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Tetrahedron 60 (2004) 11307–11315

Fluorophore-linked zinc(II)dipicolylamine coordination complexes as sensors for phosphatidylserine-containing membranes

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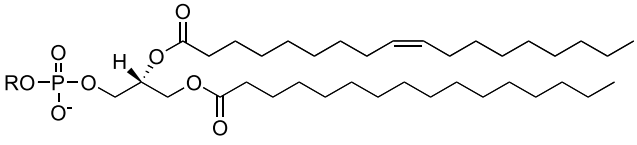
Received 19 February 2004; revised 8 July 2004; accepted 19 August 2004

Available online 15 September 2004

Abstract—A series of Zn^{2+}–2,2′-dipicolylamine (Zn^{2+}–DPA) coordination complexes with an attached NBD fluorophore are synthesized and evaluated as fluorescent sensors. The sensors do not respond to vesicles composed of zwitterionic phosphatidylcholine, but the NBD fluorescence emission is enhanced in the presence of anionic vesicles. A sensor with two Zn^{2+}–DPA units and a hydrophilic tris(ethyleneoxy) linker produced a larger emission enhancement than an analogue with a butyl linker, and titration with 1:1 POPC:POPS vesicles lead to an apparent phospholipid association constant of 5.3×10^4 M^{-1}. The sensor can detect the presence of vesicles containing as little as 5% phosphatidylserine. The sensing effect apparently requires a membrane surface because the sensors do not respond to a phosphatidylserine derivative that is monodispersed in aqueous solution.

1. Introduction

New and improved assays for the in vitro and in vivo detection of apoptosis (programmed cell death) would be a useful contribution to research in cell biology and clinical medicine. Current methods used in detecting apoptosis focus on changes in cytosolic biochemical events, degradation of cell nuclear material, and changes in the cell plasma membrane structure.[1–4] Each assay has its own inherent advantages and shortcomings. For example, monitoring of cytosolic caspase activity and detection of nucleic acid fragmentation are classic assays for apoptosis.[5–8] However, these processes often occur too late in the apoptosis process to allow other important events to be detected. Monitoring the appearance of phosphatidylserine (PS, Fig. 1) on a cell surface is a more suitable assay for the early stages of apoptosis.[9–11] PS is maintained almost exclusively in the inner monolayer of the plasma membrane of healthy animal cells, but a scrambling effect is initiated early in apoptosis which results in externalization of PS.



Formula for R	Phospholipid
-H	POPA
$-CH_2CH_2N(CH_3)_3^+$	POPC
$-CH_2CH(NH_3^+)COO^-$	POPS
$-CH_2CH(OH)CH_2OH$	POPG

Figure 1. Head group structure of common phospholipids.

Detection of externalized PS is currently achieved using annexin V, a member of a class of Ca^{2+}-dependent phospholipid binding proteins.[12,13] Annexin V exhibits high affinity for PS, and binding of fluorescent labeled annexin V (e.g., annexin-FITC) to the cell surface is currently used in microscopic and flow cytometric assays for the detection of apoptosis. However, use of annexin V is not without limitations. PS binding by annexin V is a Ca^{2+}-dependent process, which raises the concern of

Keywords: Molecular recognition; Phospholipid binding; Phosphate recognition; Chemosensor; Apoptosis; Phospholipid asymmetry; Phosphatidylserine.

Abbreviations: DHPC, 1,2-dihexanoyl-*sn*-glycero-3-phosphocholine; DHPS, 1,2-dihexanoyl-*sn*-glycero-3-[phospho-L-serine]; NBD, 7-nitro-1,3-benz-2-oxa-1,3-diaza-4-yl; PC, phosphatidylcholine; POPA, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphate; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; POPG, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-[phospho-L-serine]; PS, phosphatidylserine; TEO, tris(ethyleneoxy); TES, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid.

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doi:10.1016/j.tet.2004.08.052

activation of non-specific Ca^{2+}-dependent phospholipid scramblases within the membrane bilayer when Ca^{2+}-rich annexin binding buffers are used. Further, the protein is susceptible to degradation and exhibits slow binding kinetics.[14,15] Low molecular weight synthetic mimics of annexin V that bind rapidly to PS-enriched membrane surfaces in a Ca^{2+}-independent manner would be an attractive alternative.

An X-ray crystal structure of an annexin–glycerophosphoserine complex shows that the phosphoserine head group is coordinated to one of the four canonical binding sites of the protein through two bridging Ca^{2+} ions.[16] This picture suggested to us that synthetic metal complexes with appropriate charge, geometry, and spatial orientation may bind to the head group of anionic phospholipids preferentially over zwitterionic phospholipids. In other words, rationally designed coordination complexes may be useful as mimics of annexin V. Several phosphate chemosensors with Zn^{2+}-coordinated binding sites have been described in the literature.[17–19] For example, Hamachi and co-workers recently described an anthracene-based sensor with two zinc dipicolylamine (Zn^{2+}–DPA) units (Fig. 2).[20] The sensor binds to the dianionic phosphotyrosine groups in phosphorylated peptides with association constants of up to $10^7\ M^{-1}$.

We subsequently demonstrated that this sensor, which we call PSS-380, can be used to detect the presence of anionic phospholipids, specifically PS, on the surface of vesicles and cells.[21] While the fluorescence emission of PSS-380 was unchanged upon treatment with vesicles composed entirely of zwitterionic phosphatidylcholine (PC), a 10-fold increase in fluorescence emission was observed upon addition of anionic vesicles composed of 1:1 POPC:POPS (Fig. 1). PSS-380 was successfully used to detect apoptosis in Jurkat cells treated with camptothecin (an apoptosis inducing agent) via fluorescence microscopy and flow cytometry. Although PSS-380 is an attractive alternative to annexin V-FITC, its wavelength of excitation (380 nm) is in the UV range, which is not compatible with the lasers in most flow cytometers. Replacing the anthracene unit with fluorophores that absorb at longer wavelengths would alleviate this drawback.

In an effort to develop second generation sensors, we have chosen to pursue a modular design which allows us to

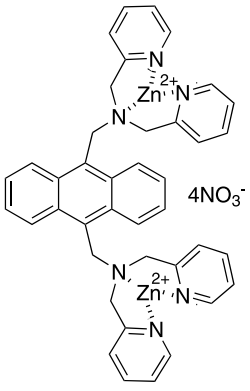


Figure 2. Structure of PSS-380.

systematically alter the three molecular components shown in Figure 3: the PS binding group, the linker, and the fluorophore. Herein, we report the design and synthesis of the first examples of this series, namely, coordination complexes **1**–**4** (Fig. 4). The design retains the Zn^{2+}–DPA affinity units, attached in a meta orientation to a phenyl ring,[18] which is in turn linked to a 7-nitrobenz-2-oxa-1,3-diaza-4-yl (NBD) fluorophore. The NBD fluorophore is excited at 470 nm and exhibits a broad emission centered at 530 nm, which limits its use in microscopy and flow cytometry applications; nevertheless, its environmental sensitivity makes it useful for the mechanistic experiments performed here.

