Phosphate bioisostere containing amphiphiles: a novel class of squaramide-based Lipids

Abhishek Saha,† Subhankar Panda,† Saurav Paul,† and Debasis Manna*  

Department of Chemistry, Indian Institute of Technology Guwahati, Assam 781039, India. Fax: (+) 91-361 258 2349; Tel: (+) 91-361 258 2325; E-mail: dmanna@iitg.ernet.in

Table of contents

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Contents</th>
<th>Page No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Materials and methods</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>Synthesis and characterization of the compounds</td>
<td>3-18</td>
</tr>
<tr>
<td>3</td>
<td>Liposome preparation</td>
<td>19</td>
</tr>
<tr>
<td>4</td>
<td>Transmission electron microscopy</td>
<td>19</td>
</tr>
<tr>
<td>5</td>
<td>Anisotropy measurements</td>
<td>20-21</td>
</tr>
<tr>
<td>6</td>
<td>Microviscosity measurements</td>
<td>21-22</td>
</tr>
<tr>
<td>7</td>
<td>pH dependent zeta potential measurements</td>
<td>23</td>
</tr>
<tr>
<td>8</td>
<td>Calcium-induced zeta potential measurements</td>
<td>23-24</td>
</tr>
<tr>
<td>9</td>
<td>Vesicle leakage study</td>
<td>24</td>
</tr>
<tr>
<td>10</td>
<td>Preparation of giant unilamellar vesicles (GUVs) by electroformation method</td>
<td>25</td>
</tr>
<tr>
<td>11</td>
<td>HPLC Analysis of SA lipids stability against PLA2 and PLC enzymes</td>
<td>26-27</td>
</tr>
<tr>
<td>12</td>
<td>Anisotropic curve of SA2, SA3, SA4 and SA9 lipids</td>
<td>27</td>
</tr>
<tr>
<td>13</td>
<td>Temperature dependent fluorescence lifetime data of DPH in liposomal solution and microviscosity parameter values</td>
<td>28</td>
</tr>
<tr>
<td>14</td>
<td>Microviscosity parameter ($\tau_\eta$) of DPH under the liposomal environment at different temperatures.</td>
<td>29</td>
</tr>
<tr>
<td>15</td>
<td>TEM image of the liposomes generated from 100% SA lipids.</td>
<td>30</td>
</tr>
<tr>
<td>16</td>
<td>Schematic representation for proposed partial aggregation mechanism of SA1 lipid</td>
<td>31</td>
</tr>
<tr>
<td>17</td>
<td>TEM image of the liposomes generated from the lipid mixtures of SA lipids/DPPC/DPPS/cholesterol</td>
<td>32</td>
</tr>
<tr>
<td>18</td>
<td>Fluorescence microscopic images of GUVs generated from pure SA9 lipid</td>
<td>33</td>
</tr>
<tr>
<td>19</td>
<td>Dynamic light scattering measurements</td>
<td>34-37</td>
</tr>
<tr>
<td>20</td>
<td>CF and R6G release profiles from the liposomes</td>
<td>38</td>
</tr>
<tr>
<td>21</td>
<td>R6G release profile from the liposomes of 100 % SA lipids</td>
<td>39</td>
</tr>
<tr>
<td>22</td>
<td>TLC images of the lipids</td>
<td>40</td>
</tr>
<tr>
<td>23</td>
<td>LC-MS analyses of the PLA2 and PLC treated DPPC lipid</td>
<td>41</td>
</tr>
<tr>
<td>24</td>
<td>HPLC-UV signals for PLA2 and PLC treatment of SA1 and SA9 lipids</td>
<td>42</td>
</tr>
<tr>
<td>25</td>
<td>NMR and Mass spectra of the compounds</td>
<td>43-60</td>
</tr>
<tr>
<td>26</td>
<td>References</td>
<td>60</td>
</tr>
</tbody>
</table>
Materials and methods

All reagents were purchased from Sigma (St. Louis, MO), Merck (Mumbai, India), Himedia (Mumbai, India) and used directly without further purification. Dry solvents were obtained according to the reported procedures. Column chromatography was performed using 60–120 mesh silica gel. Reactions were monitored by thin-layer chromatography (TLC) on silica gel 60 F254 (0.25 mm). $^1$H NMR and $^{13}$C NMR spectra were recorded at 600, 400, 151 and 100, respectively using Bruker-600 and Varian AS400 spectrometer. Coupling constants (J values) are reported in Hertz, and chemical shifts are reported in parts per million (ppm) downfield from tetramethylsilane using residual chloroform (d = 7.24 for $^1$H NMR, d = 77.23 for $^{13}$C NMR) as an internal standard. Multiplicities are reported as follows: s (singlet), d (doublet), t (triplet), m (multiplet), and br (broadened). Mass spectra were recorded using a Waters Q-TOF Premier mass spectrometry system, and data were analyzed using the built-in software. 1,2-dipalmitoylsn-glycero-3-phospho-L-serine (DPPS), 1,2-dipalmitoyl-sn-glycero-3-phosphate (DPPA) and 1,2-dipalmitoyl-sn-glycero-3-phospho-(10-rac-glycerol) (DPPG) were purchased from Avanti Polar Lipids (Alabaster, AL). Ultrapure water (Milli-Q system, Millipore, Billerica, MA) was used for the preparation of buffers.
Synthesis and characterization of the compounds:

**Scheme 1.** Synthetic routes to compounds SA1 and SA5 —

Synthesis of benzyl (2,3-dihydroxypropyl) carbamate (1): Benzylchloroformate (1.0 equiv.) was added dropwise to a suspension of 3-amino-1,2-propanediol (1.0 equiv.) and NaHCO₃ (2.0 equiv.) in THF/H₂O (2:1, 75 mL) at 0 °C. The resulting solution was then warmed to room temperature and stirred for 12 h. The reaction mixture was then acidified to pH ≤ 2 with HCl (1 M aq.) and extracted with EtOAc (3 × 100 mL). Combined organic layer was further washed
with brine and dried over anhydrous Na₂SO₄. Removal of the organic solvent under reduced pressure yielded a crude product which was purified by recrystallization (yield 2.39 g, 90%). The compound was characterized by \(^1\)H and \(^{13}\)C NMR and in accordance with the literature.¹

Synthesis of 3-(((benzyl oxy) carbonyl) amino) propane-1,2-diyl dipalmitate (2a): Palmitic acid (2.0 equiv.), dicyclohexylcarbodiimide (2.2 equiv.) and N,N-dimethylaminopyridine (0.1 equiv.) were added to a solution of N-Cbz protected diol (1.0 equiv.) in anhydrous dichloromethane (30 mL) under the N₂ atmosphere. The reaction mixture was then allowed to stir for 12 h at room temperature. After completion of the reaction, the reaction mixture was filtered and washed (3 × 20 mL) with dichloromethane. The filtrate was concentrated under reduced pressure and column chromatography with silica gel and a gradient solvent system of 0–10% ethyl acetate to hexane yielded the corresponding esters.² Characterization of the compound 2a: white solid (85% yield); \(^1\)H NMR (600MHz, CDCl₃) \(\delta_{ppm}\) 7.32-7.28 (m, 5H), 5.06 (s, 2H), 5.01-4.98 (m, 1H), 4.26-4.22 (m, 1H), 4.12-4.05 (m, 1H), 3.48-3.35 (m, 2H), 2.32-2.24 (m, 4H), 1.57-1.55 (m, 4H), 1.27-1.14 (m, 48H), 0.86-0.83 (m, 6H) ; \(^{13}\)C NMR (151 MHz, CDCl₃) \(\delta_{ppm}\) 173.7, 173.4, 156.6, 136.5, 128.8, 128.4, 128.3, 128.7, 70.5, 67.2, 62.7, 41.8, 34.4, 34.3, 32.2, 29.92, 29.89, 29.86, 29.7, 29.6, 29.5, 29.34, 29.31, 25.1, 24.9, 22.9, 14.4; HRMS (ESI) calcd. for C₄₃H₇₅NO₆[M + H]⁺: 702.5594, found: 702.5590.

