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Controlled membrane translocation provides a mechanism for signal transduction and amplification

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Abstract

Transmission and amplification of chemical signals across lipid bilayer membranes is of profound significance in many biological processes, from the development of multi-cellular organisms to information processing in the nervous system. In biology, membrane-spanning proteins are responsible for transmission of chemical signals across membranes, and signal transduction is often associated with an amplified signaling cascade. The ability to reproduce such processes in artificial systems has potential applications in sensing, controlled drug delivery and communication between compartments in tissue-like constructs of synthetic vesicles. Here we describe a new mechanism for transmitting chemical signals across membranes based on controlled translocation of a synthetic molecular transducer from one side of a lipid bilayer membrane to the other. The controlled molecular motion has been coupled to activation of a catalyst on the inside of a vesicle, which leads to a signal amplification process analogous to the biological counterpart.

Main

Lipid bilayer membranes in biological systems enable the formation of compartments, allowing chemical processes on either side of the bilayer to be physically decoupled. An essential feature of such compartmentalized systems is the communication of chemical information between the two sides of a bilayer, mediated by membrane spanning proteins, without uncontrolled mixing of the two solutions.[1] In nature there are two mechanisms by which these signaling proteins transduce and amplify chemical signals across bilayer membranes. Binding of a ligand to the extra-cellular region of a membrane-spanning protein receptor induces either a global conformational change (Fig. 1a), as exemplified by the G-coupled receptors,[2] or dimerization of two proteins (Fig. 1b), such as observed in tyrosine kinase receptors.[3] These changes in protein organisation activate an enzyme-catalysed reaction inside the cell leading to signal amplification. Whilst a number of biomimetic synthetic systems have been developed that allow chemical signals, in the shape of ions or molecules, to physically cross lipid membranes via pores or transporters,[4–10] signal transduction without this physical exchange of matter has proved to be more challenging,[11–15] and amplification of such a signal remains uniquely the domain of biology. Here we introduce membrane translocation as an alternative mechanism for signal transduction, providing a conceptually simple approach to addressing this challenge.

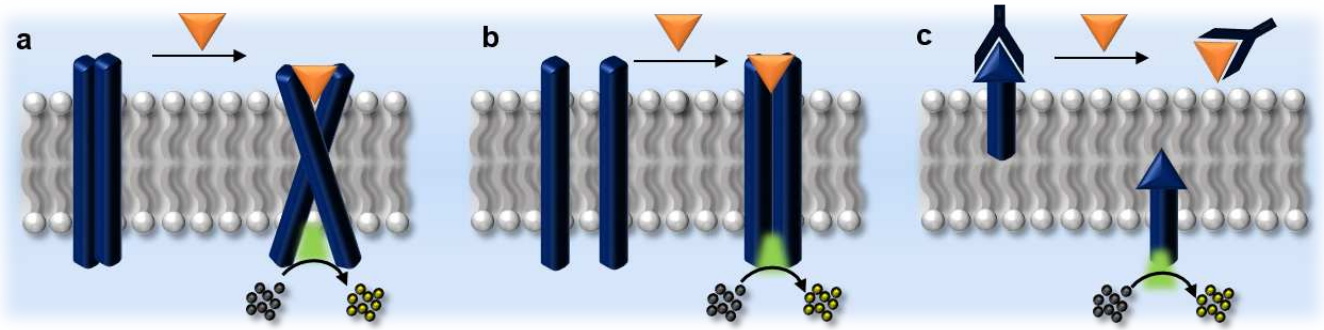

Figure 1. Mechanisms of transmembrane signal transduction. (a) Conformational change. Recognition of a signal on one side of a membrane (orange) induces a conformational change in a transmembrane protein (blue), generating an active enzyme (green), which turns over a substrate on the other side of the membrane. (b) Oligomerisation. Recognition of a signal on one side of a membrane (orange) leads to dimerization of a transmembrane protein (blue), generating an active enzyme (green), which turns over a substrate on the other side of the membrane. (c) Translocation. Recognition of a signal on one side of a membrane (orange) releases a membrane bound transducer (blue) which crosses the bilayer. This exposes an active catalyst (green), which turns over a substrate on the other side of the membrane.

Fig. 1c shows how a recognition event on one side of a membrane could be used to release a membrane bound transducer, so that it can translocate across the bilayer. If the transducer is equipped with a catalyst, translocation will expose the catalyst to the other side of the membrane, where it can act on a substrate to generate an amplified signal.