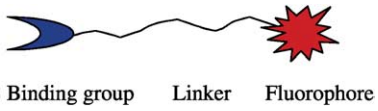


Figure 3. Modular design of PS sensor.

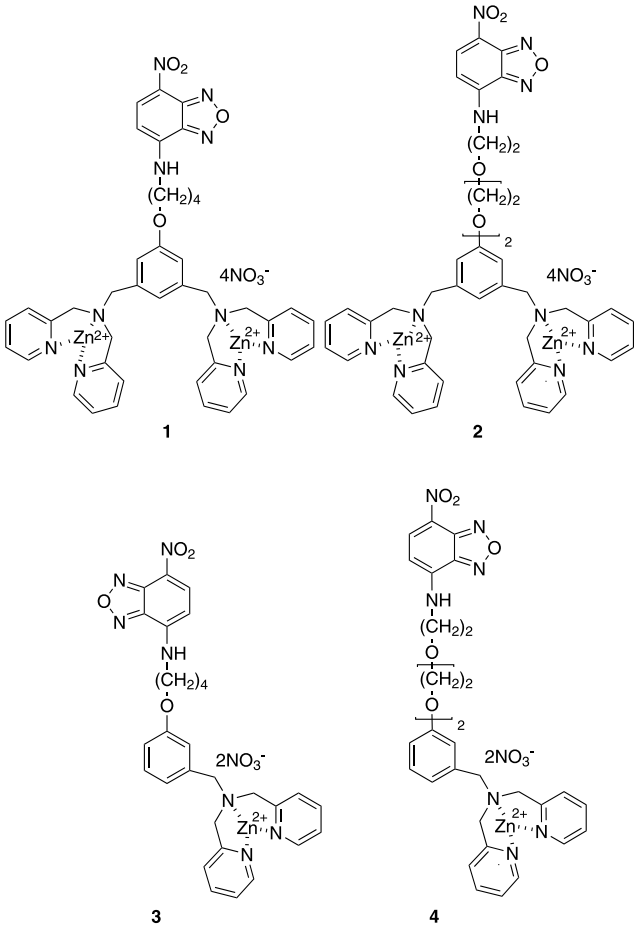


Figure 4. Coordination complexes **1**–**4** as potential PS sensors.

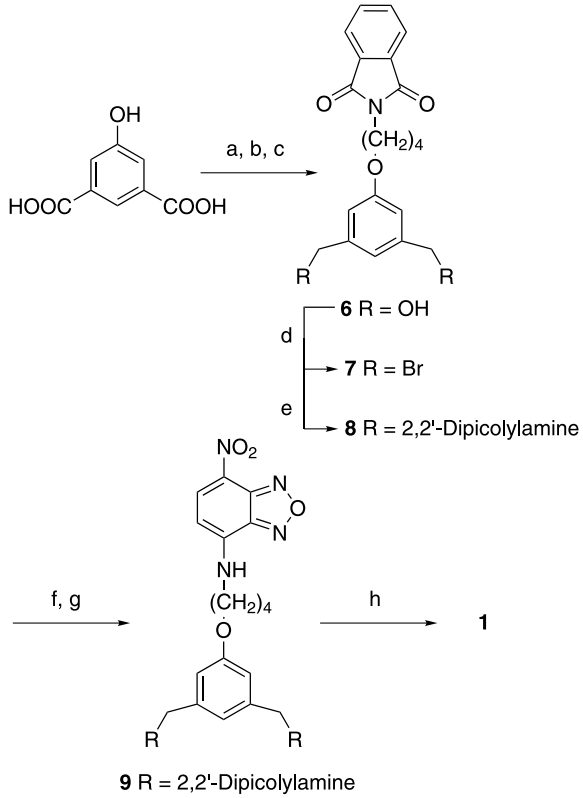
We examined two types of linkers, butyl (relatively hydrophobic) and tris(ethyleneoxy) (TEO, relatively hydrophilic), anticipating that upon binding, the nature of the linker may influence the interaction between the fluorophore and the bilayer membrane. We illustrate how sensor

response to PS-enriched membrane surfaces is altered by changes in the number of Zn^{2+}–DPA binding units, as well as the structure of the linker.

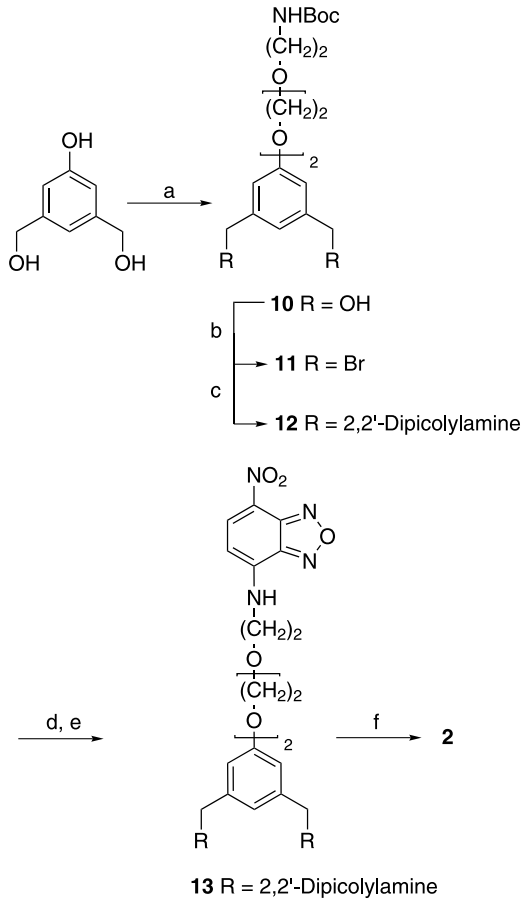
2. Results and discussion

2.1. Synthesis

The synthesis of **1** was achieved in eight steps starting from commercially available 5-hydroxyisophthalic acid (Scheme 1). The butyl linker was attached to 3,5-bis(hydroxymethyl)phenol,[22,23] using *N*-(4-bromobutyl)phthalimide and K_2CO_3 in acetonitrile, and the product converted in high yield to the corresponding dibromide **7** using CBr_4 and Ph_3P. The zinc binding groups were introduced in the next step by treating **7** with DPA in the presence of K_2CO_3 in DMF. The phthalimide protecting group was removed using hydrazine hydrate and the resulting amine was treated with NBD-Cl in the presence of K_2CO_3 in dry THF to afford **9** in 70% yield. The complexation of **9** with $Zn(NO_3)_2$ in methanol/water mixture was quantitative and the complex was used for the binding studies without further purification. Compound **2**, with a TEO linker was synthesized using a similar synthetic strategy (Scheme 2). In addition, the syntheses of control compounds **3** and **4**, each with one Zn^{2+}–DPA unit are depicted in Schemes 3 and 4.