Synthesis of 3-(((benzyl oxy)carbonyl)amino)propane-1,2-diyl (9E,9'E)-bis(octadec-9-enoate) (2b): Oleic acid (2.0 equiv.), dicyclohexylcarbodiimide (2.2 equiv.) and N,N-dimethylaminopyridine (0.1 equiv.) were added to a solution of N-Cbz protected diol (1.0 equiv.) in anhydrous dichloromethane (30 mL) under the N₂ atmosphere. The reaction mixture was then
allowed to stir for 12 h at room temperature. After completion of the reaction, the reaction mixture was filtered and washed (3 × 20 mL) with dichloromethane. The filtrate was concentrated under reduced pressure and column chromatography with silica gel and a gradient solvent system of 0–10% ethyl acetate to hexane yielded the corresponding esters.\textsuperscript{2}

**Characterization of the compound 2b:** colorless oil (80% yield); \textsuperscript{1}H NMR (600MHz, CDCl\textsubscript{3}) \(\delta_{\text{ppm}} 7.35-7.31\) (m, 5H), 5.37-5.34 (m, 4H), 5.09 (s, 2H), 5.05-5.04 (m, 1H), 4.28-4.26 (m, 1H), 4.14-4.11 (m, 1H), 3.48-3.39 (m, 2H), 2.35-2.28 (m, 4H), 2.06-2.00 (m, 8H), 1.64-1.60 (m, 4H), 1.29-1.26 (m, 44H), 0.89-0.86 (m, 6H); \textsuperscript{13}C NMR (151 MHz, CDCl\textsubscript{3}) \(\delta_{\text{ppm}} 173.7, 173.4, 156.6, 136.5, 130.4, 130.2, 129.9, 128.4, 128.3, 70.5, 67.2, 62.7, 41.5, 34.4, 34.2, 34.1, 32.1, 31.7, 30.0, 29.9, 29.8, 29.7, 29.5, 29.4, 29.32, 29.29, 29.26, 27.4, 27.36, 25.8, 25.0, 24.9, 22.9, 22.8, 14.3; HRMS (ESI) calcd. for C\textsubscript{47}H\textsubscript{79}NO\textsubscript{6} [M + H]\textsuperscript{+}: 754.5907, found: 754.5923.

**Synthesis of 3-aminopropane-1,2-diyl dipalmitate (3a):** 3-Aminopropane-1,2-diyl dipalmitate compound was synthesized by the hydrogenation of compound 2a in the presence of 10 mol % Pd/charcoal (stirred for 14 hours at room temperature) according to the literature procedure and used without further purification.\textsuperscript{1}

**Synthesis of 3-aminopropane-1,2-diyl (9E,9'E)-bis(octadec-9-enoate) (3b):** 3-Aminopropane-1,2-diyl (9E,9'E)-bis(octadec-9-enoate) compound was synthesized by the hydrogenation of compound 2b in the presence of 10 mol % Pd/charcoal (stirred for 14 hours at room temperature) according to the literature procedure and used without further purification.\textsuperscript{1}
Synthesis of tert-butyl (2-aminoethyl)carbamate (4): To a solution of di-tert-butyl dicarbonate (1.0 equiv.) in 1,4-dioxane (10 mL) a solution of ethylenediamine (3.5 equiv.) in 30 mL 1,4-dioxane was added dropwise at 0 °C. After that the solution was warmed up to room temperature and allowed to stir for 12 hours. The white precipitate was removed by filtration and clear oil was obtained by removing the organic solvent under reduced pressure (yield, 90 %). This mono-Boc protected ethylene diamine was used without further purification. The compound was characterized by $^1$H and $^{13}$C NMR and HRMS and in accordance with the literature.

Synthesis of 2-((tert-butoxycarbonyl)amino)-N,N,N-trimethylethan-1-aminium (5): To a solution of mono-boc protected ethylene diamine (1.0 equiv.) in MeCN (30 mL) and K$_2$CO$_3$ (4.0 equiv.) was added and mixture was continued to stir for 30 min at room temperature. After that methyl iodide (3.3 equiv.) was added and the reaction mixture was heated under reflux condition for 20 hours. After that the reaction mixture was cooled down to room temperature and K$_2$CO$_3$ was removed by filtration. Removal of organic solvent under reduced pressure yielded an oily crude product. Addition of diethyl ether to this oily crude product yielded the target product as colorless solid, which was filtered (yield, 80 %). The compound was characterized by $^1$H and $^{13}$C NMR and HRMS and in accordance with the literature.

Synthesis of 2-amino-N,N,N-trimethylethan-1-aminium (6): To a solution of compound 5 (1.10 g, 1 equiv.) in dichloromethane (9 mL) TFA (1 mL) was added dropwise at 0 °C (using an ice-salt bath) and stirred for 30 min. After that the reaction mixture was warmed up to room temperature and stirring was continued for another 3 hours. The solvent was removed under reduced pressure and then diethyl ether (10 mL) was added into the oily liquid and the solid was
filtered out to get the target product (yield, 80%). The compound was characterized by $^1$H and $^{13}$C NMR and HRMS and in accordance with the literature.

**Synthesis of 2-((2-ethoxy-3,4-dioxocyclobut-1-en-1-yl)amino)-N,N,N-trimethylethan-1-aminium (7):** In an oven dried round bottom flask 2-amino-N,N,N-trimethylethan-1-aminium (1.0 equiv.) was first added and then it was dissolved in 4 ml of ethanol. Diethyl squarate (1.1 equiv.) was then added drop wise into the reaction mixture and allowed to stir at room temperature for 4 hours. After completion of the reaction, the mixture was washed with diethyl ether and then it was dried under reduced pressure, which yielded a white solid compound. \(^4\)

**Characterization of compound 7:** white solid (yield, 80%); $^1$H NMR (600MHz, DMSO-$d_6$) $\delta$ ppm 9.78 (br s, 1H), 4.56-4.48 (m, 4H), 3.22-3.08 (m, 2H), 1.20-1.18 (m, 12H); $^{13}$C NMR (151 MHz, DMSO-$d_6$) $\delta$ ppm 194.5, 53.0, 51.7, 45.9, 8.7. HRMS (ESI) calcd. for C$_{11}$H$_{19}$N$_2$O$_3$ [M$^+$]: 227.1396, found: 227.1325.

**Synthesis of 2-((2-((2,3-bis(palmitoyloxy)propyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)-N,N,N-trimethylethanaminium (SA1) :** In an oven dried round bottom flask compound 7 (1.0 equiv.) and compound 3a (1.1 equiv.) were dissolve in 3.5 ml of ethanol. Then DIPEA (5.5 equiv.) was added to the reaction mixture and the reaction mixture was allowed to stir at room temperature for 12 hours. After completion of the reaction, the solvent was removed under reduced pressure. Then the reaction mixture was washed with a mixture of diethyl ether and dichloromethane (for several times) and the solid residue was dried under reduced pressure.

**Characterization of the compound SA1:** white solid (yield, 70%); $^1$H NMR (400MHz, CDCl$_3$) $\delta$ ppm 6.24 (br s, 1H), 4.16-4.12 (m, 1H), 4.08-4.03 (m, 1H), 3.96-3.93 (m, 1H), 3.77 (br s, 1H),
3.57-3.51 (m, 1H), 3.25-3.16 (m, 3H), 2.35-2.19 (m, 6H), 1.65-1.57 (m, 4H), 1.43 (s, 9H), 1.29-1.22 (m, 48H), 0.89-0.86 (t, 6H); \(^{13}\)C NMR (151 MHz, CDCl\(_3\)) \(\delta_{ppm}\) 199.2, 175.2, 69.5, 65.9, 53.7, 42.9, 42.1, 36.8, 34.4, 32.1, 29.9, 29.8, 29.7, 29.6, 25.9, 22.9, 14.3. MS (ESI) calcd. for C\(_{44}H_{82}N_3O_6\) [M + H]\(^+\): 748.6198, found: 748.5309.

Synthesis of 2-((2-(2,3-bis(((E)-octadec-9-enoyl)oxy)propyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)-N,N,N-trimethylethan-1-aminium (SA5): In an oven dried round bottom flask compound 7 (1.0 equiv.) and compound 3b (1.1 equiv.) were dissolve in 3.5 ml of ethanol. Then DIPEA (5.5 equiv.) was added to the reaction mixture and the reaction mixture was allowed to stir at room temperature for 12 hours. After completion of the reaction, the solvent was removed under reduced pressure. Then the reaction mixture was washed with a mixture of diethyl ether and dichloromethane (for several times) and the solid residue was dried under reduced pressure.

