Indicator displacement assays that detect bilayer membranes enriched in phosphatidylserine

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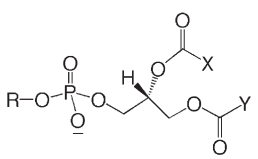
Three indicator displacement assays are described for the detection of phosphatidylserine in a bilayer membrane. A series of Zn^{2+}-dipicolylamine coordination compounds are used to bind selectively to the phosphatidylserine and act as a colorimetric chemosensing ensemble when combined with the UV-Vis indictor pyrocatechol violet. A similar displacement assay uses a coumarin methylsulfonate derivative as a fluorescent indicator, and a third assay involves quenching of calcein fluorescence by Cu^{2+} and subsequent fluorescence restoration upon addition of phosphatidylserine. In the best case, vesicle membranes containing as little as 5% phosphatidylserine could be detected under physiologically relevant conditions using as little as 10 μM sensing ensemble, and two of the three systems allow vesicles containing 50% phosphatidylserine to be detected by the naked eye.

Introduction†

The development of high throughput screening assays is often an important step in the process of identifying lead compounds for drugs and sensors.[1–4] A well-designed assay not only allows many compounds to be screened simultaneously, but also yields an easily detectable result on a short time scale. As a part of our ongoing effort to develop high throughput screens for apoptotic cells, we are investigating different ways of detecting the appearance of phosphatidylserine (see Fig. 1 for representative phospholipid structures) on the surface of animal cell membranes.[5] The plasma membrane of typical animal cells contains about 10% phosphatidylserine, although the amount can differ based on cell type. In healthy cells, all of the phosphatidylserine is located almost exclusively in the membrane inner monolayer,[6] but when cells begin to undergo apoptosis, phosphatidylserine externalization is one of the earliest observable indicators of the process.[7] Initiation of apoptosis in cancerous tissue is a desirable goal in cancer chemotherapy, and simple assays that detect externalized phosphatidylserine would be valuable contributions to anti-cancer research. As a first step we are evaluating the ability of various synthetic receptors to bind vesicle membranes enriched in phosphatidylserine, with the subsequent goal of incorporating these synthetic receptors into indicator displacement assays.

Displacement assays employing fluorescent and chromogenic indicators are becoming an increasingly popular method of detecting metal cations,[8–10] organic[11] and inorganic[12–19] anions, amino acids,[20,21] and other biomolecules.[22–24] In this approach, a receptor with an affinity for a given analyte is used to form a reversible complex with an indicator, usually a fluorescent or UV-Vis dye. Upon complexation with the receptor, the spectral properties of the indicator undergo some measurable change (fluorescence quenching, UV-Vis absorbance decrease, shift in absorbance maximum, *etc.*). Treatment of this indicator–receptor complex with the analyte results in displacement of the indicator from the receptor and a restoration of the indicator's original spectral properties.

In the present study, we utilize fluorescence and UV-Vis spectroscopy in three displacement assays (Fig. 2). In each case, the spectral properties of an indicator are changed in response to association with a receptor, either the Zn^{2+}-coordination compounds **1**–**4** (Fig. 3) or a Cu^{2+} cation. Treatment of this receptor–indicator complex with phosphatidylserine-enriched vesicles results in displacement of the indicator from the receptor and restoration of the original indicator spectral properties. The best of the following three assays is able to detect phosphatidylcholine vesicles containing as little as 5% phosphatidylserine.



Phospholipid	R	X	Y
POPA	H-	$C_{17}H_{33}$	$C_{15}H_{31}$
POPC	$(CH_3)_3N^+CH_2CH_2$-	$C_{17}H_{33}$	$C_{15}H_{31}$
POPS	$(^-OOC)(H_3N^+)CHCH_2$-	$C_{17}H_{33}$	$C_{15}H_{31}$
POPG	$HOCH_2CH(OH)CH_2$-	$C_{17}H_{33}$	$C_{15}H_{31}$
DHPS	$(^-OOC)(H_3N^+)CHCH_2$-	C_5H_{11}	C_5H_{11}
DHPC	$(CH_3)_3N^+CH_2CH_2$-	C_5H_{11}	C_5H_{11}

Fig. 1 Phospholipid structures.