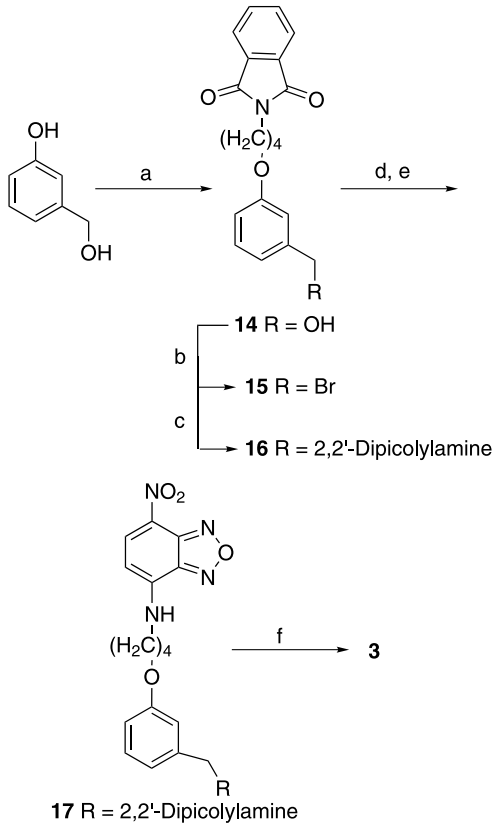
Scheme 1. (a) EtOH, concd H_2SO_4, reflux, (96%); (b) $LiAlH_4$, THF, 0 °C to rt, (96%); (c) *N*-(4-bromobutyl)phthalimide, K_2CO_3, acetonitrile, reflux, (90%); (d) CBr_4, Ph_3P, CH_2Cl_2, (89%); (e) 2,2′-dipicolylamine, K_2CO_3, DMF, (91%); (f) hydrazine hydrate, CH_2Cl_2/EtOH, reflux, (90%); (g) NBD-Cl, K_2CO_3, THF (70%); (h) zinc nitrate, MeOH/H_2O, (100%).



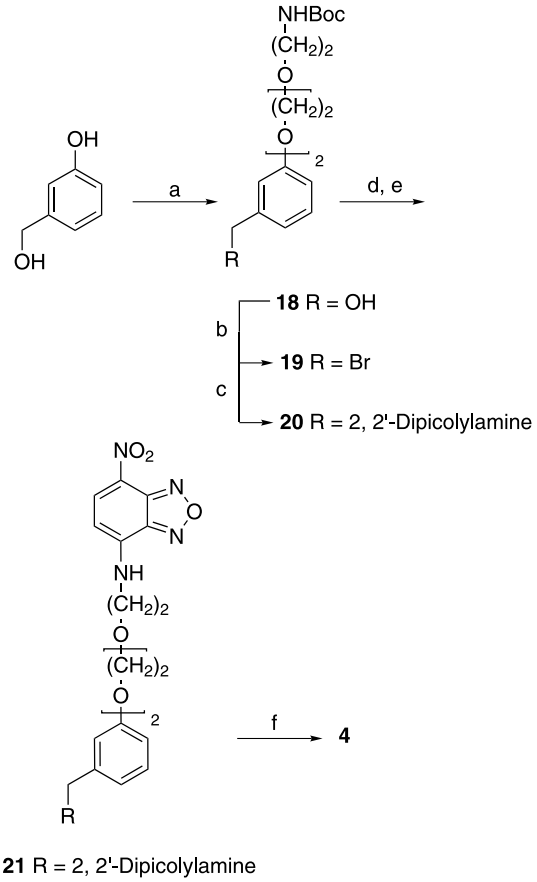
Scheme 2. (a){*N*-Boc-{2-[2-(2-bromoethoxy)-ethoxy]-ethyl}monoamine, K_2CO_3, acetonitrile, reflux (89%); (b) CBr_4, Ph_3P, CH_2Cl_2 (70%); (c) 2,2′-dipicolylamine, K_2CO_3, DMF (87%); (d) TFA, CH_2Cl_2 (85%); (e) NBD-Cl, K_2CO_3, THF (70%); (f) zinc nitrate, MeOH/H_2O (100%).

2.2. Evaluation of vesicle sensing properties

The ideal candidate for apoptosis detection via externalized PS should have a strong affinity for anionic PS embedded in a membrane that is primarily composed of zwitterionic phospholipids. The sensor binding, however, must not disrupt the membrane structure. The effect of **1** on membrane integrity was measured in two ways. The first was vesicle leakage, where **1** was found to be incapable of inducing bilayer permeabilization. Specifically, addition of **1** at concentrations up to 10 μM to vesicles composed of either 100% POPC or 1:1 POPC:POPS failed to induce carboxyfluorescein leakage. Furthermore, **1** and **2** do not penetrate into vesicles. This was proved by adding sensors **1** or **2** at concentrations up to 2.0 mol% to a dispersion of vesicles (25 μM total phospholipid) consisting either of 100% POPC or 1:1 POPC:POPS. Subsequent addition of the membrane-impermeable reducing agent sodium dithionite at 2 h after sensor addition resulted in greater than 95% quenching of fluorescence. The near total quenching of NBD fluorescence by sodium dithionite indicates that **1** and **2** do not cross the vesicle membrane and become protected from chemical reduction. The ability of **1** and **2** to bind to PS-enriched membranes (see below) and not disrupt the membrane structure establishes the utility of the *meta*-aryl bis (Zn^{2+}–DPA) unit as a PS affinity group in modular designs of PS-sensors.



Scheme 3. (a) *N*-(4-Bromobutyl)phthalimide, K_2CO_3, acetonitrile, reflux (67%); (b) CBr_4, Ph_3P, CH_2Cl_2 (85%); (c) 2,2′-dipicolylamine, K_2CO_3, DMF (93%); (d) hydrazine hydrate, EtOH/CH_2Cl_2, reflux (90%); (e) NBD-Cl, K_2CO_3, THF (72%); (f) zinc nitrate, MeOH/H_2O (100%).



Scheme 4. (a){*N*-Boc-{2-[2-(2-bromoethoxy)-ethoxy]-ethyl}monoamine, K_2CO_3, acetonitrile, reflux (89%); (b) CBr_4, Ph_3P, CH_2Cl_2 (65%); (c) 2,2′-dipicolylamine, K_2CO_3, DMF (87%); (d) TFA, CH_2Cl_2 (85%); (e) NBD-Cl, K_2CO_3, THF (76%); (f) zinc nitrate, MeOH/H_2O (100%).