Characterization of the compound SA5: white solid (yield, 60%); \(^1\)H NMR (600 MHz, CDCl\(_3\) + MeOD\(-d_4\) + DMSO\(-d_6\)) \(\delta_{ppm}\) 7.84 (br s, 1H), 5.38 (br s, 1H), 5.12-5.08 (m, 4H), 4.09-4.02 (m, 1H), 3.70-3.62 (m, 1H), 3.39-3.31 (m, 2H), 3.23-3.19 (m, 2H), 2.64-2.62 (m, 2H), 2.34-2.30 (m, 4H), 1.62-1.58 (m, 4H), 1.39-1.22 (m, 61H), 0.91-0.87 (m, 6H); \(^{13}\)C NMR (100 MHz, CDCl\(_3\) + MeOD\(-d_4\) + DMSO\(-d_6\)) \(\delta_{ppm}\) 197.0, 173.6, 173.3, 137.2, 128.7, 128.1, 70.6, 66.5, 63.1, 54.5, 42.7, 34.3, 34.2, 32.1, 29.8, 29.6, 29.5, 29.2, 25.0, 22.8, 14.2. MS (ESI) calcd. for C\(_{48}H_{86}N_3O_6\) [M + 3]\(^+\): 803.6558, found: 803.3310.
Scheme 2. Synthetic routes to compounds SA2 and SA6 —

**Synthesis of (benzyloxy) carbonyl asparagine (8):** To a stirred and ice-cooled suspension of L-asparagine (1.0 equiv.) in THF/H₂O (2:1, 75 mL) at 0 °C, NaHCO₃ (2.0 equiv.) and benzylchloroformate (1.1 equiv.; added dropwise). The resulting solution was then warmed to room temperature and stirred for 12 h. The reaction mixture was then acidified to pH ≤ 2 with HCl (1 M aq.) and extracted with EtOAc (3 × 100 mL). Combined organic layer was further washed with brine and dried over anhydrous Na₂SO₄. Removal of the organic solvent under reduced pressure yielded the solid product which and crystallized from methanol (yield, 80 %). This product was used for next reaction without further purification.
Synthesis of 3-amino-2-(((benzyloxy) carbonyl) amino) propanoic acid (9): To the suspension of (benzyloxy) carbonyl) asparagine (1.0 equiv.) in a solvent mixture of EtOAc/CH₃CN/H₂O (12:12:6 mL) at 16 °C, iodobenzene diacetate (1.2 equiv.) was added and stirred for 30 min. Then the reaction mixture was warmed to room temperature and stirring was continued for another 4 hours. After completion of the reaction the mixture was cooled to 5 °C and the solid product was filtered, washed with ethyl acetate (10 mL), and dried under reduced pressure to give the target compounds with 85 % yield. The compound was characterized by ¹H and ¹³C NMR and in accordance with the literature.

Synthesis of 2-(((benzyloxy)carbonyl)amino)-3-((2-ethoxy-3,4-dioxocyclobut-1-en-1-yl)amino) propanoic acid (10): To an oven dried round bottom flask compound 9 (1.0 equiv.) was first added and then it was dissolve in 5 ml ethanol. After that diethyl squarate (1.1 equiv.) was added drop wise to the reaction mixture allowed to stir at room temperature for 4 hours. After completion of the reaction the mixture was washed with diethyl ether and then dried under reduced pressure to give a white semisolid compound. Characterization of the compound 10: yellowish-white semi solid (yield, 70%); ¹H NMR (600MHz, DMSO-d₆) δ ppm 7.36-7.32 (m, 5H), 6.55 (s, 1H), 5.04 (s, 2H), 4.67-4.63 (m, 4H), 4.47-4.46 (m, 1H), 1.38-1.36 (m, 3H); ¹³C NMR (151 MHz, DMSO-d₆) δ ppm 189.8, 184.3, 129.0, 128.4, 128.37, 70.8, 40.7, 16.0. HRMS (ESI) calcd. for C₁₇H₁₈N₂O₇ [M + H]⁺: 363.1145, found: 363.1160.

Synthesis of 2-(((benzyloxy) carbonyl) amino)-3-((2-((2,3-bis (palmitoyloxy) propyl) amino)-3,4-dioxocyclobut-1-en-1-yl) amino) propanoic acid (11a): In an oven dried round bottom flask, compound 9 (1.0 equiv.), and compound 3a (1.1 equiv.), were dissolved in 2.5 ml of
ethanol. Then DIPEA (5.5 equiv.) was added to the reaction mixture and allowed to stir at room temperature for 12 hours. After completion of the reaction, the solvent was removed under reduced pressure. Then the solid residue was washed with diethyl ether and dichloromethane (1:1) for several times. After that the remaining solid residue was dried under reduced pressure and the target product was obtained. **Characterization of the compound 11a:** white solid (yield, 65 %); $^1$H NMR (600MHz, CDCl$_3$) $\delta_{ppm}$ 7.33-7.31 (m, 5H), 5.08 (s, 2H), 4.74-4.68 (m, 1H), 4.27-4.26 (m, 1H), 4.12-4.10 (m, 1H), 3.46-3.48 (m, 1H), 3.16-3.14 (m, 4H), 2.29-2.28 (m, 4H), 1.62-1.57 (m, 4H), 1.29-1.20 (m, 52H), 0.87-0.85 (m, 6H) ; $^{13}$C NMR (151 MHz, CDCl$_3$) $\delta_{ppm}$ 189.6, 173.7, 173.4, 156.5, 136.4, 128.7, 128.4, 128.3, 70.4, 67.1, 62.7, 46.0, 41.5, 34.4, 34.2, 32.1, 29.9, 29.8, 29.7, 29.53, 29.45, 29.3, 26.5, 25.0, 22.9, 14.3. MS (ESI) calcd. for C$_{50}$H$_{81}$N$_{3}$O$_{10}$[M + H]$^+$: 884.5968, found: 884.5309.

**Synthesis of 2-(((benzyloxy)carbonyl) amino) -3- ((2-((2,3-bis (((E) - octadec-9-enoyl)oxy)propyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)propanoic acid (11b):** In an oven dried round bottom flask, compound 9 (1.0 equiv.), and compound 3b (1.1 equiv.), were dissolved in 2.5 ml of ethanol. Then DIPEA (5.5 equiv.) was added to the reaction mixture and allowed to stir at room temperature for 12 hours. After completion of the reaction, the solvent was removed under reduced pressure. Then the solid residue was washed with diethyl ether and dichloromethane (1:1) for several times. After that the remaining solid residue was dried under reduced pressure and the target product was obtained. **Characterization of the compound 11b:**

As a white solid with 55% yield; $^1$H NMR (600 MHz, CDCl$_3$ + MeOD-$d_4$) $\delta_{ppm}$ 7.32-7.27 (m, 5H), 5.06 (s, 2H), 4.68-4.61 (m, 4H), 4.32-4.31 (m, 1H), 4.09-4.01 (m, 1H), 3.90-3.83 (m, 1H), 3.72-3.64 (m, 2H), 3.42-3.38 (m, 1H), 3.19-3.15 (m, 2H), 2.34-2.27 (m, 4H), 1.60-1.57 (m, 4H),
1.40-1.35 (m, 8H), 1.29-1.26 (m, 44H), 0.88-0.86 (m, 6H); $^{13}$C NMR (151 MHz, CDCl$_3$ + MeOD-$d_4$) $\delta_{ppm}$ 188.9, 183.2, 176.9, 173.4, 156.0, 136.7, 128.44, 128.38, 128.0, 127.9, 127.7, 127.68, 69.7, 66.6, 66.57, 53.7, 42.0, 31.9, 29.7, 29.6, 29.5, 29.3, 22.7, 15.8, 15.3, 14.3; MS (ESI) calcd. for C$_{54}$H$_{85}$N$_3$O$_{10}$ [M + H]$^+$: 936.6235, found: 936.6438.

Synthesis of 2-amino-3-((2-(2,3-bis(palmitoyloxy)propyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)propanoic acid (SA2): Compound SA2 was synthesized by the hydrogenation of compound 11a in the presence of 10 mol % Pd/charcoal (stirred for 14 hours at room temperature) according to the literature procedure.\textsuperscript{1} Characterization of the compound SA2: white solid (yield, 70 %); $^1$H NMR (600MHz, CDCl$_3$) $\delta_{ppm}$ 12.10 (br, s, 1H), 5.25 (br, s, 2H), 4.63-4.59 (m, 1H), 4.06-3.99 (m, 1H), 3.63-3.55 (m, 1H), 2.96-2.93 (m, 4H), 2.48-2.25 (m, 4H), 1.58-1.46 (m, 4H), 1.28-1.19 (m, 56H), 0.87-0.77 (m, 6H); $^{13}$C NMR (151 MHz, CDCl$_3$) $\delta_{ppm}$ 194.9, 188.9, 177.2, 174.4, 69.9, 65.2, 46.6, 34.3, 34.0, 31.8, 29.58, 29.55, 29.4, 29.3, 29.2, 29.1, 24.7, 22.6, 13.9.; MS (ESI) calcd. for C$_{42}$H$_{75}$N$_3$O$_8$ [M]$^+$: 749.5554, found: 749.5329.