The basic design principles are illustrated in Fig. 2a. Two different head groups (blue and red) are attached to a spacer (grey) that is too short to span the bilayer: one head group acts as an external sensor and the other is a pro-catalyst. When the head groups are polar they prefer to sit in the aqueous phase, and when they are non-polar they can enter the membrane. If both of the head groups can be switched between polar and non-polar states, the system will be bistable. Concerted switching of the head-group polarities of an asymmetric transducer therefore provides a mechanism for controlled translocation of the molecule across a bilayer membrane. In the OFF state, the sensor head group is polar (blue) and sits in the external aqueous solution, whilst the pro-catalyst head-group is non-polar (red) and embedded in the membrane, where it is inactive. The input signal switches the external head group from polar to non-polar (blue to purple), allowing the transducer to translocate across the lipid bilayer. Binding of a cofactor from the internal aqueous solution switches the internal head group from non-polar to polar (red to green), simultaneously activating the catalyst and providing a driving force for the directional translocation, locking the system in the ON state. In this ON state, the polar catalyst is exposed to the internal aqueous phase and turns over encapsulated substrate molecules to generate the output signal. By coupling each input molecular signal to the activation of a catalyst, the output signal is amplified, because each catalyst can turn over a large number of substrate molecules.

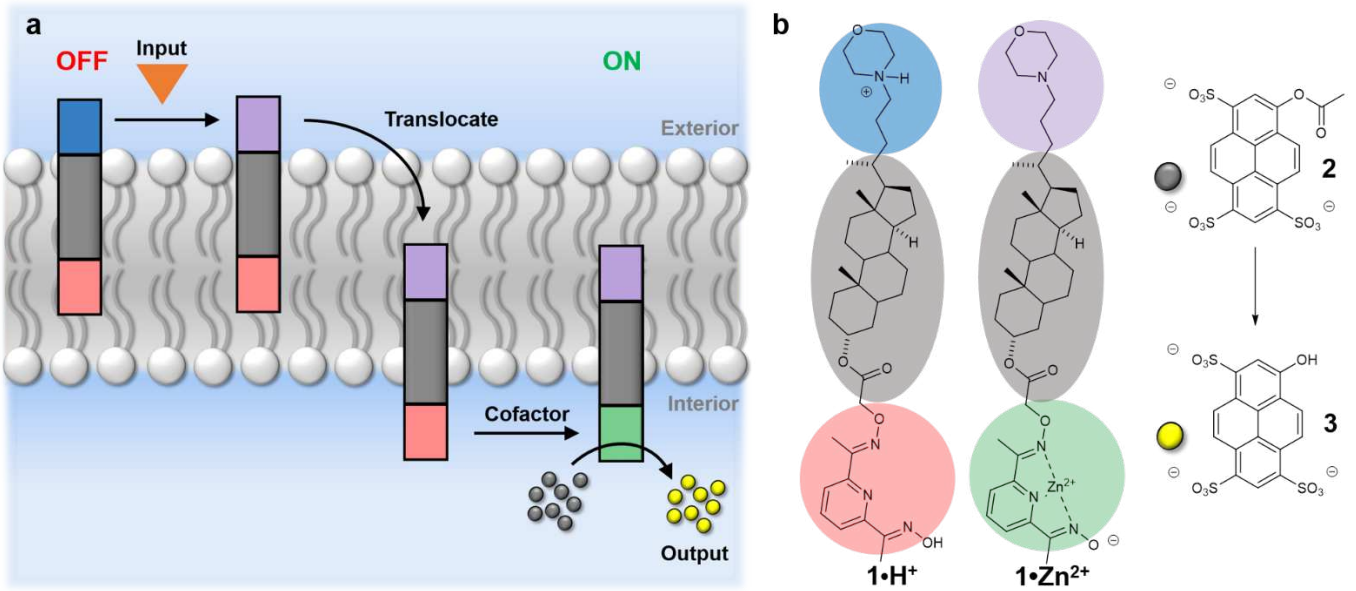

Figure 2. Membrane translocation as a signal transduction mechanism. (a) Cartoon representation showing the behaviour of a synthetic transducer embedded in a lipid bilayer membrane. When the head groups are polar (blue or green), they prefer to sit in the aqueous phase, and when they are non-polar (red or purple), they can enter the membrane. The input signal switches the external head group from polar to non-polar, and the cofactor switches the internal head group from non-polar to polar. Concerted switching of the head group polarities drives translocation of the transducer across the bilayer. The internal head group is a pro-catalyst that is activated by cofactor binding. In the OFF state, the pro-catalyst is embedded in the membrane and is inactive (red). In the ON state, the activated catalyst is exposed to the internal aqueous phase and turns over an encapsulated substrate to generate the output signal. (b) Molecular structures of protonated signal transducer **1•H**$^{+}$ (OFF state), the activated catalyst **1•Zn**$^{2+}$ (ON state), substrate **2** and fluorophore product **3**.