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† Abbreviations: DHPC: (1,2-dicaproyl-*sn*-glycero-3-phosphocholine), DHPS: (1,2-dicaproyl-*sn*-glycero-3-[phospho-L-serine] sodium salt), DPA: 2,2′-dipicolylamine, POPA: (1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphatidic acid), 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] sodium salt).

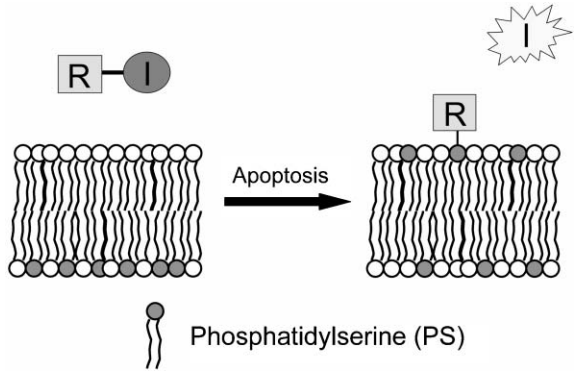

Fig. 2 Displacement assay for apoptosis: following phosphatidylserine externalization to the outer membrane monolayer, the indictor (I) is displaced from the receptor–indicator complex (R–I) by the phosphatidylserine headgroup, generating a detectable signal by restoration of the spectral properties of the indicator.

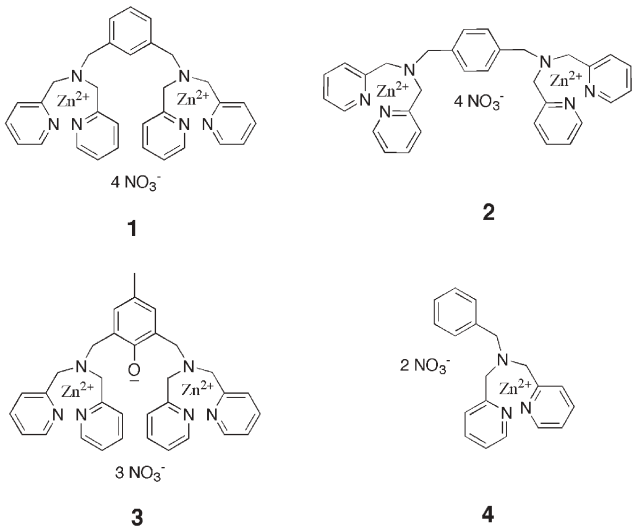

Fig. 3 Structure of the Zn^{2+}-DPA coordination complexes used as receptors for phosphatidylserine in the displacement assays.

Results and discussion

Assay 1: Displacement of UV-Vis dye 5

The Zn^{2+}–DPA coordination compounds **1**–**4** were prepared as described elsewhere.[25] The high solubility of these compounds in aqueous solution makes them ideally suited for the present sensing application. Previous work has shown that in aqueous solution, Zn^{2+}–DPA complex **3** will bind the UV-Vis dye pyrocatechol violet **5** (Fig. 4), resulting in an increased absorption band at 600–630 nm.[19] Subsequent treatment of this receptor–indicator complex with phosphate oxyanions decreases the absorption intensity due to displacement of the indictor from the receptor by the anionic species. We observe similar effects on the absorption spectrum of **5** following titration with **1**, **2**, and **4** (Fig. 5). Plots of absorbance as a function of receptor concentration were fit to a 1 : 1 binding model[26] and the derived binding constants are presented in Table 1. The binding of **1** and **2** to **5** was too tight to be determined, perhaps resulting from the additional +1 charge carried by these compounds relative to **3**.

