Supplementary information for:

*Triggered release from lipid bilayer vesicles by an artificial transmembrane signal transduction system*

Matthew J. Langton, Lorel M. Scriven, Nicholas H. Williams* and Christopher A. Hunter*

*a Department of Chemistry, University of Cambridge, Lensfield Road, Cambridge CB2 1EW, United Kingdom

b Department of Chemistry, University of Sheffield, Sheffield S3 7HF, United Kingdom

**Experimental procedures**

- Materials
- Fluorescence experiments
- Dynamic Light Scattering (DLS) Experiments
- General procedure for vesicles preparation
- Synthetic procedures

**Additional experiments**

- Calcein release assays
- Analysis of vesicles post-release experiments

**References**

S2  S4  S6
Experimental procedures

$^1$H-NMR and $^{13}$C-NMR spectra were recorded on a 400-MHz Bruker spectrometer. Chemical shifts are reported as δ values in ppm. GPC purification of vesicles was carried out using GE Healthcare PD-10 desalting columns prepacked with Sephadex G-25 medium. Fluorescence spectroscopic data were recorded using a Cary Eclipse fluorescence spectrophotometer (Agilent). pH measurements were conducted using a Mettler-Toledo “Seven Compact” pH meter equipped with an “In-lab Micro” electrode. Vesicles were prepared as described below using Avestin “LiposoFast” extruder apparatus, equipped with polycarbonate membranes with 200 nm pores.

Materials

Triethylamine was distilled and stored over KOH prior to use. All other reagents and solvents were used without further purification. Lipids were purchased from Sigma Aldrich and used without further purification.

Fluorescence experiments

a) Membrane signaling experiments monitoring formation of 4.

Fluorescence excitation experiments were recorded using the following parameters: emission wavelength = 510 nm, excitation range 380–480 nm, recorded at 2 minute intervals, with the excitation and emission slits set at 5 nm.

b) Calcein release experiments.

Fluorescence emission experiments were recorded using the following parameters: excitation wavelength = 365 nm, emission range 380–600 nm, recorded at 2 minute intervals, with the excitation and emission slits set at 2.5 nm and 5 nm, respectively.

All experiments were repeated a minimum of two times on independently prepared samples to confirm reproducibility.

Dynamic Light Scattering (DLS) Experiments

DLS experiments were undertaken using a Malvern Zetasizer Nano S90 apparatus, equipped with a 4 mW HeNe laser source (633 nm). The sample was placed in a thermostat-controlled cell-holder maintained at 298 K and the scattered light collected at an angle of 173°. Unimodal intensity weighted distributions of the hydrodynamic diameters were obtained and the averaged values of three measurements reported.
General procedure for vesicles preparation

Vesicle suspensions were prepared as follows:

To a round bottom flask was added chloroform/ethanol solutions of the lipids (1,2-Dioleoyl-sn-glycero-3-phosphocholine DOPC and 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine DOPE, in a 3:2 ratio), and a chloroform solution of 1, to obtain final concentrations of 2 mM lipids, and 50 µM 1. The solvent was removed in vacuo and dried under high vacuum for 2 h. A 250 mM solution of HEPES buffer at pH 7 (2.5 mL) and aqueous solutions of ester 3 and ZnCl₂ (to obtain final concentrations of 250 µM) were added to the flask containing the lipids, and sonicated for 1 min. For calcein release experiments, the lipids were hydrated in 2.5 mL of a 70 mM calcein solution, containing 25 µM 3 in 250 mM solution of HEPES buffer at pH 7. The suspension was subjected to 5 cycles of freeze-thaw using liquid nitrogen. The suspension was extruded 19 times through a polycarbonate filter with 200 nm pores in an Avestin Lipofast apparatus,¹ and then the vesicles were separated from the bulk solution using prepacked GPC columns eluting with a 250 mM NaCl buffer solution at pH 7 (for calcein release experiments, the vesicles were eluted with 400 mM NaCl).

Synthetic procedures

Compound 1 was prepared as previously described.²

Compound 3

To a stirred solution of pyranine (500 mg, 0.953 mmol) in DMF (30 mL) at 0°C was added triethylamine (1.93 g, 19.1 mmol) and 2-naphtoyl chloride (908 mg, 4.77 mmol). The reaction was then stirred for 20 h under nitrogen and then poured onto diethyl ether (700 mL) to form a cloudy white suspension. The product was then collected under vacuum filtration, then dissolved in the minimum volume of water. Addition of acetone precipitated the product as a yellow powder (490 mg, 76%). ¹H NMR (400 MHz, D₂O) δ (ppm): 8.93 (s, 1H), 8.73 (d, ³J = 9.5 Hz, 1H), 8.65 (d, ³J = 9.7 Hz, 1H), 8.55 (s, 1H), 8.51 (d, ³J = 9.7 Hz, 1H), 8.11 (d, ³J = 9.5 Hz, 1H), 7.59 (s, 1H), 7.22 (d, ³J = 8.4 Hz, 1H), 6.61 (m, 1H), 6.51 (m, 3H), 6.42 (m, 1H). ¹³C-NMR (100 MHz, CDCl₃) δ (ppm): 166.1, 143.5, 137.5, 136.6, 135.5, 133.5, 130.7, 129.6, 128.9, 128.2, 127.8, 127.2, 126.1, 125.7, 125.5, 125.4, 124.6, 124.2, 124.0, 123.9, 123.6, 123.1, 123.0, 122.3, 119.3. HRMS calc. for C₂7H₁₅O₁₁S₃: 610.9782, found: 610.9780.
Additional transmembrane signalling experiments