To demonstrate the viability of this concept, we designed and prepared a bistable pH-responsive signal transducer, able to both transduce and amplify an input signal across a vesicle bilayer membrane by catalysing the hydrolysis of an encapsulated substrate. The artificial signal transduction system is assembled from three components:

Transducer 1 (Fig. 2b) has two switchable head-groups linked by a steroid core. The steroid (grey moiety in Fig. 2b) anchors the transducer in the bilayer membrane in a perpendicular orientation. One head group is a protonated morpholine (blue moiety in Fig. 2b), which acts as the sensor for the input signal. When the pH on the outside of the vesicle is below the pK_a of morpholine (≈ 8), the charged head group will be held in the external aqueous phase (the OFF state). Raising the external pH will remove the charge and allow the neutral morpholine head group (purple in Fig. 2b) to enter the membrane. The other head group is a neutral pyridine-oxime pro-catalyst (red in Fig. 2b), which is soluble in the hydrophobic lipid membrane. This head group can be switched to a charged state by coordination of a zinc ion cofactor (green in Fig. 2b), which pulls the catalyst into the internal aqueous phase. Zinc binding also lowers the pK_a of the oxime from 11 to less than 6. If the pH on the inside of the vesicle is maintained at 7 by a buffer, cofactor binding will lead to deprotonation of the oxime and activation of the catalyst (the ON state). The deprotonated zinc-oxime complex catalyses the hydrolysis[16] of encapsulated substrate **2** to generate the output signal.

Substrate 2 (Fig. 2b) is highly charged at neutral pH, so it does not cross lipid bilayer membranes and can be efficiently encapsulated inside vesicles. The substrate is non-fluorescent, but the ester group can be hydrolysed by the zinc complex of the oxime catalyst at neutral pH to give a fluorescent product, **3**, which is also highly charged and does not cross lipid bilayer membranes. The conversion of **2** to **3** inside vesicles can therefore be monitored using fluorescence spectroscopy. In addition, the fluorescence excitation spectrum of **3** can be used to quantify local pH changes independently of the total product concentration, providing a mechanism for monitoring the integrity of the vesicles (see Supplementary Figure 2).[17]

Phospholipids used for vesicle preparation must generate membranes that are capable of maintaining a pH gradient, so that the pH on the outside and inside of the vesicles can be changed independently. In addition, the vesicles must be impermeable to substrate **2**, product **3**, the buffer and the zinc ion cofactor. A mixture of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) lipids was found to perform this function effectively.

Results and Discussion

Signal transduction

Transducer **1** was synthesised in six steps from lithocholic acid and substrate **2** was prepared in one step from **3** (see Supplementary Information). The artificial signal transduction system was assembled by sonicating a mixture of transducer **1**, DOPC and DOPE in a solution of **2**, zinc chloride and HEPES buffer (pH 7) and then extruding the suspension through a polycarbonate membrane to afford unilamellar vesicles with an average diameter of 200 nm (see Supplementary Figure 5).[18] A control system was prepared in an identical manner, except that transducer **1** was omitted. The vesicles were separated from the external solution using size exclusion chromatography and then suspended in a non-buffered sodium chloride solution (pH 7). This procedure affords vesicles with a statistical distribution of transducer **1** in the inner and outer leaflets of the membrane with half of the molecules in the orientation shown in Fig. 2a and the other half inverted with the recognition group on the inside of the vesicle. However, only transducer molecules that have inserted in the correct orientation will produce a signal. For inverted transducer molecules, the protonated morpholine head-group is locked at pH 7 in the aqueous phase inside of the vesicle, the pro-catalyst is inactive, and external pH changes will have no effect.