Synthesis of 2-amino-3-((2-((2,3-bis( (E)-octadec-9-enoyl)oxy )propyl) amino)-3,4-dioxocyclobut-1-en-1-yl)amino)propanoic acid (SA6): Compound SA6 was synthesized by the hydrogenation of compound 11b in the presence of 10 mol % Pd/charcoal (stirred for 14 hours at room temperature) according to the literature procedure.\textsuperscript{1} Characterization of the compound SA6: As a white solid with 50% yield; $^1$H NMR (600 MHz, CDCl$_3$) $\delta_{ppm}$ 10.38 (br s, 1H), 5.11-5.01 (m, 4H), 4.70-4.55 (m, 2H), 4.30-4.27 (m, 1H), 3.84-3.80 (m, 1H), 3.66-3.60 (m, 2H), 3.48-3.33 (m, 2H), 3.08-3.02 (m, 4H), 1.45-1.32 (m, 12H), 1.29-1.16 (44H), 0.87-0.83 (6H); $^{13}$C NMR (151 MHz, CDCl$_3$ + MeOD-$d_4$) $\delta_{ppm}$ 191.8, 187.8, 183.0, 173.4, 172.2, 128.7, 128.1, 70.1, 67.0,
Scheme 3. Synthetic routes to compounds SA3 and SA7 —

Synthesis of 3-((2,3-dihydroxypropyl)amino)-4-ethoxycyclobut-3-ene-1,2-dione (12): To an oven dried round bottom flask 3-aminopropane-1,2-diol (1 equiv.) and 5 mL of ethanol were added sequentially. Then diethyl squarate (1.1 equiv.) was added dropwise to the reaction mixture and allowed to stir at room temperature for 4 hrs. After completion of the reaction, the reaction mixture was successively washed with diethyl ether and then dried under reduced pressure, which produced a yellow sticky compound. Characterization of the compound 12: white semi solid (yield, 75%); $^1$H NMR (600MHz, CDCl3 + MeOD-d4 + DMSO-d6) $\delta_{ppm}$ 8.24 (br s, 1H), 3.77-3.64 (m, 1H), 3.48-3.39 (m, 2H), 3.11-3.06 (m, 4H), 1.19-1.15 (m, 3H); $^{13}$C NMR (151 MHz, CDCl3 + MeOD-d4 + DMSO-d6) $\delta_{ppm}$ 204.3, 75.7, 72.8, 72.2, 68.5, 67.9, 50.8, 13.2; MS (ESI) calcd. for C$_9$H$_{13}$NO$_5$ [M+H]$^+$: 215.0794, found: 215.0766.

Synthesis of 3-((2,3-dihydroxypropyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)propane-1,2-diyl dipalmitate (SA3): In an oven dried round bottom flask, compound
12 (1.0 equiv.), and compound 3a (1.1 equiv.), were dissolved in 2.5 ml of ethanol. Then DIPEA (5.5 equiv.) was added to the reaction mixture and allowed to stir at room temperature for 12 hours. After completion of the reaction, the solvent was removed under reduced pressure. Then the solid residue was washed with diethyl ether and dichloromethane (1:1) for several times. After that the remaining solid residue was dried under reduced pressure and the target product was obtained. **Characterization of the compound SA3:** As a white solid with 55 % yield; \(^1\)H NMR (600MHz, CDCl\(_3\) + DMSO-\(d_6\)) \(\delta\)ppm 6.74 (br s, 1H), 4.92 (br s, 1H), 4.06-3.99 (m, 1H), 3.63-3.55 (m, 1H), 2.96-2.93 (m, 4H), 2.48-2.25 (m, 4H), 1.58-1.46 (m, 4H), 1.28-1.19 (m, 56H), 0.87-0.77 (m, 6H); \(^{13}\)C NMR (151 MHz, CDCl\(_3\) + DMSO-\(d_6\)) \(\delta\)ppm 197.4, 174.6, 174.0, 68.9, 65.9, 53.6, 42.8, 42.1, 36.6, 34.2, 32.0, 29.73, 29.69, 29.6, 29.5, 29.46, 29.39, 29.3, 29.2, 25.8, 25.0, 22.7, 18.3, 14.2; MS (ESI) calcd. for C\(_{42}\)H\(_{76}\)N\(_2\)O\(_8\) [M+H]+: 737.5635, found: 737.2932.

**Synthesis of 3-((2-((2,3-dihydroxypropyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)propane-1,2-diyl (9E,9'E)-bis(octadec-9-enoate) (SA7):** In an oven dried round bottom flask, compound 12 (1.0 equiv.), and compound 3b (1.1 equiv.), were dissolved in 2.5 ml of ethanol. Then DIPEA (5.5 equiv.) was added to the reaction mixture and allowed to stir at room temperature for 12 hours. After completion of the reaction, the solvent was removed under reduced pressure. Then the solid residue was washed with diethyl ether and dichloromethane (1:1) for several times. After that the remaining solid residue was dried under reduced pressure and the target product was obtained. **Characterization of the compound SA7:** As a white solid with 50% yield; \(^1\)H NMR (600 MHz, CDCl\(_3\) + MeOD-\(d_4\)) \(\delta\)ppm 7.50 (br, s, 1H), 5.28 (br, s, 1H), 5.04-5.00 (m, 4H), 4.24-4.22 (m, 1H), 4.02-4.01 (m, 1H), 3.66-3.64 (m, 2H), 3.51-3.49 (m, 1H), 3.35-3.33 (m, 1H), 3.10-3.08 (m, 2H), 2.24-2.21 (m, 4H), 1.54-1.51 (m, 4H), 1.32-1.30 (m, 8H),
1.25-1.17 (m, 44H), 0.80-0.78 (m, 6H); $^{13}$C NMR (151 MHz, CDCl$_3$ + MeOD-$d_4$) $\delta_{ppm}$ 199.9, 173.6, 173.4, 136.2, 128.0, 127.4, 70.1, 66.3, 62.5, 53.8, 45.7, 42.0, 40.6, 33.8, 31.5, 29.3, 29.1, 28.9, 28.7, 24.5, 22.2, 18.4, 13.4; MS (ESI) calcd. for C$_{46}$H$_{80}$N$_2$O$_8$ [M+4]$^+$: 792.5915, found: 792.6092.

**Scheme 4.** Synthetic routes to compounds SA4 and SA8 —

**Synthesis of 3-((2-hydroxy-3,4-dioxocyclobut-1-en-1-yl) amino) propane-1,2-diyl dipalmitate (SA4):** Squaric acid (0.40 mmol) and compound 3a (0.44 mmol) were placed in an oven dried round bottom flask. Then, the reaction mixture was dissolved in 3.5 ml of toluene and allowed to reflux at 70 °C for 16 hrs. The progress of the reaction was monitored by thin layer chromatography. After completion of the reaction the organic solvent was removed under reduced pressure. The obtained white solid product was washed with diethyl ether and further column chromatography with silica gel and a gradient solvent system of 0–10% ethyl acetate to hexane yielded the target product. The isolated product was characterized by $^1$H and $^{13}$C NMR and LCMS analysis. **Characterization of the compound SA4:** white solid (75% yield); $^1$H NMR (400 MHz DMSO-$d_6$) $\delta_{ppm}$ 3.92-3.87 (m, 2H), 3.71-3.68 (m, 1H), 3.19-3.07 (m, 2H), 2.25-
2.23 (m, 2H), 2.07-2.03 (m, 2H), 1.50-1.47 (m, 4H), 1.31-1.12 (m, 56H) 0.83-0.80 (m, 6H); $^{13}$C NMR (100 MHz, CDCl$_3$ + MeOD-$d_4$) $\delta_{ppm}$ 194.4, 187.2, 180.5, 174.4, 167.3, 62.2, 52.3, 46.0, 45.9, 38.0, 34.2, 31.7, 29.7, 29.3, 29.1, 29.0, 24.8, 22.4, 20.8, 13.7; MS (ESI) calcd. for C$_{39}$H$_{69}$NO$_7$ [M]$^+$: 663.5074, found: 663.4414.