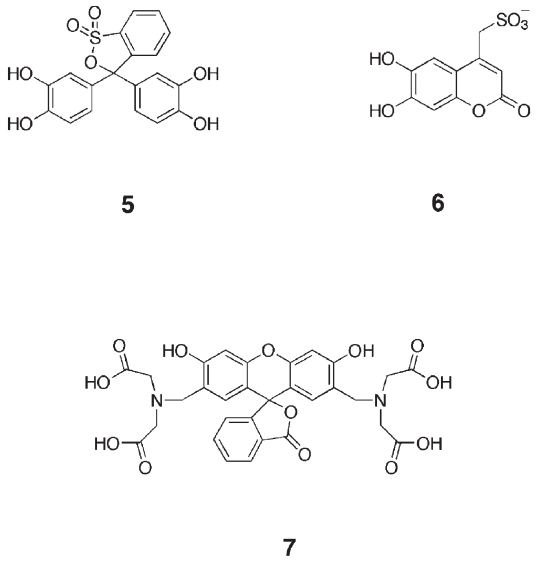

Fig. 4 Structure of pyrocatechol violet (**5**), the coumarin methylsulfonate (**6**), and calcein (**7**) used as indicators in the displacement assays.

The next goal was to determine if PS-rich vesicles can displace **5** from the Zn^{2+}–DPA receptors. By mixing equimolar amounts of **1**–**4** with **5** (50 μM each), a sensing ensemble was formed which was then titrated with POPC vesicles containing between 0 and 50% POPS. Vesicles containing 50% POPS were easily detected by the sensing ensemble formed between all four receptors and **5**, but the ensembles of receptors **1** and **2** failed to respond to vesicles containing a lesser POPS fraction. The **3** : **5** ensemble was successfully used to detect vesicles containing 20% POPS with the naked eye (Fig. 6), and as little as 5% POPS could be detected spectroscopically (Fig. 7). The overall lower cationic charge of **3** compared to that of **1** and **2** may facilitate the easier displacement of the indicator from the **3** : **5** ensemble. The **4** : **5** ensemble failed to respond to any system containing any fraction of POPS less than 50%. None of these ensembles responded to treatment with POPC vesicles containing 50% POPA or POPG (See Fig. 1 for structures). Further, a 1 : 1 suspension of the short-chain phospholipids DHPC and DHPS also failed to displace the indicator. These phospholipids are known to exist as a monodisperse suspension in aqueous solution, so the lack of response to DHPS clearly signifies the importance of a membrane surface for phosphatidylserine recognition by the displacement method.

Assay 2: Displacement of fluorescent dye 6

We have previously shown that Zn^{2+}–DPA compounds **1**–**4** could be used in a chemosensing ensemble with the coumarin methylsulfonate **6** for detection of small phosphate oxyanions.[27] We hypothesized that the anionic headgroup of phosphatidylserine may act in the same way, thereby producing a fluorescent displacement assay for PS-rich membranes that could be used similarly to assay 1, but with the advantage of lower concentrations due to the increased sensitivity. The coumarin methylsulfonate **6** was prepared as previously described.[27] Titration of Zn^{2+}–DPA compounds **1**–**4** into an aqueous solution of **6** resulted in a concentration-dependent quenching of the indicator fluorescence (Fig. 8). In the case of