The results of the signaling experiment using this system are shown in Fig. 3. For the control system lacking the transducer, a small increase in fluorescent emission intensity was observed over a period of hours, which reflects the slow background rate for solvolysis of the substrate (black data in Fig. 3a).[19] For the signaling system including the transducer, the behaviour was identical to the control system when the external pH was maintained at 7 (red data in Fig. 3a). However, when sodium hydroxide was added to raise the external pH to 9, a sharp increase in fluorescence was observed (green data in Fig. 3a). Fluorescence excitation spectroscopy showed that the pH of the internal vesicle solution was unchanged at 7 (Supplementary Figure 1), so the rapid hydrolysis of **2** must be due to catalysis of the reaction inside the vesicle. In other words, addition of the hydroxide signal on the outside of the vesicles leads to membrane translocation of transducer **1** and activation of the oxime catalyst by coordination to zinc ions on the inside of the vesicles. The compartmentalised nature of the signal transduction process was visualised using Total Internal Reflection Fluorescence Microscopy.[20] For the signaling system including the transducer at pH 7, no emission was observed from the vesicles (Fig. 3b, bottom). When the pH of the external solution was raised to 9 by adding sodium hydroxide, localised spherical regions of fluorescence emission were

observed due to **3** encapsulated inside the vesicles (Fig. 3b, top). This observation shows that the reaction is localised on the inside of the vesicles and that neither **2** nor **3** leak out under the reaction conditions.

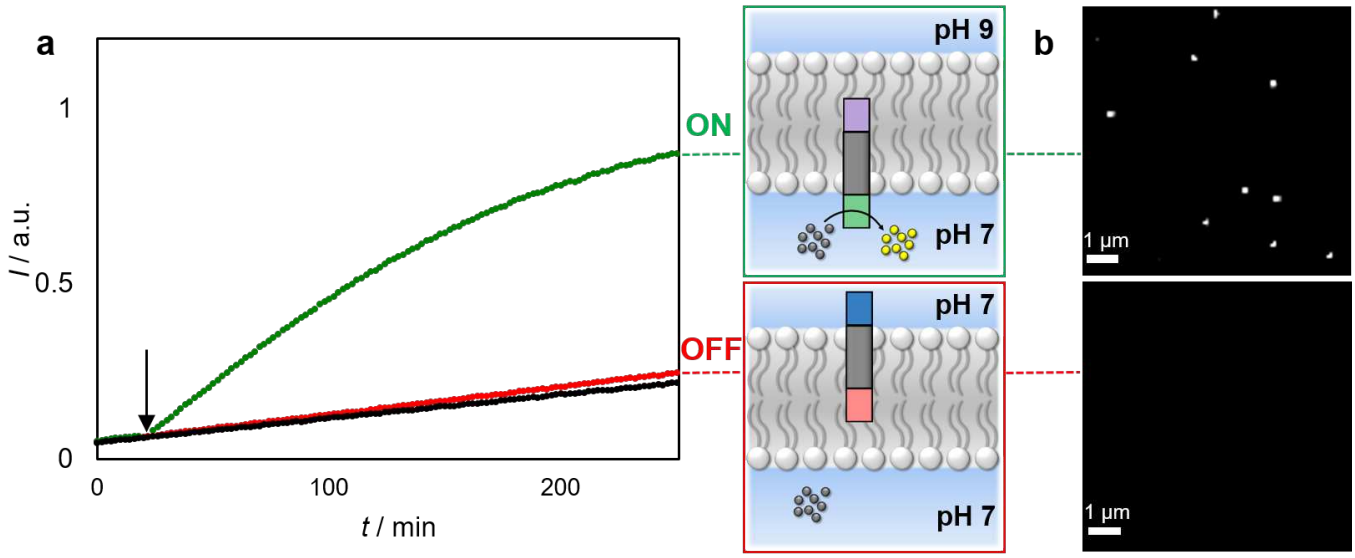

Figure 3. Transmembrane signal transduction (a) Time dependence of the relative fluorescence emission intensity at 510 nm (exciting at 415 nm). Red data: vesicles composed of lipids with 2.5 mol% **1** incubated at an external pH of 7. Green data: vesicles composed of lipids with 2.5 mol% **1** initially incubated at an external pH of 7, and then raised to an external pH of 9 after 20 minutes (indicated by the black arrow). Black data: control vesicles prepared without **1** incubated at an external pH of 7. All experiments were conducted in 200 nm DOPC/DOPE vesicles containing 250 µM **2**, 250 µM $ZnCl_2$ and 100 mM HEPES buffer at pH 7. (**b**) Total internal reflection fluorescence microscopy (TIRFM) images of vesicles composed of lipids with 2.5 mol% **1** under the same conditions: incubated at an external pH of 7 (bottom) and an external pH of 9 (top). Vesicles were adsorbed onto a poly-L-lysine-coated glass slide and excited at 405 nm.