**Synthesis of 3-((2-hydroxy-3,4-dioxocyclobut-1-en-1-yl)amino)propane-1,2-diyl (9E,9'E)-bis(octadec-9-enoate) (SA 8):** Squaric acid (0.40 mmol) and compound 3b (0.44 mmol) were placed in an oven dried round bottom flask. Then, the reaction mixture was dissolved in 3.5 ml of toluene and allowed to reflux at 70 °C for 16 hrs. The progress of the reaction was monitored by thin layer chromatography. After completion of the reaction the organic solvent was removed under reduced pressure. The obtained white solid product was washed with diethyl ether and further column chromatography with silica gel and a gradient solvent system of 0–10% ethyl acetate to hexane yielded the target product. The isolated product was characterized by $^1$H and $^{13}$C NMR and LCMS analysis. **Characterization of the compound SA8:** white solid (70% yield); $^1$H NMR (600 MHz, CDCl$_3$) $\delta_{ppm}$ 5.37 (br s, 1H), 5.12-5.09 (m, 4H), 5.02-4.98 (m, 1H), 4.29-4.25 (m, 1H), 4.15-4.10 (m, 1H), 3.49-3.47 (m, 2H), 2.32-2.27 (m, 4H), 1.98-1.94 (m, 4H), 1.83-1.59 (m, 8H), 1.30-1.18 (m, 44H), 0.89-0.86 (m, 6H); $^{13}$C NMR (100 MHz, CDCl$_3$) $\delta_{ppm}$ 175.8, 175.5, 162.4, 117.9, 116.0, 71.3, 63.6, 63.0, 52.8, 45.8, 42.2, 41.0, 36.6, 32.0, 29.8, 29.7, 29.5, 29.4, 25.9, 22.8, 22.6, 14.2; MS (ESI) calcd. for C$_{43}$H$_{73}$NO$_7$ [M$^{+3}$+NH$_4$]$^+$: 736.5731, found: 736.4954.
Scheme 5. Synthetic routes to compound SA9 —

**Synthesis of benzyl (2,3-bis (hexadecyloxy) propyl) carbamate (13):** To a solution of NaH (5 equiv.) in DMF, 1-bromo hexadecane (4 equiv.) was added and stirred for 30 min at room temperature under N₂ atmosphere. After that compound 1 (1 equiv.) was added to the reaction mixture and stirred for 16 hours at 45 °C. After completion of the reaction the reaction mixture was quenched with water and the compound was extracted with EtOAc (3 × 50 mL) and dried over anhydrous Na₂SO₄. The organic layer was concentrated under reduced pressure and column chromatography with silica gel and a gradient solvent system of 0–10% ethyl acetate to hexane yielded the corresponding ether. **Characterization of the compound 13:** colorless oil (yield, 65
Synthesis of 2,3-bis(hexadecyloxy)propan-1-amine (14): 2,3-bis(hexadecyloxy)propan-1-amine was synthesized by the hydrogenation of compound 13 in the presence of 10 mol % Pd/charcoal (stirred for 14 hours at room temperature) according to the literature procedure and used without further purification.

Synthesis of 2-((2-((2,3-bis(hexadecyloxy)propyl)amino)-3,4-dioxocyclobut-1-en-1-yl)amino)-N,N,N-trimethylethanaminium (SA9): In an oven dried round bottom flask 1.0 equiv. of compound 7, 1.1 equiv. of compound 14 were dissolve in 3.5 ml of ethanol. Then, 5.5 equiv. of anhydrous DIPEA was added to the reaction mixture and the reaction mixture was allowed to stir at room temperature for 12 hrs. After completion of the reaction, the solvent was removed under reduced pressure. The reaction mixture was then washed for several times with a 1:1 mixture of diethyl ether and dichloromethane solvents. Then the left over solid residue was dried under reduced pressure and characterized by $^{1}H$ and $^{13}C$ NMR and LCMS analysis. The product yield was 65 % for this reaction. Characterization of the compound SA9: white solid (yield, 65 % ); $^{1}H$ NMR (400 MHz, CDCl$_3$ + MeOD-$d_4$) $\delta_{ppm}$ 6.25 (br s, 1H), 3.65-3.62 (m, 1H), 3.35-3.38 (m, 2H), 2.93-2.75 (m, 6H), 1.01-0.97 (m, 78H), 0.93-0.88 (m, 6H); $^{13}C$ NMR (151 MHz, CDCl$_3$ + MeOD-$d_4$) $\delta_{ppm}$ 199.3, 65.8, 54.3, 51.9, 46.3, 42.4, 37.4, 32.3, 31.7, 29.4, 29.4,
29.4, 29.3, 29.1, 25.6, 22.4, 11.9; MS (ESI) calcd. for C₄₄H₈₆N₃O₄ [M+3]+: 723.6613, found: 723.3215.

**Liposome preparation**

Liposome was prepared by thin film hydration method in 25 mM HEPES buffer, pH 7.2, containing 100 mM KCl. First 40-50 µL solution of the lipid was taken from 10 mM lipid stock and dried for 3h to prepare the lipid film. Then 500 µL of buffer was added into the lipid film and heated to 60-70 °C for 10 min (final concentration of the liposome ~1 mM). After that the solution was vortexed well until the dry lipid film was disappeared. Finally, the solution was sonicated for 10 times (30 s of sonication followed by 30 s of cooling on ice). A hand-held mini-extruder (Avanti Polar Lipids, Alabaster, AL) with polycarbonate membrane (diameter of 200 nm) was used to prepare small unilamellar vesicles (at room temperature) whenever required. We observed that hydration of the dried film of the SA lipids over 60-70 °C only resulted in the formation of bilayer membranes.

**Transmission electron microscopy**

For transmission electron microscope (TEM) imaging liposome was prepared by the above mention method in 25 mM HEPES buffer, pH 7.2, containing 100 mM KCl (no extrusion method was used). Identity of the unsaturated lipids under this experimental condition was confirmed by ¹H NMR spectroscopy (spectra not shown here). First, 10 µL solution of liposome was placed onto a carbon-coated copper grid and allowed to absorb for 1 minute. Then the grid was carefully blotted with filter paper. After that uranyl acetate (1% solution in water) was added on the grid and allowed to wait for another 1 minute. The excess uranyl acetate solution was then
removed and the grid was washed with water and excess water was removed, and the grid was allowed to dry at 37 °C for overnight. The images of the liposomes formed on the carbon-coated copper grid were collected using a JEOL JEM 2100 transmission electron microscope (operated at a maximum accelerating voltage of 200 kV).²

**Anisotropy measurements**

Temperature dependent steady-state anisotropy measurement was used to determine the gel to liquid-disordered phase transition temperature (Tₘ) values of the lipid bilayers. Environment sensitive fluorescence probe 1,6-diphenyl-1,3,5-hexatriene (DPH) was used for this study. For liposome preparation, first 35-50 µL of the lipid from its stock solution (15 mM solution in chloroform) was added to a 3 mL sample vial and dried for 3h to prepare the lipid film. ⁶ Then 800 µL of 25 mM HEPES buffer, pH 7.2, containing 100 mM KCl was added into the lipid film and heated to 60-70 °C for 10 min (final concentration of the liposome 2 mM). After that the solution was vortexed well until the dry lipid film was disappeared. Finally, the solution was sonicated for 10 times (30 s of sonication followed by 30 s of cooling on ice). A hand-held mini-extruder with polycarbonate membrane (diameter of 200 nm) was used to prepare large unilamellar vesicles (at room temperature). Then 7 µL of 1 mM DPH solution in THF was added to the extruded vesicles final concentration of DPH ~ 8.5 µM was maintained < 1% v/v]. This liposomal solution was kept under shaking or tumbling condition at room temperature for overnight for maximum incorporation of the DPH into the membrane. Steady-state anisotropy measurements were performed on a fluoromax-4 spectrofluorometer (Horiba Scientific, Singapore) with a refrigerated system for temperature control. The temperature was regulated using a peltier temperature controller (without removing the cuvette throughout the experiment).
All anisotropy values of the DPH probe are the mean values of three individual determinations. The degree (r) of anisotropy in the DPH fluorescence (\(\lambda_{ex} = 350\) nm; \(\lambda_{em} = 429\) nm) was calculated using eq 1 at the peak of the fluorescence spectrum, where \(I_{VV}\) and \(I_{VH}\) are the fluorescence intensities of the emitted light polarized parallel and perpendicular to the excited light, respectively, and \(G = I_{VH}/I_{HH}\) is the instrumental grating factor.

\[
r = \frac{(I_{VV} - G I_{HH})}{(I_{VV} + 2GI_{HH})}
\]  

(1)

Anisotropy values were collected at every 3-4 °C temperature differences, apart from near to the \(T_m\) value of the lipid; in that case the anisotropy values were collected at every 1 or 2 °C. Plot of degree (r) of anisotropy values of the DPH probe as a function of temperature was used to determine the \(T_m\) value of the lipid. The stability of lipid was examined by TLC measurements before and after the anisotropy measurements. However, the \(T_m\) values of SA5-8 lipids with dioleic-alkyl chains could not be measured because of the lowest temperature setup for our steady-state fluorimeter was 5 °C. The anisotropy values decreased continuously for these lipids within the range of 5-90 °C, without any sharp change in the anisotropy value within this temperature range (data not shown here).