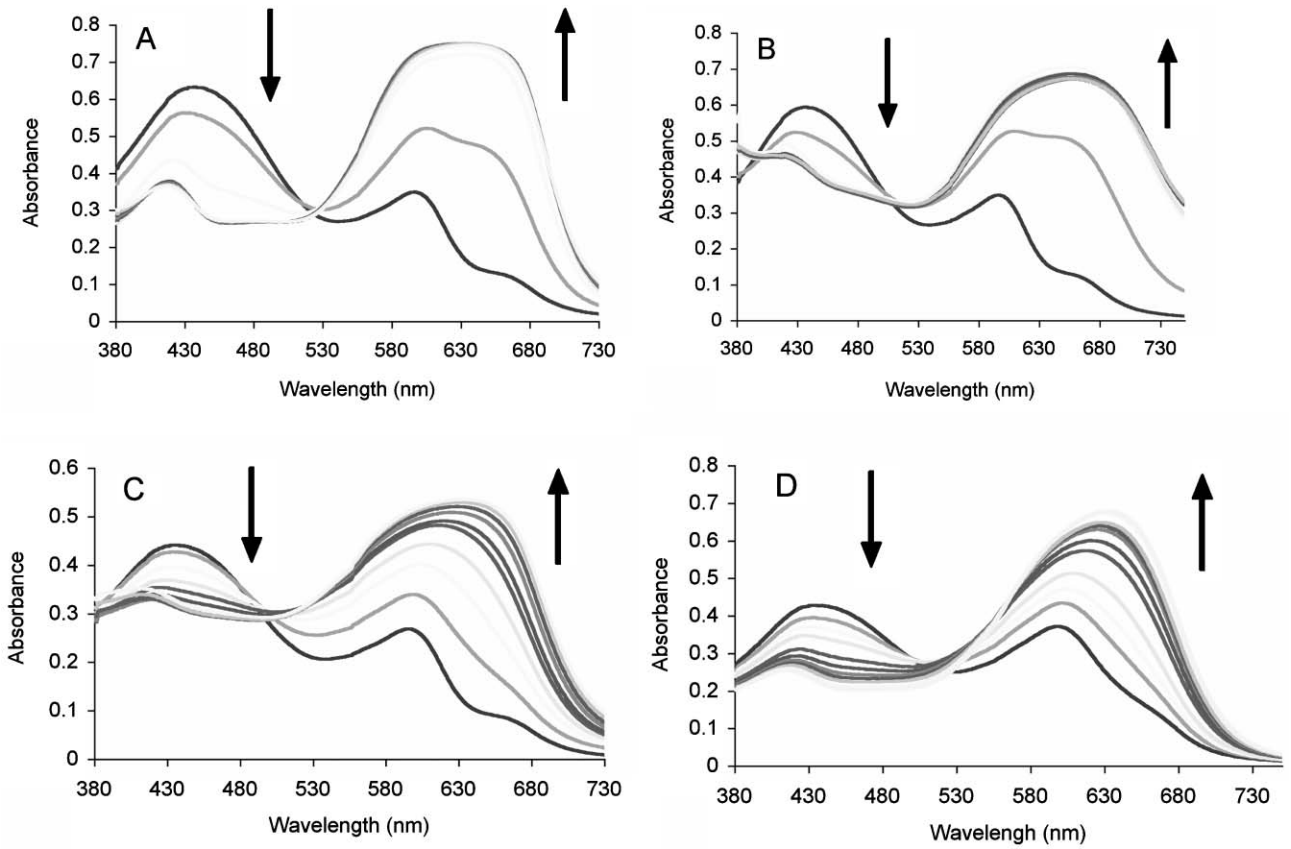

Fig. 5 UV-Vis absorbance spectra of **5** (50 μM) when titrated with Zn^{2+}–DPA complexes **1** (A), **2** (B), **3** (C), or **4** (D) over the concentration range 0 to 400 μM. Titrations were performed at 25 °C in 5 mM TES buffer, 145 mM NaCl, pH 7.4.

Table 1 $K_{association}$ ($\times 10^4$ M^{-1}) for receptor–indicator binding[a]

Indicator		
Receptor	**5**	**6**
1	>17	231 ± 160
2	>17	60.2 ± 15.4
3	5.6 ± 2.2	17.8 ± 3.0
4	17.6 ± 5.4	10.3 ± 0.6

[a] Each value represents the average of at least three separate experiments. All binding constants were determined at 25 °C in aqueous solutions of 5 mM TES, 145 mM NaCl, pH 7.4.

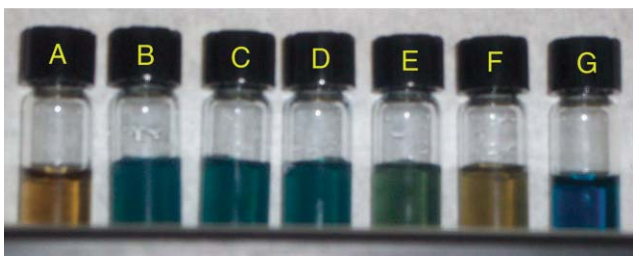

Fig. 6 Aqueous solutions of the **3** : **5** ensemble (50 μM each) in the presence of POPC vesicles containing various amounts of POPS. (A) Indicator **5** only. (B)–(F) are solutions of the **3** : **5** ensemble treated with POPC vesicles containing 0, 5, 10, 20, and 50% POPS, respectively. (G) The **3** : **5** ensemble in the absence of any phospholipids. All solutions contain 5 mM TES, 145 mM NaCl, pH 7.4.