The NBD fluorophore is known to exhibit an enhancement in fluorescence intensity upon transfer from a polar environment to an apolar environment.[24,25] Thus, the fluorescence emission from sensors **1** and **2** was expected to increase upon binding to the surface of a bilayer membrane. Indeed, titration of both sensors with anionic vesicles produced moderate to large fluorescence enhancements. While such an enhancement is not a necessary feature for employment in apoptosis detection, the observed NBD fluorescence enhancement upon membrane binding provides insight into the interactions between the sensor and the membrane surface. The titration experiments involved addition of unilamellar vesicles composed of 1:1 POPC:POPS, 1:1 POPC:POPA, 1:1 POPC:POPG, or 100% POPC to a 1 μM solution of **1** or **2**. The resulting isotherms (Fig. 5) were fitted to a 1:1 binding model, which allowed calculation of apparent phospholipid binding constants.[†] With sensors **1** and **2**, the order of binding affinities to vesicles was 1:1 POPC:POPS > 1:1 POPC:POPG ~ 1:1 POPC:POPA ≫ 100% POPC (Table 1). The sensors were not expected to bind the zwitterionic POPC head group, and this titration was performed as a control to determine specificity of the sensors for anionic PS. Although **1** binds to anionic vesicles with slightly higher binding constants than **2**, the more important sensing property is the difference in emission intensity for anionic vesicles versus 100% POPC. In this case, the response with sensor **2** is much more selective. For example, when the total phospholipid concentration is 60 μM, the emission of butyl-linked sensor **1** with 1:1 POPC:POPS vesicles is about two times that observed with 100% POPC vesicles, whereas, the intensity ratio with the TEO-linked sensor **2** is about five.

Sensors **1** and **2** only respond to membrane-bound phospholipids, as evidenced by the lack of response to the short acyl chain phospholipids, dihexanoylphosphatidylcholine (DHPC) and dihexanoylphosphatidylserine (DHPS), which exist as monomeric dispersions in aqueous media. For example, addition of DHPC (100 μM) or a 1:1 mixture of DHPC:DHPS to a 1 μM solution of **2** results in no detectable increase in NBD fluorescence intensity (data not shown).

The effect of only one Zn^{2+}–DPA unit in the sensor structure was evaluated using control compounds **3** and **4**. Vesicles composed of 100% POPC or 1:1 POPC:POPS were added to a 1 μM solution of **3** or **4**. As in the case above, the emission of butyl-linked sensor **3** with 1:1 POPC:POPS vesicles (60 μM phospholipid) is about two times that observed with 100% POPC vesicles, whereas the intensity

[†] At this point it is not known if the sensors induce lateral phospholipid aggregation; however, control experiments showed that the meta-aryl bis (Zn^{2+}–DPA) unit does not promote phospholipid transmembrane flip flop. The binding constants in Tables 1 and 2 are considered apparent because they do not consider Zn^{2+} dissociation from the DPA units in **1** and **2** in the absence of vesicles.

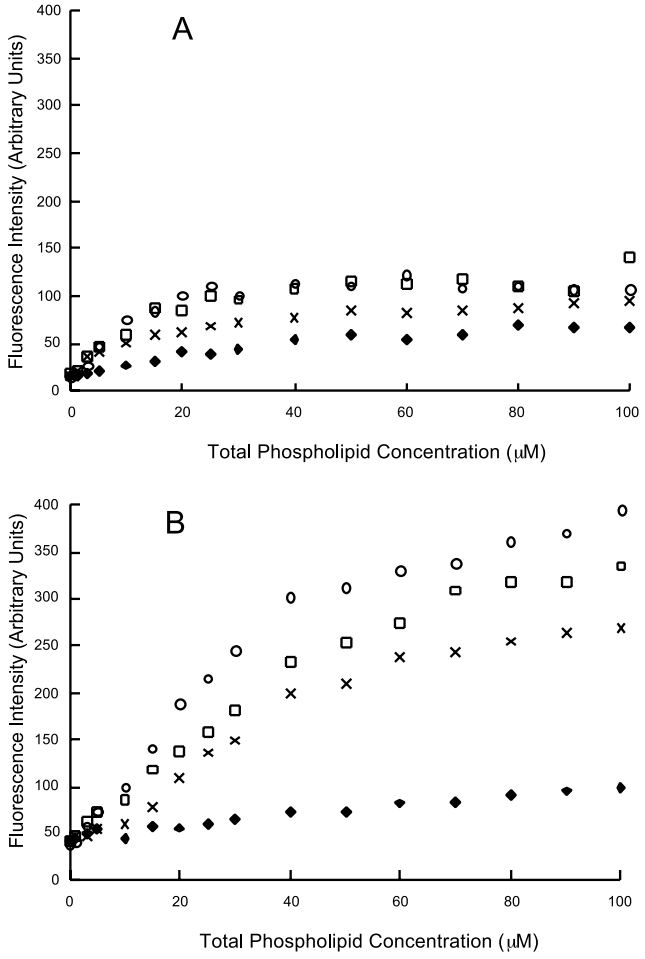


Figure 5. Fluorescence intensity upon addition of vesicles to a 1 μM solution of **1** or **2** in 5 mM TES buffer, 145 mM NaCl, pH 7.4 at 25 °C. (A) Addition of 1:1 POPC:POPS (○), 1:1 POPC:POPG (□),1:1 POPC:POPA (×), or 100% POPC (♦) to **1**. (B) Addition of 1:1 POPC:POPS (○), 1:1 POPC:POPG (□),1:1 POPC:POPA (×), or 100% POPC (♦) to **2**.

ratio with TEO-linked sensor **4** is about five. Thus, it is clear that the hydrophilic TEO-linker is more useful for detecting PS-containing membranes over PC-only membranes. The emission of sensor **2** with two Zn^{2+}–DPA units is significantly higher than that obtained with **4**, indicating that the second Zn^{2+}–DPA unit increases the polar interactions that produce a stronger response to PS-containing membranes.

The Hamachi group has measured association constants for the two Zn^{2+} in PSS-380 and found $K_1 \sim 7\times10^6\ M^{-1}$ and $K_2 \sim 7\times10^4\ M^{-1}$.[26] It is reasonable to think that sensors **1**–**4** have similar Zn^{2+} association constants which means that at 1 μM, the sensor concentration used in this study, a

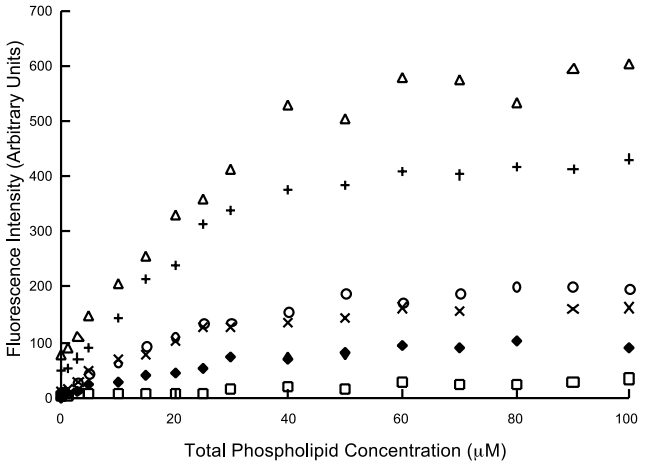


Figure 6. Fluorescence intensity of a 1 μM solution of either **3** or **4** upon addition of phospholipid vesicles consisting of 100% POPC (♦ for **3**, □ for **4**), or 1:1 POPC-POPS (○ for **3**, × for **4**) in 5 mM TES buffer, 145 mM NaCl, pH 7.4 at 25 °C. For comparison, the fluorescence intensity for **2** upon addition of vesicles composed of 1:1 POPC:POPS is represented by (+). Fluorescence intensity of **2** upon addition of vesicles composed of 1:1 POPC:POPS in the presence of 1 μM $Zn(NO_3)_2$ is denoted by (△). All experiments were reproduced at least three times.