**Microviscosity measurements**

Microviscosity of DPH molecule (a polarity probe) in the vicinity of lipid headgroup was calculated from steady state fluorescence anisotropy and fluorescence lifetimes measurements. The microviscosity, \(\eta\) can be calculated using Perrin’s equation.\(^7\)\(^8\)

\[
\frac{r_0}{r_s} = 1 + C(r) \frac{T}{\eta} \tau
\]  

(1)
Where $r_0$ is the limiting steady state fluorescence anisotropy of DPH in the absence of any depolarizing methods such as diffusion or energy transfer, $r_{ss}$ is the observed steady state fluorescence anisotropy of DPH at any temperature, $T$ is the temperature in Kelvin, $\tau$ is the fluorescence lifetime of DPH in seconds and $C(r)$ is a molecular shape parameter having a precalibrated value for each value of $r$. $C(r)$ is also related with the location of the transition dipoles of the rotating DPH molecule. $C(r)$ takes care of the non-spherical nature of the molecule. $C(r) = k/v$; where $k$ is Boltzman constant $(1.38 \times 10^{-23} \text{ m}^2 \text{ kg s}^{-2} \text{ K}^{-1})$ and $v$ is the volume $(\text{m}^3)$ of the DPH molecule. Obtaining a precise value of $C(r)$ in the heterogeneous microenvironments (such as gels, micelles, emulsions and others) is often intricate for fluorescent molecules including DPH. Hence equation (1) can also be written as-

$$\eta = \frac{r_{ss} C(r) \tau}{\delta r}; \delta r = r_0 - r_{ss}$$  \hspace{1cm} (2)

$$\eta = \frac{r_{ss} k \tau}{\delta r}; \quad k = C(r)T$$  \hspace{1cm} (3)

$$\eta \propto \frac{r_{ss} \tau}{\delta r}$$  \hspace{1cm} (4)

The term $\frac{r_{ss} \tau}{\delta r}$ is called the microviscosity parameter ($\tau_\eta$).\textsuperscript{7, 8} This $\tau_\eta$ also reflects the microviscosity of the environment around the fluorescent molecule. The measurements of $\tau_\eta$ values are advantageous since precise and difficult measurement of $C(r)$ is crucial for the exact value of $\eta$. In this regards, we calculated the $\tau_\eta$ values of the DPH molecules under the liposomal environment at different temperatures. The limiting anisotropy ($r_0$) of DPH is 0.362.\textsuperscript{9} Liposomes were prepared according to the mentioned procedure. DPH solution (14 $\mu$L of 1 mM solution) in THF was added to the extruded vesicles final concentration of DPH $\sim$ 8.5 $\mu$M was maintained.
Temperature was varied from 5 to 90 °C. Microviscosity parameter, \( \tau_\eta = \frac{r_{st} \tau}{\delta r} \) was used to compare the microviscosities of DPH molecule through the liposome of **SA1, SA9** and DPPC lipids.

**pH dependent zeta potential measurements**

For zeta potential measurements liposomes were prepared according to the previous method in 5 mM Tris buffer, pH 8.6, containing 5 mM NaCl. The liposomes were then extruded through a polycarbonate membrane (200 nm) using a handheld mini-extruder. Isosmotic buffers consisting of 10 mM buffering agent and 10 mM salt at different pH values were newly prepared and used for the measurements. Depending on the pK\(_a\) values of the buffering agent buffer solutions at different pH were prepared. Citric acid and trisodium-citrate was used for pH 3.0–6.5; 3-(N-morpholino)propanesulfonic acid (MOPS) was used for pH 7.0, tris(hydroxymethyl)aminomethane (Tris)–HCl was used for pH 7.5–8.5. Zeta potential measurements of the liposomes in buffer at different pH were carried out using a Zetasizer Nano ZS90 (Malvern, Westborough, MA) instrument. Liposomal solution was diluted into 1 mL of isosmotic buffer (final lipid concentration was 150 nM) in a disposable capillary cell (DTS1061) for zeta potential measurements. All the measurements were performed three times per sample.\(^2\)

**Calcium-induced zeta potential measurements**

Liposome preparation used in the pH dependent zeta potential measurements were also used for the calcium-induced zeta potential measurements in 20 mM of 2-(N-morpholino)-ethanesulfonic acid (MES) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, pH 7.2. Liposomal solution was added into 1 mL of 10 mM HEPES-MES buffer, pH 7.2 (final lipid
concentration was 100 nM) with various concentration of CaCl\(_2\) and NaCl. NaCl was added along with CaCl\(_2\) to maintain a constant ionic strength across all Ca\(^{2+}\) ion concentrations. Ionic strengths of the solutions were calculated according to the Debye–Huckel model, where the ionic strength (I) = 0.5(4[Ca\(^{2+}\)] + [Cl\(^-\)] + [Na\(^+\)]). Zeta potential measurements of liposomes were performed using a Zetasizer Nano ZS90 (Malvern, Westborough, MA) instrument.\(^2,10\)

**Vesicle leakage study**

Liposomes were prepared according to the previous method (without passing through the extruder) in 10 mM HEPES buffer, pH 7.2, in the presence of 2 mM 5(6)-carboxyfluorescein (CF) or 4 mM rhodamine 6G (R6G).\(^2,10\) Free CF or R6G was removed by size exclusion chromatography on a PD-10 sephadex column (Sigma, St. Louis, MO) by eluting with 10 mM HEPES buffer, pH 7.2, containing 92 mM NaCl and 8 mM NaN\(_3\). Then, purified liposome solution was added into 1 mL of the elution buffer (final lipid concentration was 1.7 mM). The release of CF and R6G was measured by monitoring the emission signals at 516 nm (\(\lambda_{ex} = 485\) nm) and 554 nm (\(\lambda_{ex} = 530\) nm), respectively. Steady state fluorescence measurements were performed using Fluoromax-4 spectrofluorometer (Horiba Scientific, Singapore) at room temperature. Finally liposomes were lysed using TRITON X-100 surfactant (final concentration was 2 mM) to measure the total fluorescence of CF/R6G. Percent release was calculated using the following relation: percentage release at time, (t) = (measured fluorescence at time, (t)) / (total fluorescence from lysed liposomes) × 100. All measurements were performed at 37 °C.
Preparation of giant unilamellar vesicles (GUVs) by electroformation method

Giant unilamellar vesicles (GUVs) were prepared using a home-made electroformation unit on indium tin oxide coated (ITO) glass slide (surface resistivity 70-100 Ω/sq).\textsuperscript{11, 12} For the preparation of GUV of the lipids, chloroform/methanol (8:2) stock solutions containing 1.0 mM of the lipid (i.e., SA1 and SA9) were prepared. Then, 10 µL of 2 mM 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS) in methanol solution was added into 50 µL of chloroform-methanol stock solutions of the lipids and mixed thoroughly. After that 10 µL of this lipid mixture was spread in a snakelike pattern without overlap over a 2.5 × 2.5 cm\textsuperscript{2} area using a 10 µL Hamilton syringe (on the conductive surface of both the ITO-coated glass plates). Following the deposition of the lipid film onto the ITO-coated glass, the solvent was evaporated by passing a stream of nitrogen gas over the glass for 2 hours and then the electroformation chamber was assembled. Using two ITO-coated plates (both ITO-coated surfaces were facing each other) the chamber was constructed and the plates were connected using copper tapes. The ITO coated plates were separated by a silicone ‘O’-ring (20 mm × 3 mm). The resulting electroformation chamber was then slowly filled with 500 µL of 25 mM HEPES buffer, pH 7.2 containing 100 mM glucose. Silicone high vacuum grease was used to seal the chamber. Both ITO-coated cover-slips were then connected with copper tapes and a sinusoidal AC electric field of 10 Hz and 2.0 V (rms) was then applied to
the system. This electroformation process was continued for 12h to generate GUVs at temperature greater than that of the phase transition temperature ($T_m$) of the lipid using a temperature regulated hotplate (60-70 °C for both the lipids). For membrane labeling, 0.1 µL of 100 µM Texas Red DHPE (Invitrogen, CA) solution in EtOH was added to 10 µL of GUV solution prior to microscope observation. Finally, the GUV solution was spread on a glass slide and covered with a 17 mm cover slip and fluorescence microscopic images were collected using Olympus BX51y fluorescence microscope. In general, large amounts of the GUVs with a diameter $> 10$ µm was obtained under this condition. The GUVs were prepared around 60-70 °C because of relatively high $T_m$ value of the SA1 and SA9 lipid. The efficiency of GUV preparations at lower temperature was extremely poor.