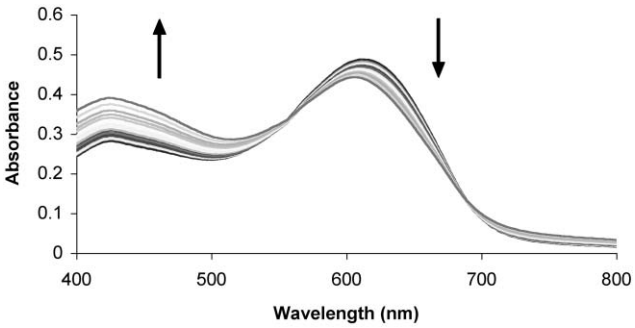

Fig. 7 UV-Vis absorbance spectra for titration of a 50 μM 1 : 1 complex of **3** and **5** with phospholipid vesicles containing 95% POPC, 5% POPS. Only the **3** : **5** ensemble was able to detect vesicles containing 5% POPS. Titrations were performed at 25 °C in 5 mM TES buffer, 145 mM NaCl, pH 7.4.

1, the fluorescence intensity of **6** was quenched by approximately 75% after addition of a single equivalent of **1**, and by approximately 90% when a second equivalent was added. Compounds **2**–**4** each exhibited similar, though less dramatic, effects on the fluorescence of **6**. The curves of fluorescence intensity of **6** as a function of receptor concentration were fit to a 1 : 1 binding model[26] to determine the association constants given in Table 1.

Equimolar amounts of receptor and indicator **6** were mixed in a cuvette (10 μM each) and stirred for approximately one

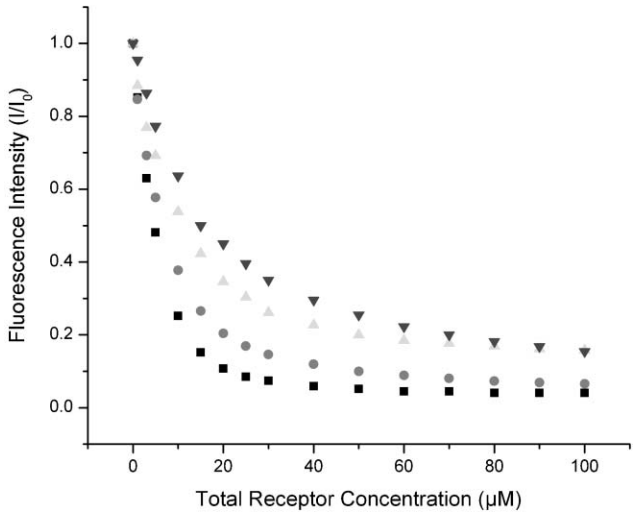

Fig. 8 Fluorescence intensity at 480 nm (I/I_0) of a 10 μM aqueous solution of **6** in 5 mM TES, 145 mM NaCl, pH 7.4. Fluorescence intensity was quenched by addition of **1** (■), **2** (●), **3** (▲), and **4** (▼) over the concentration range of 0 to 100 μM.

minute. POPC vesicles containing from 0 to 50% POPS were then titrated into the solution and the fluorescence intensity was observed (λ_{ex} = 347 nm, λ_{em} = 480 nm). The indicator–Zn^{2+}–DPA complexes of **1**–**3** responded to addition of vesicles containing POPS with an increase in fluorescence intensity, while the complex of **4** failed to respond with detectable enhancements. In each case, vesicles containing 50% phosphatidylserine elicited the most dramatic enhancement in fluorescence intensity (Fig. 9). The increase in fluorescence intensity upon addition of vesicles containing 50% POPS was sufficient to generate saturated binding curves in the case of **1** and **3**, while **2** exhibited a linear enhancement with increasing phospholipid concentration. After addition of 2.5 equivalents of phospholipid to the **1** : **6** ensemble, a 1.8 fold enhancement in fluorescence intensity was observed. Similarly, 2.5 equivalents of phospholipid resulted in 1.2 and 1.1 fold enhancements for the ensembles of **2** : **6** and **3** : **6**, respectively. Vesicles containing lesser amounts of POPS were detectable by the **1** : **6** ensemble, which exhibited enhancements in fluorescence intensity when treated with vesicles containing as little as 5% POPS. The **2** : **6** ensemble could detect vesicles containing as little as 20% POPS, while the **3** : **6** ensemble exhibited no significant response to vesicles containing less than 50% POPS. Phospholipid–Zn^{2+}–DPA complex binding constants derived from theses titrations[28] are summarized in Table 2. Importantly, these fluorescent ensembles also failed to respond to treatment with vesicles enriched in the anionic phospholipids POPA and POPG, and again required a membrane surface for POPS recognition as evidenced by the lack of response to treatment with a DHPS suspension.