significant fraction of **1** and **2** may exist as the corresponding mononuclear Zn^{2+} complexes. To test if Zn^{2+} dissociation induces a measurable effect on sensor performance, the titration of sensor **2** with 1:1 POPC:POPS vesicles was repeated in the presence of 1 μM $Zn(NO_3)_2$. The resulting titration isotherm (Fig. 6) shows a 20% increase in fluorescence enhancement, but no measurable change in binding constant. This is consistent with an equilibrium picture that converts a less responsive mononuclear Zn^{2+} form of sensor **2** into a more responsive binuclear Zn^{2+} form.

The PS sensitivity of sensor **2** was evaluated by conducting additional titration experiments with POPC vesicles enriched with various amounts of POPS. Specifically, a solution of **2** (1 μM) was titrated with vesicles composed of POPC and 0–50% POPS (Fig. 7). The emission response of **2** increases with increasing fraction of PS, until about 20% PS, after which there is a plateau. This trend is also reflected by the apparent binding constants listed in Table 2. The data in Figure 7 shows that sensor **2** can readily detect the presence of bilayer membranes containing 5% PS. It is worth noting that this is approximately the fraction of PS that is externalized during the early-to-intermediate stages of cell apoptosis.[27]

In conclusion, we have shown that coordination complexes with Zn^{2+}–DPA units do not respond to anionic

Table 1. Sensor/phospholipid binding constants (K_a)

Sensor	$K_a\times10^4\ (M^{-1})^a$			
	100% POPC	1:1 POPC:POPS	1:1 POPC:POPG	1:1 POPC:POPA
1	<1	23.3±1.7	14.2±3.8	11±6.0
2	<1	5.3±2.0	2.0±0.5	2.0±0.3
3	<1[b]	11.5±5.5	—	—
4	<1[b]	7.7±3.3	—	—

[a] Values are average of at least three independent measurements.
[b] Values are average of two independent measurements.

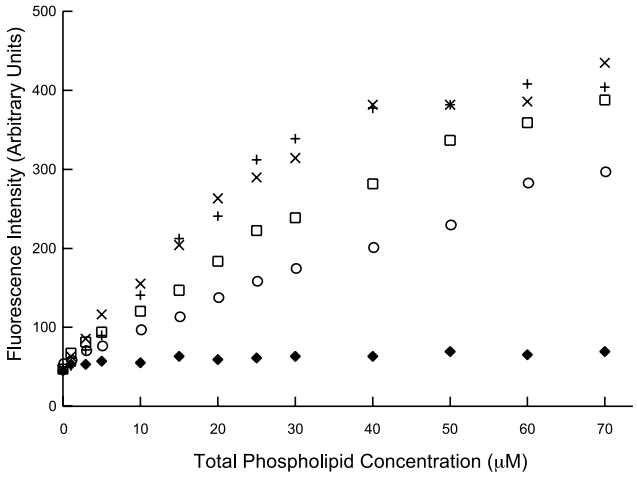


Figure 7. Fluorescence intensity of **2** (1 μM) upon addition of POPC vesicles enriched with 0% (♦), 5% (○), 10% (□), 20% (×), or 50% (+) POPS in 5 mM TES buffer, 145 mM NaCl, pH 7.4 at 25 °C.

Table 2. Sensor **2**/phospholipid binding constants (K_a) for vesicles with varying PS content

	95:5 POPC:POPS	90:10 POPC:POPS	80:20 POPC:POPS	50:50 POPC:POPS
$K_a \times 10^4$ (M^{-1})[a]	1.0±0.3	2.2±0.5	5.7±0.5	5.3±2.0

[a] Values are averages of at least three independent measurements.

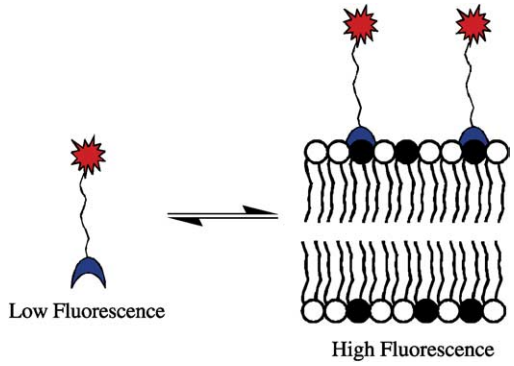


Figure 8. Sensing of PS-containing membranes. PS is represented with filled head group, PC is unfilled head group.

phospholipids (such as PS) when they are monodispersed in aqueous solution, but there is a significant fluorescence enhancement when the anionic phospholipids are part of a vesicle membrane (Fig. 8). The magnitude of the enhancement depends on the structure of the linker between the Zn^{2+}–DPA affinity units and the NBD fluorophore. The more hydrophilic TEO linker produces a sensor with significantly enhanced emission response to anionic PS-enriched membranes compared to zwitterionic PC-only membranes. The results reported here show that the NBD fluorophore can be used as a probe for rapid testing of new sensor designs, and for investigating fundamental questions in molecular recognition at the membrane surface. Future studies will determine if analogous sensors with more practically useful fluorophores can detect PS externalization and cell apoptosis.

3. Experimental

3.1. Synthesis

3.1.1. Synthesis of compound 6. 3,5-Bis-hydroxymethyl phenol (1.06 g, 6.88 mmol), *N*-(4-bromobutyl)phthalimide (2.13 g, 7.57 mmol) and K_2CO_3 (4.75 g, 34.4 mmol) were mixed in acetonitrile and refluxed for 24 h. The reaction mixture was cooled to room temperature and the solvent removed. The residue was redissolved in CH_2Cl_2 and the insoluble residue filtered off. The filtrate was washed with water, dried over $MgSO_4$ and the solvent removed under vacuum. The pure product was obtained as a semi-solid after purification on a silica column using EtOAc. Yield 90%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.82–1.88 (br, 4H), 3.73–3.78 (m, 2H), 3.99–4.03 (m, 2H), 4.63 (s, 4H), 6.81 (s, 2H), 6.90 (s, 1H), 7.70–7.73 (m, 2H), 7.83–7.85 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 25.3, 26.6, 37.7, 64.7, 67.3, 112.0, 117.5, 123.2, 132.1, 133.9, 143.0, 159.2, 168.5.

