.27 (d, 6H); δ 3.6-3.87 (m, 3H); δ 4.00 (m, 1H); δ 4.13 (m, 1H); δ 4.50 (m, 1H); δ 4.36 (m, 4H); δ 5.64 (m, 1H). MALDI-MS calc'd mass 771.20, found 772.88. Elemental Analysis: 68.61 %C expected, 67.4 % C observed, 10.86 % H expected, 10.95 % H observed, 1.82 % N expected, 1.85 % observed.

Carboxyfluorescein (CF) release

The CF encapsulation procedure was adapted from Ohno[1]. Lipid formulations were dried down from chloroform solutions in a 10:3 molar ratio (DiC_{16}Lipid:cholesterol) in a test tube to form a thin film. The thin film was rehydrated in a 10 mM Tris, 100 mM CF, 500 mM NaCl, pH 7.4 to a final concentration of 25 mM DiC_{16}Lipid. Each preparation was sonicated for 10 minutes at 80 °C under argon and then cooled to room temperature. Free carboxyfluorescein was removed by size exclusion chromatography with a PD-10 sephadex column (GE Healthcare) with approximately 10% dilution of the liposome volume. Ten microliters of each liposome solution was added to a well with 200 uL buffer in a 96 well plate. Two isotonic buffers, 10 mM HEPES, 605 mM NaCl, pH 7.4 ("equal salt") and 840 mM HEPES, 150 mM NaCl, pH 7.4 ("low salt"), the first one equal in ionic strength and second with a lower ionic strength were used. Leakage was measured with a FLUOstar plate reader (BMG Labtech) with Ex 485 nm and Em 518 nm over the course of 48 hours. Percent leakage values were calculated by measuring the total CF per well by liposome lysis with $C_{12}E_{10}$ surfactant. All buffer/lipid formulation combinations were run in triplicate. Percent Release was calculated as follows: % Release at time, (t) = (measured fluorescence at time, (t)) / (total fluorescence from lysed liposomes) X 100.

Calcium-Induced Zeta-Potential Shift

Liposome preparations used in the CF release study were also used for these measurements. Twenty microliters of each liposome formulation were added to 3 mL of a 10 mM HEPES, pH 7.4 buffer with various amounts of $CaCl_2$ and NaCl added. NaCl was added along with $CaCl_2$ to maintain a constant ionic strength across all Ca^{2+} concentrations. Ionic strengths were calculated according to the Debye-Hückel model, where the ionic strength (I) = ½(4[Ca^{2+}] + [Cl^-] + [Na^+]), where brackets denote the total concentration of the enclosed ion. Zeta potential measurements were performed on a Malvern Nanosizer using the Smoluchowski modelprovided by Malvern's Nanosizer Software and run in triplicate.

Liposome vesicle formation at various salt types and concentrations

Liposome preparations were rehydrated from a thin film in a test tube to a final concentration of 26 mM lipid with the specified buffer. Liposome preparations were heated with gentle agitation in an 80 °C heat bath for 2 minutes and then sonicated for 7 minutes at 80 °C under argon. Diameter and zeta potential were measured on a Malvern Nanosizer. Mark-

Houwink parameters were used for size measurements and the Smoluchowski model was used for zeta potential measurements as provided by Malvern's Nanosizer software package.

Differential scanning calorimetry, T_m measurements

Liposome formulations were prepared as above for size measurements, with 3, 20 second bursts of sonication instead of a 7 minute sonication to disperse the lipid bilayer fragments[2]. Then 200 uL of each preparation were added into each calorimeter chamber with buffer used in the specific liposome preparation as the standard. The measurements were run on a MC-DSC 4100 (Calorimetry Sciences Corp.) from 10 to 80 °C at 1 degree/minute with a heat-cool-heat cycle where the last heating cycle is reported. Data was processed with CpCalc software and transferred to Excel to be graphed.