Assay 3: Displacement of Cu^{2+} from fluorescent dye 7

The fluorescence emission of calcein **7** is quenched in the presence of Cu^{2+}. The resulting Cu^{2+}–calcein complex has been shown to act as a sensing ensemble for α-amino acids.[29] Since the headgroup of phosphatidylserine is formally an α-amino acid, it occurred to us that the Cu^{2+}-calcein ensemble may be a candidate for selective recognition of phosphatidylserine-rich phospholipid vesicles. Titration of an aqueous solution of calcein with Cu^{2+} lead to a Cu^{2+}–calcein binding constant of $(3.24 \pm 0.64) \times 10^5$ M^{-1}. The Cu^{2+}–calcein complex (1.4 μM Cu^{2+}, 1 μM calcein) was then titrated with phospholipid vesicles containing between 0 and 50% POPS. Vesicles consisting of POPC only were not able to restore calcein fluorescence to any significant level (Fig. 10). A concentration-dependent restoration of calcein fluorescence was observed upon addition of vesicles containing POPS. Restoration of fluorescence was observed upon addition of vesicles containing as little as 5% POPS; however, more significant enhancements in fluorescence intensity and tighter binding to the phospholipid membrane were observed at a membrane composition of $\geqslant$20% POPS. Cu^{2+}–membrane binding constants of $(2.85 \pm 1.50) \times 10^4$ M^{-1} and $(4.59 \pm 1.45) \times 10^4$ M^{-1} were determined for vesicles containing 50% and 20% POPS, respectively. These experiments allowed us to calculate a Cu^{2+}–PS binding affinity of $(1.43 \pm 0.51) \times 10^5$ M^{-1}, which is remarkably high given that the divalent cations Ni^{2+}, Co^{2+}, Mn^{2+}, Ba^{2+}, Sr^{2+}, Ca^{2+}, and Mg^{2+} interact with phosphatidylserine with association constants of 40, 28, 25, 20, 14, 12, and 8 M^{-1}, respectively.[30] The total enhancement was greater than that observed in fluorescence assay 2, and naked eye detection of vesicles containing 50% POPS was possible (Fig. 11). As with assays 1 and 2, this ensemble responded only to treatment with POPC vesicles enriched with POPS, and vesicles containing 50% POPA or POPG did not induce a signal. The previously identified requirement for a membrane surface was also observed in this system because treatment with a suspension of short-chain DHPS failed to enhance fluorescence intensity.

Summary

Three indicator displacement assays are described that allow vesicle membranes enriched in the anionic phospholipid POPS to be detected under physiological conditions, in the best cases with the naked eye. Importantly, none of the sensing ensembles respond to monodisperse phosphatidylserine, underscoring the necessary requirement of a membrane surface for successful indicator displacement, and hence phosphatidylserine recognition. Two of the assays can detect phosphatidylcholine vesicles containing as little as 5% phosphatidylserine. Therefore, these displacement assays may be useful for detecting the onset of cell apoptosis, where the fraction of phosphatidylserine exposed on the cell surface increases to around 10% (clustering may lead to patches of membrane with significantly higher localized concentrations).[31,32] Further, the simple non-covalent assembly of these ensembles makes high throughput screening a clear possibility. Finally, we intend to employ the displacement assays described here in discovery processes that identify new types of phosphatidylserine recognition compounds.