Figure S1. Time dependence of the normalized fluorescence emission intensity at 510 nm (exciting at 415 nm) of vesicles composed of lipids with 2.5 mol% 1 encapsulating 250 μM 3, at pH 7 (grey), pH 7.5 (blue) and pH 8.0 (black). All experiments were conducted in 200 nm DOPC/DOPE vesicles (2 mM final lipid concentration) containing 250 μM ZnCl₂ and 250 mM HEPES buffer and suspended in 250 mM NaCl.

Figure S2. Fluorescence excitation spectrum response for 3 as a function of pH. (a) Excitation spectrum of 4 (emission at 510 nm) at pH 7, 8 and 9. (b) pH vs $I_{460}/I_{405}$. The data was fitted to the Henderson-Hasselbach type equation ($r^2 = 0.995$), to obtain the calibration curve, using the following parameters: a = 4.03; b = 7.81; where $x = I_{460}/I_{405}$. Vesicles formed from 3:2 DOPC/DOPE and 2.5% 1, encapsulating 250 μM 4 and 250 μM ZnCl₂ in HEPES buffer was lysed with Triton X-100, and the pH adjusted by adding small aliquots of dilute NaOH$_{(aq)}$. After each addition, the pH of the solution was measured using a pH electrode and the excitation spectrum recorded.
**Calcein release assays**

Vesicles containing 70 mM calcein were prepared as described above, and suspended in 400 mM NaCl solution. In initial experiments, 4 µL of a methanol solution of naphthoic acid (final concentration 50 µM) was added to a cuvette containing the vesicle suspension and shaken rapidly. The calcein emission spectra were recorded as described above at 2 minute intervals. The vesicles were lysed with Triton X-100 at the end of experiment to calibrate the release assay, using equation S1, where \( I \), \( I_{100} \), and \( I_0 \) are the fluorescence intensity values observed after the addition of naphthoic acid surfactant, after the addition of excess Triton X-100, and before any addition, respectively.

**Equation S1**

\[
\text{\% release} = \frac{100 (I - I_0)}{I_{100} - I_0}
\]

Release assays for the signal transduction system with transducer 1 and substrate 3 were interpreted in the same manner.

**Analysis of vesicles post-release experiments**

**Table S1: Dynamic Light Scattering (DLS).**

<table>
<thead>
<tr>
<th>Sample</th>
<th>(d/\text{nm})</th>
<th>PdI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vesicles after 1000 mins at pH 7 (OFF state)</td>
<td>173 ± 11</td>
<td>0.16 ± 0.06</td>
</tr>
<tr>
<td>Vesicles after 1000 mins at pH 9.0 (ON state)</td>
<td>176 ± 10</td>
<td>0.21 ± 0.04</td>
</tr>
</tbody>
</table>

Average vesicles dimensions determined by DLS and the corresponding poly-dispersity index. Data averaged over 3 measurements (errors denote 2σ), for DOPC/DOPE vesicles prepared by passing through 200 nm membranes as described above, and recorded after the signal transduction experiment (after \( t = 1000 \) min).

**NMR phospholipid concentration assay**

The concentration of phospholipids in the vesicle samples following the release experiments were determined using the NMR integration method. A vesicle samples containing initially transducer 1, 25 µM 3, 70 mM calcein and 250 µM ZnCl₂, was subjected to an increase in extra-vesicle pH to initiate transmembrane signal transduction (Fig. 5 green data). A separate sample was left in the OFF state at pH 7 (Fig. 7, red data). After 1000 minutes, the sample were passed through size exclusion columns to separate intact vesicles from non-encapsulated materials. Aliquots of these samples were dissolved in CD₃OD / CDCl₃ and an internal standard 3-(Trimethylsilyl)propionic-2,2,3,3-d₄ acid sodium salt (TMSP). Integration of the terminal CH₃ phospholipid signal at ~ 0.88 ppm, which is well separated from the other peaks in the spectrum, enables the concentration to be determined by reference to the integration of the internal standard signal (Figure S3 and Table S2).
Figure S3. Partial $^1$H NMR spectrum of an aliquot of vesicles used for phospholipid determination: (a) pH 7 OFF state and (b) pH 9 ON state (400 MHz, 9:2:3 CD$_3$OD/CDCl$_3$/H$_2$O, 298 K). TMSP = 3-(Trimethylsilyl)propionic-2,2,3,3-d$_4$ acid sodium salt.

Table S2: NMR phospholipid quantification

<table>
<thead>
<tr>
<th>Sample</th>
<th>Phospholipid concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vesicles after 1000 mins at pH 7 (OFF state)</td>
<td>0.5</td>
</tr>
<tr>
<td>Vesicles after 1000 mins at pH 9 (ON state)</td>
<td>0.5</td>
</tr>
</tbody>
</table>

References