TEM imaging

Preparations of DPSB/Cholesterol (10:3 molar ratio) were prepared in the specified buffer through thin film rehydration to a final DPSB concentration of 20 mM. The preparations were sonicated at 80 °C for 5 minutes. Prior to imaging, the liposome solutions were diluted to half the original concentration with the same buffer. Copper Grids with 400 mesh and Formvar/carbon coatings from Structure Probe, Inc (West Chester, PA) were glow discharged prior to use. Liposome solutions were dropped onto the grid and allowed to adsorb for 1 minute. The liposome solution was then wicked away and the grid surface was washed three times with MilliQ water. Then a 1% solution of uranyl acetate in water was dropped on the grid and allowed to sit for 1 minute. The uranyl acetate solution was then wicked away and the grid was washed once with water and excess water was removed and the grid was allowed to dry. TEM images were collected on a FEI Tecnai 12 transmission electron microscope at the Berkeley Electron Microscopy Lab at UC Berkeley, Berkeley, CA.

Thermally-Triggered CF Release

Liposome preparations of DiC_{16}Lipid:chol:PEG_{3000}DSPE 85:10:5 were dried to a thin film in a test tube and rehydrated in either 150 mM NaCl or 150 mM KBr, both with 100 mM CF and 10 mM Tris, pH 7.4 to a concentration of 20 mM DiC_{16}Lipid. The preparations were sonicated at 80 °C for 5 minutes and then cooled to room temperature. Free CF was removed by size exclusion chromatography with a PD-10 sephadex column (GE Healthcare) with mild dilution of the liposome fraction. Two elution buffers were used for each of the initial preparations to investigate the effect of asymmetry of salt form across the bilayer on liposome stability and release. Figure S2 provides a schematic of the liposome formation and purification process. The preparations made in 150 mM KBr were eluted in one of two buffers; one buffer contained 260 mM KBr, 10 mM HEPES, pH 7.4 and the second buffer was 150 mM KBr, 110 mM NaCl, 10 mM HEPES, pH 7.4. The preparations made with 150 mM NaCl were eluted with one of two buffers containing either 260 mM NaCl or 260 mM KBr, both with 10 mM HEPES, pH 7.4. The CF release was then measured for each of the purified liposome samples. Measurements were

made on a Flourolog Fluorimeter (Horiba Scientific) using the kinetic measurement function. For each measurement, 10 μL of the liposome sample was added to 2 mL of a 50% fetal bovine serum (FBS), 50% 10 mM HEPES, 150 mM NaCl, pH 7.4 (HBS) solution. Each measurement lasted for 250 seconds and the liposomes were added 15 seconds into the run to insure capture of the initial release. At 200 seconds, 30 μL of $C_{12}E_{10}$ surfactant (concentration) was added to lyse the liposomes to determine the maximum CF fluorescence. Measurements were done in triplicate and the data was exported to Excel for analysis. Percent Release was calculated as follows: % Release at a time (t) = (measured fluorescence at time (t)) / (total fluorescence from lysed liposomes) X 100.

To measure the release of CF at 37 °C over a longer period of time, release from each preparation made initially in 150 mM KBr was monitored for approximately 4 hours in a 96 well plate. To do this, following the purification of the above liposomes solutions, 10 μL of each liposome solution was added to a well with 200 uL of the 50/50 FBS/HBS buffer in a 96 well plate. The plate was kept in a 37 °C incubator and release was measured with a FLUOstar plate reader (BMG Labtech) with Ex 485 nm and Em 518 nm over the course of 4 hours. Percent release values were calculated by measuring the total CF per well by liposome lysis with 10 μL of $C_{12}E_{10}$ surfactant. All formulations were run in triplicate.

Liposome formation characterization

Table S1 Liposome diameter (nm) and polydispersity index in various NaCl concentrations

Lipid	1000 mM NaCl	500 mM NaCl	150 mM NaCl	0 mM NaCl
DLSB	57, [0.4]	42, [0.3]	1728, [0.7]	DNF
DMSB	77, [0.2]	64, [0.4]	DNF	DNF
DPSB	93, [0.3]	67, [0.3]	DNF	DNF
DSSB	138, [0.4]	123, [0.3]	DNF	DNF
DOSB	119, [0.2]	127, [0.2]	DNF	DNF

Average liposome diameter and polydispersity index, PDI (in brackets), in various concentrations of NaCl. PDI values range from 0-1 with 0 corresponding to a monodisperse population. Liposome preparations were made at 26 mM lipid in 10 mM HEPES buffer, pH 7.4 with the specified salt concentration. All preparations were rehydrated and sonicated at 80 °C for 7 minutes, allowed to cool for 5 minutes and then measured. DNF = Did Not Form, no liposomes formed.