Signal amplification

Signal amplification in biology is achieved by coupling the signal recognition process by the transmembrane receptor to an enzyme catalysed reaction. In this artificial system, deprotonation events on the outside of the vesicle turn on catalysts on the inside of the vesicle, which can turn over multiple substrate molecules. This process provides a mechanism for signal amplification: a single hydroxide ion, corresponding to the external input signal, can generate multiple copies of **3** as the internal output signal. Fig. 4a shows the ratio of the total number of moles of **3** generated inside all of the vesicles in the sample relative to the total number of moles of sodium hydroxide added externally. Under these conditions, the signal transduction process generates a 5-fold amplification relative to the hydroxide input signal. The magnitude of the amplification achievable in this system is simply governed by the concentration of input required to produce a signal and the amount of substrate which can be encapsulated inside the vesicles.

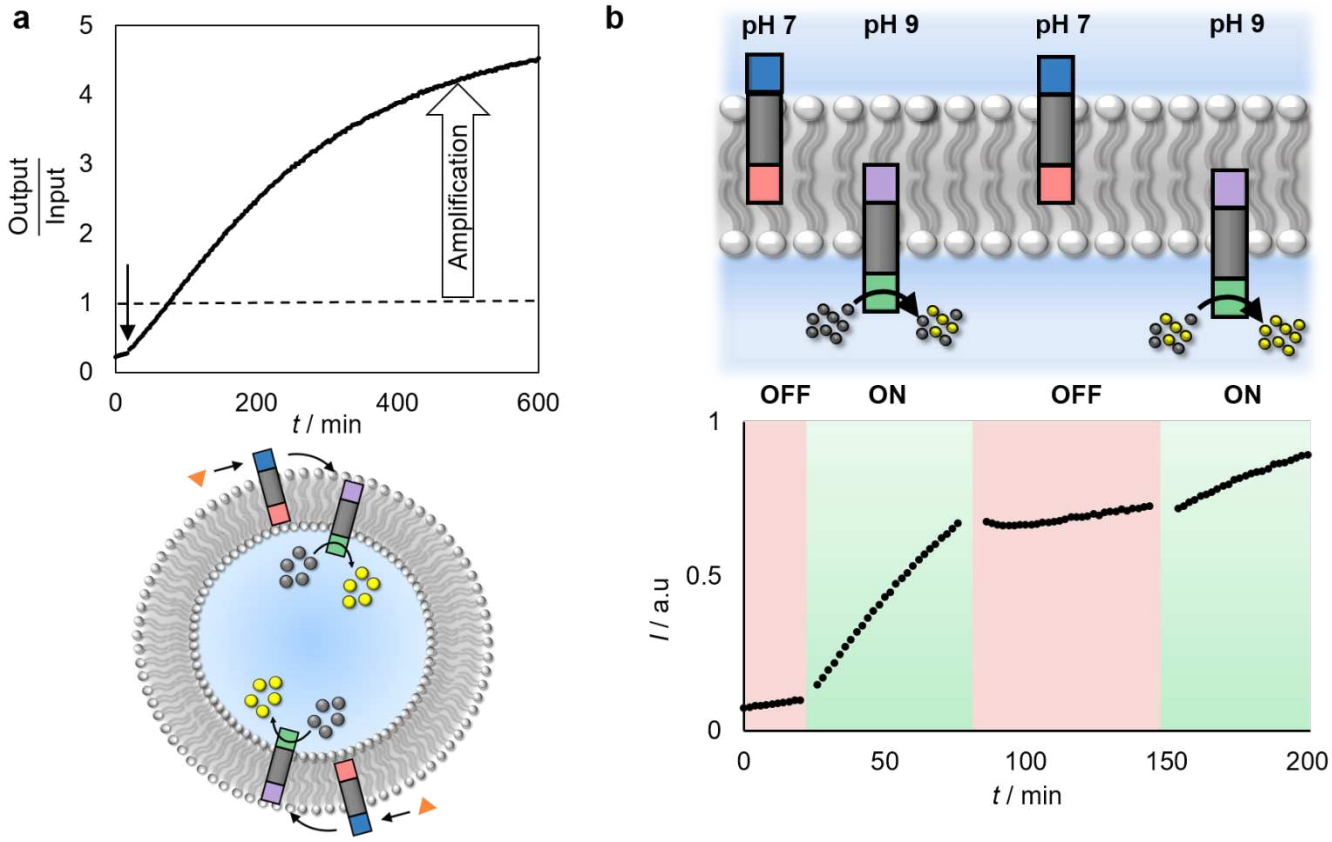

Figure 4. Transmembrane signal amplification and reversible switching of catalysis. (a) Signal amplification in vesicles incubated at an external pH of 7 and then raised to an external pH of 8 after 20 minutes (indicated by the black arrow). The plot shows the time dependence of the molar ratio of internal **3** (output) to external hydroxide (input), determined by monitoring the fluorescence emission intensity at 510 nm (exciting at 415 nm). The cartoon shows that under these conditions one input molecule (orange) generates five output molecules (yellow). (**b**) Cycling the external pH between 7 and 9 moves transducer **1** backwards and forwards across the membrane, switching the catalyst OFF and ON. The plot shows the time dependence of the relative fluorescence emission intensity at 510 nm (exciting at 415 nm) during two strokes of a reciprocating cycle in which the external pH was changed by addition of aliquots of sodium hydroxide and hydrochloric acid to the vesicle suspension. All experiments were conducted in 200 nm DOPC/DOPE vesicles with 2.5 mol% **1** that encapsulated 250 μM **2**, 250 μM $ZnCl_2$ and 100 mM HEPES buffer.

Reversible membrane translocation

The signaling process is fully reversible and can be switched on and off by raising and lowering the pH of the external solution, which generates reciprocating translocation of the transducer across the lipid bilayer. Fig. 4b shows two cycles of this process. When the pH on the outside of the vesicle is 7, the system is in the OFF state. Increasing the external pH to 9 deprotonates the morpholine head group, the transducer crosses the membrane, and the catalyst is activated by binding to zinc in the internal compartment. In this ON state, the substrate is turned over inside the vesicles, leading to an increase in fluorescence. However, it is possible to turn the signaling process off again by adding