**HPLC Analysis of SA lipids stability against PLA$_2$ and PLC enzymes**

2 µg of lipid (DPPC or SA1 or SA9) was taken in a glass vial and dried for 2 hours under continuous nitrogen gas flow. Dried lipid was then dissolve with 50 µL 100 mM Tris-base at pH 8.5 containing 10 mM CaCl$_2$ buffer solution which was vortexed for 1 min and sonicated (for 3 times interval of 1min). For PLA$_2$ activity assay 1 Unit of bovine pancreatic PLA$_2$ enzyme was added to the liposome containing buffer solution (50 µL) and the reaction mixture incubated at 37 °C for overnight. For PLC activity assay 0.2 unit of PLC enzyme (from Clostridium Perfringens) was added to the liposome containing buffer solution (50 µL) and the reaction incubated at 25 °C for 2 hours. After that the reaction mixtures were diluted with methanol and subject to HPLC/LCMS analyses. UV signals were simultaneously monitored to confirm the identity of the compounds. HRMS analyses of the peaks were also performed for further characterization of the compounds. HPLC analysis was carried out on a Thermo-Fisher C18
analytical column in Thermo-Fisher UHPLC ultimate 3000 machine. Both SA1 and SA9 lipids have a strong absorption peak at 270 nm. In this regard HPLC analysis was performed using an UV-detector at 270 nm. Because of the unavailability of the ELSD detector hydrolysis of DPPC lipid by PLA2 and PLC enzymes were investigated by TLC and LCMS analyses.

HPLC gradient: 0 min – 2 min 50% Phase A in Phase B to 5% Phase A in Phase B, 2 min – 12 min, 5% Phase A in Phase B, 12 min – 14 min, 5% Phase A in Phase B to 50% Phase A in Phase B. (Phase A: H2O with 0.1% formic acid, Phase B: MeOH with 0.1% formic acid).6

Fig. S1 (A-D) Anisotropic curve of SA2, SA3, SA4 and SA9 lipids within the temperature range from 5 to 90 °C.
Table S1. Temperature dependent fluorescence lifetime data of DPH in the liposomal solution and their microviscosity parameter values.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Temperature (K)</th>
<th>( \tau_1 ) (ns) (( \alpha_1 ))</th>
<th>( \tau_2 ) (ns) (( \alpha_2 ))</th>
<th>( \tau_3 ) (ns) (( \alpha_3 ))</th>
<th>( \tau_{\text{avg}} ) (ns)</th>
<th>( \chi^2 )</th>
<th>( \eta_{\text{ss}} )</th>
<th>( \delta \eta )</th>
<th>Microviscosity parameter (( \tau_0 )) (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPC</td>
<td>288</td>
<td>4.2 (0.08)</td>
<td>9.6 (0.92)</td>
<td>-</td>
<td>9.21</td>
<td>1.029</td>
<td>0.33910</td>
<td>0.02290</td>
<td>136.4396</td>
</tr>
<tr>
<td></td>
<td>298</td>
<td>4.2 (0.07)</td>
<td>9.8 (0.93)</td>
<td>-</td>
<td>9.44</td>
<td>1.007</td>
<td>0.33615</td>
<td>0.02585</td>
<td>122.7565</td>
</tr>
<tr>
<td></td>
<td>303</td>
<td>4.8 (0.09)</td>
<td>10.0 (0.91)</td>
<td>-</td>
<td>9.51</td>
<td>1.039</td>
<td>0.32664</td>
<td>0.03536</td>
<td>87.84916</td>
</tr>
<tr>
<td></td>
<td>313</td>
<td>5.0 (0.07)</td>
<td>10.1 (0.93)</td>
<td>-</td>
<td>9.71</td>
<td>1.019</td>
<td>0.17410</td>
<td>0.18790</td>
<td>8.996865</td>
</tr>
<tr>
<td></td>
<td>318</td>
<td>3.2 (0.06)</td>
<td>7.6 (0.94)</td>
<td>-</td>
<td>7.34</td>
<td>0.995</td>
<td>0.09897</td>
<td>0.26302</td>
<td>2.761929</td>
</tr>
<tr>
<td></td>
<td>328</td>
<td>2.4 (0.09)</td>
<td>6.9 (0.91)</td>
<td>-</td>
<td>6.50</td>
<td>1.003</td>
<td>0.08510</td>
<td>0.27689</td>
<td>1.997867</td>
</tr>
<tr>
<td></td>
<td>338</td>
<td>2.5 (0.13)</td>
<td>5.2 (0.87)</td>
<td>-</td>
<td>4.86</td>
<td>1.010</td>
<td>0.05916</td>
<td>0.30283</td>
<td>0.949481</td>
</tr>
<tr>
<td></td>
<td>348</td>
<td>2.5 (0.30)</td>
<td>4.2 (0.70)</td>
<td>-</td>
<td>3.70</td>
<td>1.001</td>
<td>0.04339</td>
<td>0.31860</td>
<td>0.503952</td>
</tr>
<tr>
<td></td>
<td>358</td>
<td>2.2 (0.32)</td>
<td>3.6 (0.68)</td>
<td>-</td>
<td>3.15</td>
<td>1.008</td>
<td>0.03893</td>
<td>0.32306</td>
<td>0.379608</td>
</tr>
<tr>
<td>SA1</td>
<td>288</td>
<td>1.4 (0.47)</td>
<td>5.5 (0.53)</td>
<td>-</td>
<td>3.57</td>
<td>1.005</td>
<td>0.26553</td>
<td>0.09647</td>
<td>9.826289</td>
</tr>
<tr>
<td></td>
<td>298</td>
<td>1.4 (0.49)</td>
<td>5.7 (0.51)</td>
<td>-</td>
<td>3.60</td>
<td>1.006</td>
<td>0.25298</td>
<td>0.10902</td>
<td>8.353770</td>
</tr>
<tr>
<td></td>
<td>308</td>
<td>1.6 (0.48)</td>
<td>5.8 (0.52)</td>
<td>-</td>
<td>3.78</td>
<td>1.014</td>
<td>0.24194</td>
<td>0.12006</td>
<td>7.617301</td>
</tr>
<tr>
<td></td>
<td>318</td>
<td>1.6 (0.50)</td>
<td>6.0 (0.50)</td>
<td>-</td>
<td>3.80</td>
<td>1.019</td>
<td>0.23060</td>
<td>0.13140</td>
<td>6.668798</td>
</tr>
<tr>
<td></td>
<td>323</td>
<td>1.3 (0.40)</td>
<td>5.1 (0.60)</td>
<td>-</td>
<td>3.58</td>
<td>1.032</td>
<td>0.14235</td>
<td>0.21965</td>
<td>2.320114</td>
</tr>
<tr>
<td></td>
<td>328</td>
<td>1.4 (0.42)</td>
<td>4.9 (0.58)</td>
<td>-</td>
<td>3.43</td>
<td>1.015</td>
<td>0.08418</td>
<td>0.27782</td>
<td>1.039297</td>
</tr>
<tr>
<td></td>
<td>333</td>
<td>1.3 (0.32)</td>
<td>4.5 (0.68)</td>
<td>-</td>
<td>3.48</td>
<td>0.995</td>
<td>0.04768</td>
<td>0.31432</td>
<td>0.527890</td>
</tr>
<tr>
<td></td>
<td>338</td>
<td>1.2 (0.30)</td>
<td>4.2 (0.70)</td>
<td>-</td>
<td>3.30</td>
<td>1.032</td>
<td>0.04499</td>
<td>0.31701</td>
<td>0.468335</td>
</tr>
<tr>
<td></td>
<td>343</td>
<td>1.1 (0.25)</td>
<td>3.7 (0.75)</td>
<td>-</td>
<td>3.04</td>
<td>0.981</td>
<td>0.04111</td>
<td>0.32089</td>
<td>0.389462</td>
</tr>
<tr>
<td></td>
<td>348</td>
<td>0.9 (0.20)</td>
<td>3.5 (0.80)</td>
<td>-</td>
<td>2.97</td>
<td>1.030</td>
<td>0.03756</td>
<td>0.32444</td>
<td>0.343833</td>
</tr>
<tr>
<td></td>
<td>358</td>
<td>0.9 (0.20)</td>
<td>3.0 (0.80)</td>
<td>-</td>
<td>2.58</td>
<td>1.000</td>
<td>0.03276</td>
<td>0.32924</td>
<td>0.256715</td>
</tr>
<tr>
<td>SA9</td>
<td>288</td>
<td>0.7 (0.80)</td>
<td>3.3 (0.20)</td>
<td>-</td>
<td>1.22</td>
<td>1.006</td>
<td>0.19252</td>
<td>0.16948</td>
<td>1.385583</td>
</tr>
<tr>
<td></td>
<td>298</td>
<td>0.7 (0.80)</td>
<td>3.8 (0.20)</td>
<td>-</td>
<td>1.32</td>
<td>1.072</td>
<td>0.18540</td>
<td>0.17660</td>
<td>1.385776</td>
</tr>
<tr>
<td></td>
<td>308</td>
<td>0.7 (0.80)</td>
<td>4.3 (0.20)</td>
<td>-</td>
<td>1.43</td>
<td>0.991</td>
<td>0.17490</td>
<td>0.18710</td>
<td>1.336756</td>
</tr>
<tr>
<td></td>
<td>318</td>
<td>0.7 (0.76)</td>
<td>4.4 (0.24)</td>
<td>-</td>
<td>1.60</td>
<td>0.999</td>
<td>0.15514</td>
<td>0.20686</td>
<td>1.199961</td>
</tr>
<tr>
<td></td>
<td>323</td>
<td>0.8 (0.77)</td>
<td>4.5 (0.23)</td>
<td>-</td>
<td>1.61</td>
<td>0.989</td>
<td>0.14067</td>
<td>0.22133</td>
<td>1.023263</td>
</tr>
<tr>
<td></td>
<td>328</td>
<td>0.8 (0.44)</td>
<td>2.8 (0.36)</td>
<td>0.1 (0.20)</td>
<td>1.36</td>
<td>1.006</td>
<td>0.11056</td>
<td>0.25144</td>
<td>0.598002</td>
</tr>
<tr>
<td></td>
<td>338</td>
<td>0.7 (0.44)</td>
<td>2.4 (0.36)</td>
<td>0.1 (0.20)</td>
<td>1.21</td>
<td>1.107</td>
<td>0.05940</td>
<td>0.30260</td>
<td>0.236933</td>
</tr>
<tr>
<td></td>
<td>348</td>
<td>0.7 (0.50)</td>
<td>2.3 (0.25)</td>
<td>0.1 (0.25)</td>
<td>1.03</td>
<td>1.095</td>
<td>0.03925</td>
<td>0.32275</td>
<td>0.125259</td>
</tr>
<tr>
<td></td>
<td>358</td>
<td>0.7 (0.55)</td>
<td>2.6 (0.20)</td>
<td>0.1 (0.25)</td>
<td>0.91</td>
<td>1.019</td>
<td>0.03401</td>
<td>0.32799</td>
<td>0.094049</td>
</tr>
</tbody>
</table>