Table S2 Liposome diameter (nm) and polydispersity index in various salts following sonication and at 24 Hours

Lipid	150 mM $NaClO_4$	150 mM $NaClO_4$ (24h)	150 mM NaI	150 mM NaI (24h)	150 mM KBr	150 mM KBr (24h)	150 mM NaF
DLSB	30, [0.2]	37, [0.3]	29, [0.2]	39, [0.2]	55, [0.3]	74, [0.3]	DNF
DMSB	42, [0.2]	44, [0.2]	35, [0.2]	39, [0.3]	29, [0.2]	32, [0.2]	DNF
DPSB	35, [0.2]	43, [0.4]	57, [0.3]	Gel	64, [0.1]	Precip	DNF
DSSB	42, [0.3]	72, [0.3]	43, [0.2]	Gel	102, [0.1]	Precip	DNF
DOSB	68, [0.3]	69, [0.2]	91, [0.2]	99, [0.3]	121, [0.3]	138, [0.4]	DNF

Average liposome diameter and polydispersity index (in brackets) were measured both five minutes after sonication and at 24 hours. PDI values range from 0-1 with 0 corresponding to a monodisperse population. Gel = Formed Gel, Precip = Liposomes formed polydisperse, large chunks and precipitated, DNF= Did Not Form liposomes.

Table S3 Variation in the diameter of DMSB liposomes

Lipid	$NaClO_4$	$NaClO_4$ (24 hrs)	NaI	NaI (24 hrs)	KBr	KBr (24 hrs)
DMSB	35 ± 9.4	40 ± 5.5	33 ± 2.5	42 ± 3	34 ± 4.3	38 ± 5.7

Liposome diameter (nm) ± one standard deviation

Comparison of transition temperatures of SB, PE and PC lipid dispersions

Table S4 Transition temperatures (°C) for hypothesized inner and mobile counter-ion salt SB forms compared to PE and PC headgroups

Chain Length	PE	SB Inner Salt Onset, Peak	PC	SB Mobile Counter-ion Salt Onset, Peak
C14	49.4	45-46, 46-48	24.2	23-24, 24-25
C16	63.5	58-59, 59-60	41.7	40-41, 43
C18	74.4	64-66, 68	55.3	53-56, 56-57

Comparison of the transition temperatures of the SBL to literature values[3] for the phase transition temperatures of PE and PC. SB values correspond to the ranges of transition onset or peak values observed across the four NaCl concentrations.

Reproducibility of DSC measurements for samples run in triplicate or duplicate

Table S5 Average T_m values with standard deviation for either duplicate or triplicate DSC scans

Lipid	NaCl Concentration (mM)	Number of DSC Runs	Low T_m Onset (°C)	Low T_m Peak	High T_m Onset	High T_m Peak
DMSB	500	3	23 ± 1	24 ± 0	45 ± 1.5	47 ± 0.6
DPSB	500	3	40 ± 0.6	42 ± 1.7	57 ± 2.6	58 ± 1.2
DPSB	1000	2	40 ± 2	43 ± 0	Not Pres.	Not Pres.
DSSB	500	2	52 ± 1.4	56 ± 0	Not Pres.	Not Pres.
DSSB	1000	2	53 ± 4	57 ± 7	Not Pres.	Not Pres.

Not Pres. = no peak was observed at the high T_m.

Temperature Jump CF Release Studies

The release of CF from DPPC and DPSB liposomes was examined at three temperatures, 37, 43, and 59 °C. The aim was to determine if liposomes prepared in the presence of 150 mM of two different salts types (NaCl and KBr) would exhibit enhanced release at the transition temperatures reported by DSC (i.e. for DPSB: 59 °C for NaCl and 43 °C for KBr). Additionally, we attempted to establish a salt asymmetry across the bilayer by preparing the liposomes in a solution with one type of salt and then exchanging the buffer with another type through during purification by size exclusion chromatography. The method is depicted in Fig. S2.