hydrochloric acid to decrease the external pH to 7. Re-protonation of the morpholine head-group pulls the catalytic oxime unit out of the internal compartment and back into the membrane to re-set the transducer. In this OFF state, there is no further increase in fluorescence (t ≈ 80−140 min region in Fig. 4b). Increasing the external pH to 9 again gives a second ON stroke of the cycle, and the fluorescence increases again. The rate constant for hydrolysis of **2**, obtained by fitting to pseudo-first order rate equations (see Supplementary Fig. 4), is identical in the two ON phases in Fig. 4b, which means that the concentration of active catalyst does not change and the translocation processes do not cause any reorientation of the transducer molecules in the membrane. These experiments demonstrate that the system acts as a reciprocating molecular switch that can transduce and amplify chemical information when in its active state.[21]

Conclusions

The results reported here demonstrate that membrane translocation is an effective mechanism for transmembrane signal transduction and amplification in synthetic systems. The simplicity of this concept suggests that it will be broadly applicable to diverse stimuli, opening up a wide range of possible applications. The ability to change the internal chemistry of membrane-bound capsules will be crucial for the development of bio-inspired nanotechnology, and these responsive vesicles may ultimately form the basis for the construction for biocompatible interfaces for communication with cellular systems.

Methods

Vesicle preparation: A 3:2 mixture of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and 2.5 mol% **1** dissolved in a chloroform / ethanol solution was evaporated and dried thoroughly under vacuum before hydrating with 100 mM HEPES buffer at pH 6.8. Following sonication the suspension was subjected to 5 cycles of freeze-thaw using liquid nitrogen. Stock solutions of ester **2** and zinc chloride were added to reach final concentrations of 250 μM. The suspension was extruded 19 times through a 200 nm polycarbonate filter in an Avestin Lipofast apparatus,[18] before separating the vesicles from the bulk solution using pre-packed GPC columns (Sephadex G-25) eluting with a 100 mM NaCl solution at pH 6.8. **Signaling experiments** were conducted on vesicle suspensions in a 2.5 mL cuvette using a Cary Eclipse fluorescence spectrophotometer in excitation spectrum mode, measuring the emission intensity at 510 nm at 2 minute intervals, using slit widths of 5 nm. For further information see the supplementary information.

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Author contributions: M.J.L., F.K. and M.C. carried out the experimental work. N.H.W. and C.A.H. supervised the project. All authors contributed to the experimental design, analysis of the results and discussion of the manuscript. M.J.L., F.K., N.H.W. and C.A.H. wrote the paper.

Additional information

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