All experiments were carried out in 25 mM HEPES buffer, pH 7.2, containing 100 mM KCl. \( r_0 \) of DPH = 0.362.
Fig. S2: Microviscosity parameter ($\tau_\eta$) of DPH under the liposomal environment at different temperatures. The DPH concentration for all the experiment sets was $\sim$ 8.5 µM. All experiments were carried out in 25 mM HEPES buffer, pH 7.2, containing 100 mM KCl. $r_0$ of DPH = 0.362.
**Fig. S3** TEM image of the liposomes generated from 100% SA lipids.
Aggregation of **SA-1** lipid in physiological Buffer media

**SA1; R = OC(CH$_2$)$_{14}$CH$_3$**

**Fig. S4** Schematic representation for the proposed partial aggregation mechanism of **SA1** lipid in salt containing buffer. A similar mechanism can be proposed for **SA5** and **SA9** lipids under the similar experimental conditions.
**Fig. S5** TEM image of the liposomes generated from the lipid mixtures of SA lipids/DPPC/DPPS/cholesterol (in the molar ratio of 2:4:2:2).
Fig. S6 Fluorescence microscopic images of GUVs generated from pure SA9 lipid. (A) Bright field. (B) Green channel illustrating HPTS encapsulation. (C) Red channel illustrating the staining of the lipid membrane with Texas Red DHPE. (D) Merge of Green and Red channels.
Fig. S7 Dynamic light scattering (DLS) measurements. Size distribution of extruded liposomes (200 nm) formed from \textbf{SA1-4} and \textbf{SA9} lipids measured at 25 °C. Content is given in percentage distribution of different sized liposome at different pH.
Fig. S8 Dynamic light scattering (DLS) measurements. Size distribution of extruded liposomes (200 nm) formed from SA1-4 and SA9 lipids measured at 25 °C. Content is given in percentage distribution of different sized liposomes at pH 7.2 in the presence of different concentration of Ca²⁺ ion.
Fig S9 (A) CF and (B) R6G release profiles from the liposomes of 100 % phospholipids. (C) CF and (D) R6G release profiles from the liposomes of mixed lipids DPPC/DPPS/cholesterol/SA1-4 and SA9 (in the molar ratio of 2:4:2:2).
Fig S10 R6G release profile from the liposomes of 100 % SA lipids in the absence and presence of different Ca$^{2+}$ concentrations (0-5 mM). (A) R6G release profile of SA1 lipid. (B) R6G release profile of SA2 lipid. (C) R6G release profile of SA3 lipid. (D) R6G release profile of SA4 lipid. (E) R6G release profile of SA9 lipid.
Fig. S11 TLC images (KMnO₄ stained) of the lipids in the absence and presence of PLA₂ and PLC enzymes. (A) only DPPC liposome. (B) DPPC liposome + PLA₂ enzyme. (C) only SA¹ liposome. (D) SA¹ liposome + PLA₂ enzyme. (E) only SA⁹ liposome. (F) SA⁹ liposome + PLA₂ enzyme. (G) only DPPC liposome. (H) DPPC liposome + PLC enzyme. (I) only SA¹ liposome. (J) SA¹ liposome + PLC enzyme. (K) only SA⁹ liposome. (L) SA⁹ liposome + PLC enzyme. Aluminum sheet TLC silica gel 60 F₂₅₄ plate was used for TLC checking. Solvent system for PLA₂ and PLC assays (TLC) was 10% MeOH / Dichloromethane. Marked circles (black color) are the hydrolyzed product of DPPC in the presence of PLA₂ and PLC enzymes.
Fig. S12. LC-MS analyses of the PLA<sub>2</sub> and PLC treated DPPC lipid. (A) LC-(+)-ESI-MS of only DPPC liposome in methanol. (B) LC-(+)-ESI-MS of PLA<sub>2</sub> treated DPPC liposome in methanol. (C) LC-(+)-ESI-MS of PLA<sub>2</sub> treated DPPC liposome in methanol. (D) LC-(+)-ESI-MS of PLC treated DPPC liposome in methanol.
Fig. S13 HPLC-UV (270 nm) signals for PLA₂ and PLC treatment of SA₁ and SA₉ lipids. SA₁/SA₉ before (black), after the treatment of PLA₂ (blue) and after the treatment of PLC (red). No hydrolysis was observed. Retention time for SA₁/SA₉ lipids is 3.9 min. The HPLC-UV signals at Rₜ = 3.9 min were further confirmed by LCMS analyses, which indicates its stability under the enzymatic assay conditions. The HPLC-UV signals at Rₜ = 9.5 min may come from buffer, which cannot be properly characterized by MS analyses.
NMR and Mass spectra of the compounds:

Figure S14: $^1$H & $^{13}$C NMR of compound 2a.
Figure S15: $^1$H & $^{13}$C NMR of compound 2b.
**Figure S16:** $^1$H & $^{13}$C NMR of compound 7.
Figure S17: $^1$H & $^{13}$C NMR of compound SA1.
Figure S18: $^1$H & $^{13}$C NMR of compound SA5.
Figure S19: $^1$H & $^{13}$C NMR of compound 10.
Figure S20: $^1$H & $^{13}$C NMR of compound 11a.
Figure S21: $^{1}$H & $^{13}$C NMR of compound 11b.
Figure S22: $^1$H & $^{13}$C NMR of compound SA2.
Figure S23: $^1$H & $^{13}$C NMR of compound SA6.
Figure S24: $^1$H & $^{13}$C NMR of compound 12.
Figure S25: $^1$H & $^{13}$C NMR of compound SA3.
Figure S26: $^1$H & $^{13}$C NMR of compound SA7.
Figure S27: $^1$H & $^{13}$C NMR of compound SA4.
Figure S28: $^1$H & $^{13}$C NMR of compound SA8.
Figure S29: $^1$H & $^{13}$C NMR of compound SA9.
Figure S30. MS (+ESI) data of compounds SA1-8.
Figure S31. MS (+ESI) data of compound SA9.

References