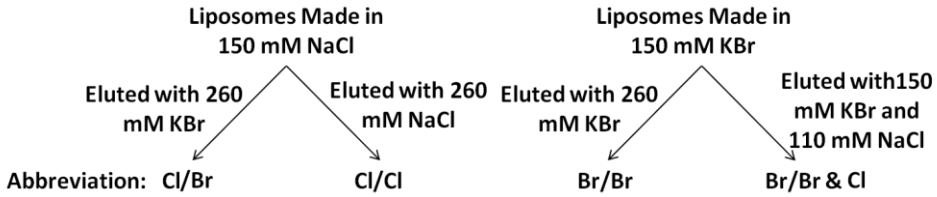

Fig. S2: Schematic explaining the nomenclature for the CF release studies in Fig. S3.

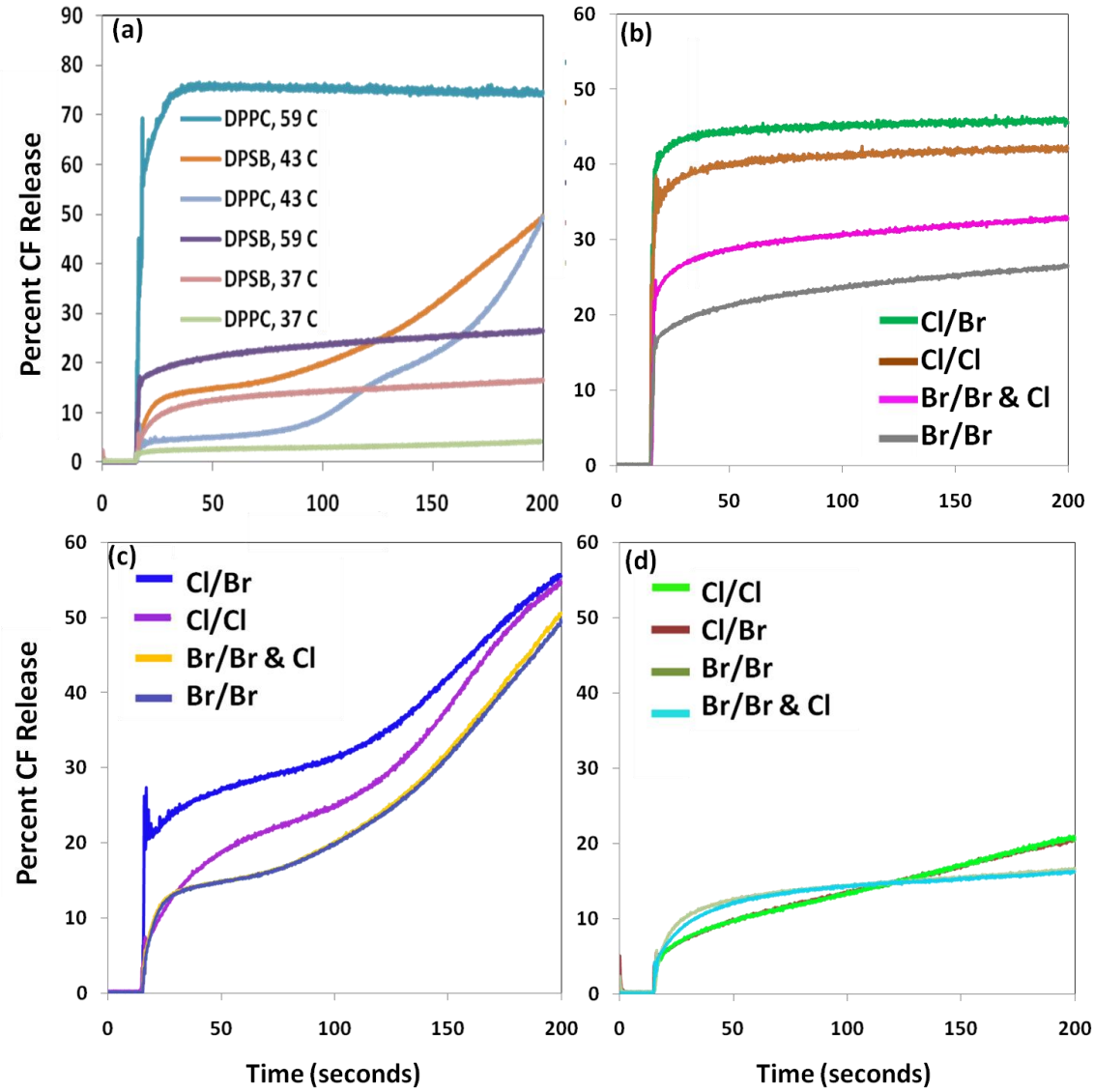

Fig. S3: Thermally-triggered CF release from DPSB and DPPC liposomes. a) Liposomes were prepared as described in the Methods and correspond to the Br/Br preparation in Figure S2. Samples were all run in triplicate with average standard deviations of less than 2% for all experiments except DPPC at 59° C, which had a standard deviation of 18.5%. b-d) DPSB Liposomes were prepared as described in the Methods section and as depicted in Figure S2. Measurements were performed at 59 °C (b), 43 °C (c), and 37 °C (d). Samples were run in triplicate with average standard deviations of less than 3% for all experiments except for "Br/Br & Cl" at 59 °C, "Cl/Br" at 59 °C, and "Cl/Br" at 43 °C which had average standard deviations between 5-7% and "Cl/Cl" at 59 °C which had an average standard deviation of 10.4%.

Fig. S3a shows the CF release profiles of "Br/Br" DPPC and DPSB liposomes at three temperatures, 37, 43 and 59 °C. Fig. S3a reveals two release profiles. Both DPSB and DPPC

show a sudden release followed by a plateau at 59 and 37 °C, but the magnitude of the initial release is greater at 59 °C than at 37 °C for both lipids. At 59 °C, DPPC releases the majority of its contents in the first seconds following liposome addition, while DPSB releases only 20 % initially. Although the cause of the sudden release remains unclear, we believe the initial increase in fluorescence is primarily due to CF release after the addition of the liposomes to the cuvette and is not a result of CF release in the liposome stock solution. We believe this because measurements at different temperatures with the same stock solution did not show the same initial increase in fluorescence (data not shown). The immediate jump in fluorescence could be due to a sudden reorganization of the headgroups and melting of the hydrocarbon chains that permit CF release, followed by the formation of stable state that allows the liposome to retain any remaining encapsulated CF. At 43 °C, both DPSB and DPPC