3.1.2. Synthesis of compound 7. To a solution of compound **6** (3.05 g, 8.62 mmol) and CBr_4 (6.3 g, 18.96 mmol) in dry CH_2Cl_2 at 0 °C, was slowly added Ph_3P (4.85 g, 18.52 mmol) in dry CH_2Cl_2. The reaction mixture was stirred at room temperature overnight. The reaction mixture was concentrated under vacuum and the pure product obtained as a white solid after silica gel chromatography (EtOAc/hexanes (1:1). Yield 89%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.86–1.89 (m, 4H), 3.78 (m, 2H), 4.00 (m, 2H), 4.42 (s, 4H), 6.83 (m, 2H), 6.98 (br, 1H), 7.71–7.74 (m, 2H), 7.84–7.87 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 25.4, 26.6, 33.0, 37.7, 67.4, 115.3, 121.9, 123.3, 132.2, 134.1, 139.7, 159.4, 168.5.

3.1.3. Synthesis of compound 8. Compound **7** (1 g, 2.08 mmol), and K_2CO_3 (1.44 g, 10.4 mmol) were mixed in dry DMF. 2,2′-Dipicolylamine (1.04 g, 5.21 mmol) was added and the reaction mixture stirred overnight. The DMF was removed and the residue dissolved in $CHCl_3$. The insoluble residue was filtered off. The filtrate was washed with water, brine and dried over $MgSO_4$. Solvent was removed and the residue chromatographed on a neutral alumina column using $CHCl_3$ as eluent to afford the pure product. Yield 91%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.85 (br, 4H), 3.62 (s, 4H), 3.74–3.79 (m, 10H), 3.97 (m 2H), 6.84 (s, 2H), 7.04 (s, 1H), 7.08–7.13 (m, 4H), 7.57–7.64 (m, 8H), 7.67–7.70 (m, 2H), 7.81–7.84 (m, 2H), 8.48–8.50 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 25.6, 26.9, 37.8, 58.7, 60.2, 67.2, 113.6, 121.6, 122.1, 122.9, 123.3, 132.2, 134.1, 136.6, 140.8, 149.1, 159.2, 159.9, 168.6; FAB MS *m/z* 718 [M+H$^+$].

3.1.4. Synthesis of compound 9.

3.1.4.1. Generation of free amine from compound 8. Compound **8** (0.550 g, 0.77 mmol) was dissolved in a mixture of CH_2Cl_2 and EtOH (25:75). Hydrazine hydrate

(0.074 g, 2.31 mmol) was added and refluxed for 1 h. The solvent was removed and the residue dissolved in CH_2Cl_2. The insoluble residue was removed by filtration. The filtrate was collected, solvent removed under high vacuum to afford the free amine as a semi-solid. The free amine was directly used in Section 3.1.4.2 without further purification. Yield (90%); ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.60–1.70 (m, 2H), 1.76–1.84 (m, 2H), 2.78–2.83 (m, 2H), 3.28 (br, 2H), 3.63 (s, 4H), 3.79 (s, 8H), 3.93–3.97 (m, 2H), 6.23 (s, 2H), 7.05 (s, 1H), 7.10–7.14 (m, 4H), 7.56–7.64 (m, 8H), 8.48–8.50 (m, 4H).

3.1.4.2. Preparation of compound 9. Amine (0.75 g, 1.28 mmol) and K_2CO_3 (0.88 g, 6.4 mmol) was stirred in dry THF under Ar atmosphere. NBD-Cl (0.28 g, 1.41 mmol) dissolved in dry THF was slowly added and the reaction mixture stirred in dark for 36 h. The reaction mixture was filtered to remove the K_2CO_3 and concentrated under vacuum. The dark green semi-solid obtained was dissolved in CH_2Cl_2, washed with water, brine and dried over $MgSO_4$. The crude product was purified on an alumina column using $CHCl_3$ as eluent. Yield 70%; ^{1}H NMR (300 MHz, CD_3OD): δ 1.86 (br, 4H), 3.52 (s, 6H), 3.66 (s, 8H), 3.98 (m, 2H), 6.20 (d, J=9 Hz, 1H), 6.73 (s, 2H), 6.92 (s, 1H), 7.13–7.18 (m, 4H), 7.54–7.68 (m, 8H), 8.30–8.33 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 25.6, 26.7, 43.9, 58.6, 60.1, 67.3, 98.7, 113.7, 113.9, 122.2, 122.3, 123.0, 136.6, 140.7, 140.9, 144.2, 144.5, 149.2, 158.8, 159.9, 168.3; FAB MS m/z 751 [M+H$^+$].

3.1.5. Synthesis of compound 1. A methanolic solution of compound **9** (0.187 g, 0.25 mmol) and an aqueous solution of zinc nitrate (0.1521 g, 0.512 mmol) were mixed and stirred for 0.5 h. The solvent was removed and the residue lyophilized to afford the complex **1** in quantitative yield.

3.1.6. Synthesis of compound 10. 3,5-Bis-hydroxymethyl phenol (1.35 g, 8.74 mmol), *N*-Boc-{2-[2-(2-bromoethoxy)-ethoxy]-ethyl}monoamine (3.0 g, 9.61 mmol) and K_2CO_3 (6.63 g, 48.05 mmol) were mixed in acetonitrile and refluxed for 24 h. The reaction mixture was then cooled to room temperature and the solvent removed. The residue was dissolved in CH_2Cl_2 and the insoluble residue filtered off. The filtrate was washed with water and dried over $MgSO_4$. The pure product was obtained as a semi-solid after purification on a silica column using EtOAc. Yield 89%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.44 (s, 9H), 3.05–3.30 (br, 2H), 3.30 (br, 2H), 3.45–3.50 (m, 2H), 3.55–3.70 (m, 4H), 3.78 (m, 2H), 4.05 (m, 2H), 4.53 (s, 4H), 5.20 (br, 1H), 6.73 (s, 2H), 6.83 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 28.6, 40.8, 60.6, 65.1, 67.6, 70.0, 70.5, 71.0, 79.6, 112.3, 117.9, 143.1, 156.3, 159.4.

3.1.7. Synthesis of compound 11. Synthesized according to procedure given in Section 3.1.2. Yield 70%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.44 (s, 9H), 3.34 (m, 2H), 3.56 (m, 2H), 3.64–3.67 (m, 2H), 3.71–3.74 (m, 2H), 3.85–3.88 (m, 2H), 4.15–4.18 (m, 2H), 4.43 (s, 4H), 5.00 (br, 1H), 6.90 (s, 2H), 7.01 (s, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 28.6, 33.0, 40.0, 67.6, 69.7, 70.5, 71.0, 115.6, 122.6, 140.0, 159.0.