show a different type of release profile characterized by a burst release followed by a slow release phase and finally a rapid CF release. This profile may result from a rapid destabilization caused by headgroup rearrangement, followed by a slower release rate as portions of the bilayer begin to transition, and then an increased release rate when the entire bilayer is at the point of transition. At 43 °C, the DPSB and DPPC preparations show similar releases at the end of the measurement.

Fig. S3b-d show the CF release rates of DPSB liposomes in which a combination of NaCl and KBr were used in the preparations according to Fig. S2. We hypothesized that the "Cl/Cl" liposomes would have the steadiest CF release at the higher T_m (59 °C) and the "Br/Br" liposomes to have the steadiest CF release at the lower T_m (43 °C). However, all the salt combinations had a steady release at 43 °C and not at 59 °C, with the "Cl/Cl" preparation releasing more than either preparation hydrated in KBr. At 59 and 43 °C, all the preparations had similar release profiles with differing maximum values. For all three temperatures, liposomes made with NaCl released more CF than those made with KBr. At 37 °C, both of the liposome preparations hydrated in NaCl exhibited a third type of release profile, with an immediate burst followed by a single steady release phase. Based on the results in Figure S3, it does not appear that the type of salt used in the preparation of the liposomes significantly affects their CF release profile, but liposomes prepared in NaCl are less stable overall than those prepared in KBr. These results do not correlate with the T_m data measured by DSC. DSC measurements were performed predominantly with bilayer fragments, not liposomes, so it is possible that the elevated T_m correspond to an interaction between stacked bilayers. However, many of the DSC measurements show only a low T_m even though bilayer fragments were used.

References

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3. C. Huang, Z.-q. Wang, H.-n. Lin, E. E. Brumbaugh and S. Li, *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1994, **1189**, 7-12.