3.1.8. Synthesis of compound 12. Synthesized according to procedure given in Section 3.1.3. Yield 87%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.42 (s, 9H), 3.32 (m, 2H), 3.54 (m, 2H), 3.63–3.66 (m, 6H), 3.70–3.73 (m, 2H), 3.80 (s, 8H), 3.86 (m, 2H), 4.14 (m, 2H), 5.05 (br, 1H), 6.89 (s, 2H), 7.07 (s, 1H), 7.10–7.15 (m, 4H), 7.56–7.65 (m, 8H), 8.49–8.52 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 28.6, 40.6, 58.8, 60.3, 67.4, 70.0, 70.5, 70.9, 113.8, 121.9, 122.2, 122.9, 136.6, 140.8, 149.2, 159.1, 160.0; FAB MS m/z 748 [M+H$^+$].

3.1.9. Synthesis of compound 13.

3.1.9.1. Generation of free amine from 12. Compound **12** (1.073 g, 1.43 mmol) was dissolved in 6.9 mL of 50% TFA in dry CH_2Cl_2 at 0 °C under N_2 atmosphere. The reaction mixture was stirred at room temperature for 2 h. After removing TFA, the residue was taken in CH_2Cl_2 and neutralized with satd Na_2CO_3. The aqueous layer was extracted with CH_2Cl_2 (3×20 mL) and the combined organic layer dried over $MgSO_4$. The solvent was removed under vacuum to yield the free amine (85%), which was directly used for the next step.

3.1.9.2. Synthesis of compound 13. Synthesized according to procedure given in Section 3.1.4.2. Yield 70%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.64 (br, 6H), 3.74–3.78 (m, 12H), 3.85–3.92 (m, 4H), 4.16–4.19 (m, 2H), 6.11 (d, J= 8.7 Hz, 1H), 6.85 (s, 2H), 7.07 (s, 1H), 7.11–7.20 (m, 5H), 7.55–7.64 (m, 8H), 8.39 (d, J=8.7 Hz, 1H), 8.49–8.51 (m, 4H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 43.9, 58.7, 60.2, 67.5, 68.5, 70.1, 70.8, 71.1, 98.9, 113.8, 122.0, 122.2, 122.9, 128.7, 130.7, 136.5, 136.6, 140.7, 144.1, 144.4, 149.2, 158.9, 159.8; FAB MS m/z 811 [M+H$^+$].

3.1.10. Preparation of compound 2. Synthesized according the procedure given in Section 3.1.5.

3.1.11. Synthesis of compound 14. 3-Hydroxy benzyl-alcohol (1 mmol), *N*-(4-bromobutyl)phthalimide (1.01 mmol) and K_2CO_3 (5 mmol) were mixed in aceto-nitrile and refluxed for 12 h. The work up and purification were done according to procedure given in Section 3.1.1. Yield 67%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.96–2.05 (m, 4H), 2.39 (br, 1H), 3.91 (m, 2H), 4.15 (t, J=5.7 Hz, 2H), 4.80 (s, 2H), 6.93–6.96 (m, 1H), 7.05–7.07 (m, 2H), 7.36–7.41 (m, 1H), 7.85–7.89 (m, 2H), 7.96–8.01 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 25.4, 26.7, 37.7, 65.2, 67.2, 113.0, 113.8, 119.2, 123.3, 129.6, 132.1, 134.1, 142.8, 159.2, 168.6.

3.1.12. Synthesis of compound 15. Compound **14** (1 mmol), CBr_4 (1.1 mmol) were dissolved in dry CH_2Cl_2 and Ph_3P (1.05 mmol) dissolved in dry CH_2Cl_2 slowly added over a period of 15 min. The reaction mixture was stirred at room temperature for 12 h. The solvent was removed and the residue chromatographed on a silica column using gradient elution with EtOAc/Hex (1:1). Yield 85%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.83–1.92 (m, 4H), 3.78 (m, 2H), 4.00 (m, 2H), 4.45 (s, 2H), 6.79–6.83 (m, 1H), 6.90 (m, 1H), 6.94–6.96 (m, 1H), 7.20–7.25 (m, 1H), 7.70–7.75 (m, 2H), 7.84–7.87 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 25.6, 26.8, 33.8, 37.9, 67.4, 114.9, 115.3, 121.5, 123.5, 130.0, 132.4, 134.2, 139.5, 159.4, 168.7.

3.1.13. Synthesis of compound 16. Compound **15** (1 mmol), K_2CO_3 (5 mmol) and 2,2′-dipicolylamine (1.2 mmol) were

mixed in dry DMF under N_2 atmosphere and the reaction mixture stirred for 5 h. The work up and purification were carried out according procedure given in Section 3.1.3. Yield 93%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.86–1.91 (m, 4H), 3.67 (s, 2H), 3.75–3.79 (m, 2H), 3.83 (s, 4H), 3.97–4.01 (m, 2H), 6.74–6.77 (m, 1H), 6.97–7.00 (m, 2H), 7.12–7.23 (m, 3H), 7.59–7.74 (m, 6H), 7.83–7.87 (m, 2H), 8.51–8.53 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 25.6, 26.9, 37.9, 58.6, 60.0, 67.3, 113.3, 115.3, 121.4, 122.3, 123.2, 123.4, 129.5, 132.3, 134.2, 136.8, 149.1, 159.3, 168.7.

3.1.14. Synthesis of compound 17. Synthesized according to procedure given in Section 3.1.4. Yield 72%; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.83 (br, 4H), 3.54 (br, 2H), 3.60 (s, 2H), 3.70 (s, 4H), 4.01 (m, 2H), 6.42 (d, $J=9$ Hz, 1H), 6.68–6.81 (m, 1H), 6.95–6.97 (m, 2H), 7.19–7.26 (m, 3H), 7.56 (d, $J=7.5$ Hz, 2H), 7.75–7.80 (m, 2H), 8.47–8.49 (m, 3H), 9.58 (m, 1H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 24.4, 26.1, 43.0, 57.3, 59.0, 66.9, 99.1, 113.0, 114.7, 120.7, 122.2, 122.5, 129.3, 136.6, 137.9, 140.1, 144.2, 144.4, 145.2, 148.8 158.6, 158.9; FAB MS m/z 540 [M+H$^+$].

3.1.15. Preparation of Zn complex 3. A methanolic solution of compound **17** (1 mmol) and an aqueous solution of zinc nitrate (1.025 mmol) were mixed and stirred for 0.5 h. The solvent was removed and the residue lyophilized to afford the complex **3** in quantitative yield.

3.1.16. Synthesis of compound 18. 3-Hydroxy benzyl-alcohol (1 mmol), *N*-Boc-{2-[2-(2-bromoethoxy)-ethoxy]-ethyl}monoamine (1.01 mmol) and K_2CO_3 (5 mmol) were mixed in acetonitrile and refluxed for 12 h. The work up and purification were done according to procedure given in Section 3.1.6. Yield 89%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.46 (s, 9H), 2.01 (br, 1H) 3.32 (m, 2H), 3.56 3.58 (m, 2H), 3.61–3.76 (m, 4H), 3.87–3.90 (m, 2H), 4.12–4.20 (m, 2H), 4.65–4.69 (m, 2H), 5.09 (br, 1H), 6.85–6.88 (m, 1H), 6.95 (m, 2H), 7.26–7.31 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 28.6, 40.8, 65.4, 67.6, 70.0, 70.5, 71.0, 79.6, 113.3, 114.1, 119.6, 129.8, 143.0, 156.3, 159.3.

3.1.17. Synthesis of compound 19. Synthesized according to procedure given in Section 3.1.12. Yield 65%; ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.43 (s, 9H), 3.20 (br, 2H), 3.55 (m, 2H), 3.63–3.66 (m, 2H), 3.70–3.74 (m, 2H), 3.85–3.88 (m, 2H), 4.13–4.17 (m, 2H), 4.46 (s, 2H), 5.01 (br, 1H), 6.84–6.88 (m, 1H), 6.95–7.00 (m, 2H), 7.23–7.27 (m, 1H); ^{13}C NMR (75 MHz, $CDCl_3$): δ 28.7, 33.7, 40.7, 67.7, 70.0, 70.6, 71.0, 115.1, 115.5, 121.8, 130.1, 139.4, 156.2, 159.2.

3.1.18. Synthesis of compound 20. Synthesized according to procedure given in Section 3.1.13. Yield 87%; ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.35 (s, 9H), 3.03–3.09 (m, 2H), 3.32–3.40 (m, 2H), 3.50–3.53 (m, 2H), 3.56–3.60 (m, 4H), 3.71–3.75 (m, 6H), 4.05–4.08 (m, 2H), 6.75–6.83 (m, 2H), 6.97–7.00 (m, 2H), 7.21–7.28 (m, 3H), 7.57–7.60 (m, 2H), 7.76–7.82 (m, 2H), 8.48–8.50 (m, 2H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 28.2, 39.7, 57.3, 59.1, 66.9, 68.9, 69.2, 69.5, 69.9, 112.9, 114.6, 120.8, 122.2, 122.4, 129.3, 136.6, 140.4, 148.8, 155.6, 158.5, 159.1.

3.1.19. Synthesis of compound 21. Synthesized according to procedure given in Section 3.1.9. Yield 76%; ^{1}H NMR (300 MHz, DMSO-d_6): δ 3.59–3.72 (m, 16H), 4.01–4.04 (m, 2H), 6.44 (d, $J=9$ Hz, 1H), 6.75–6.78 (m, 1H), 6.94–6.97 (m, 2H), 7.18–7.26 (m, 3H), 7.56 (d, $J=7.8$ Hz, 2H), 7.75–7.80 (m, 2H), 8.45–8.49 (m, 3H), 9.44 (br, 1H); ^{13}C NMR (75 MHz, DMSO-d_6): δ 43.4, 57.3, 59.0, 66.9, 68.0, 69.0, 69.9, 99.5, 112.8, 114.6, 120.8, 122.2, 122.4, 129.3, 136.6, 137.8, 140.3, 144.4, 145.3, 148.8, 158.4, 159.1; FAB MS m/z 600 [M+H$^+$].

3.1.20. Synthesis of compound 4. Synthesized according to procedure given in Section 3.1.15.

3.2. Vesicle preparations

All lipids were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL) and stored as 10 mg/mL stock solutions in $CHCl_3$ at -20 °C. Lipids were added in the appropriate ratios to a 10 mL round bottom flask and solvent removed by rotary evaporation. Residual solvent was removed under oil pump vacuum for ≥ 1 h. Dried lipid was then rehydrated in TES buffer (5 mM TES, 145 mM NaCl, pH 7.4). A glass pyrex ring was added to the flasks to ensure complete removal of lipid from the flask walls, and then flasks were vortexed vigorously. The resulting lipid dispersion was then extruded approximately 30 times through a 19 mm diameter polycarbonate membrane with 200 nm pore diameter. All vesicles were prepared at room temperature and used the same day.

3.3. Fluorescence spectroscopy

All fluorescence spectroscopy was performed on a Perkin Elmer LS50B fluorimeter with FT WinLab software using standard $1\times1\times5$ cm^3 cuvettes. NBD fluorescence was measured using excitation and emission wavelengths of 470 and 530 nm, respectively. A 515 nm cutoff filter was used in all NBD acquisitions. Carboxyfluoresecin fluorescence was measured with monochromater excitation and emission wavelengths of 495 and 520 nm, respectively, with an open filter. All measurements were performed at 25 °C without degassing of samples.

3.4. Carboxyfluorescein leakage

Vesicles of the appropriate composition were prepared as described, with the following exceptions. Lipids were rehydrated after drying with TES buffer (5 mM TES, 100 mM NaCl, pH 7.4) containing 50 mM (5–6)carboxy-fluorescein. Following extrusion, vesicles were separated from excess carboxyfluorescein using a Sephadex G-45 column. The fluorescence intensity of a 3 mL sample of 50 μM vesicles was monitored for 400 s. At time 100 s, an appropriate volume of a 1 mM solution of **1** in TES buffer was added to the cuvette, followed by Triton X-100 detergent (0.5%) at time 300 s. Zero and 100% leakage was determined from the initial fluorescence and the fluorescence after addition of detergent, respectively.

3.5. Vesicle titrations

Stock solutions of compounds **1**, **2**, **3** or **4** were diluted in TES buffer to a final concentration of 1 μM in a 5 mL cuvette. With stirring, aliquots of 10 mM phospholipid

vesicles of the appropriate composition were sequentially added to the solution to give the desired phospholipid concentration over the range 0–100 μM. After each addition, the fluorescence intensity was measured after a twenty-second incubation. Curves of fluorescence intensity (λ_{530}) versus available phospholipid concentration (taken as 60% of the total phospholipid concentration) were generated and fitted to a 1:1 binding model.[28] An iterative curve-fitting method yielded the apparent binding constants listed in Tables 1 and 2, all of which represent the average of at least three independent measurements.

3.6. Sensor permeation

To a 45 mL solution of 25 μM phospholipid vesicles composed either of 100% POPC or 1:1 POPC-POPS, compound **1** or **2** was added to a final concentration of either 0.5 or 2.0 mol%. A 3.0 mL aliquot of the resulting solution was removed at predetermined intervals over a two-hour period and placed in a 5 mL cuvette. Fluorescence intensity of each aliquot was monitored over a 200 s interval. At 50 s, sodium dithionite was added to the cuvette to a final concentration of 55 mM. At 150 s, 0.2% octaethylene glycol monododecyl ether was added to disrupt vesicle membranes and expose any internalized NBD-labeled compounds to the aqueous environment.

Acknowledgements

This work was supported by the National Institutes of Health, the Department of Defense, and the Phillip Morris External Research Program.

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