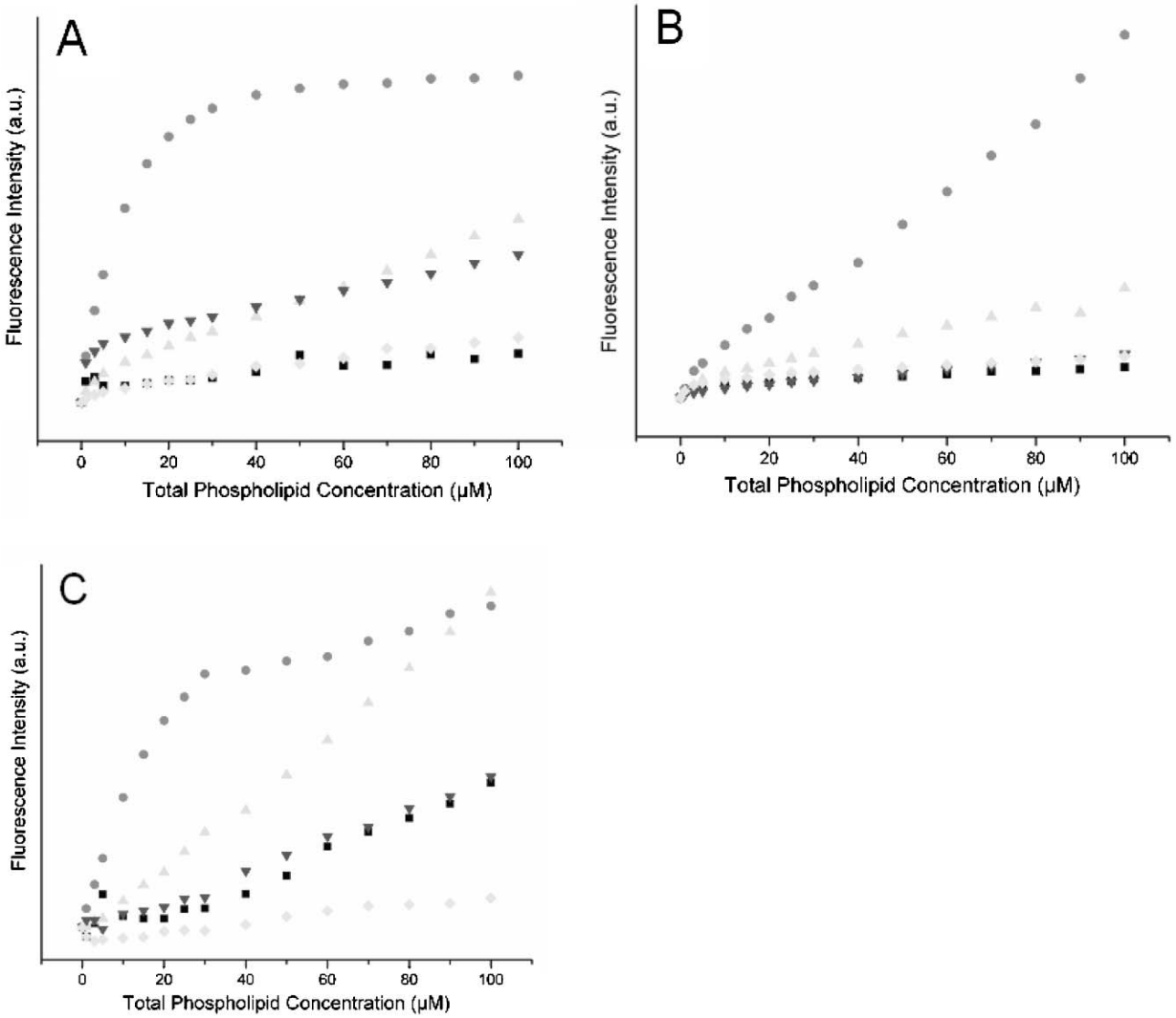

Fig. 9 Fluorescence intensity of **6** as a function of total phospholipid concentration when vesicles of the indicated composition were titrated into a 10 μM solution of a 1 : 1 complex with **1** (A), **2** (B), or **3** (C). POPC liposomes contained 50% (■), 20% (●), 10% (▲), 5% (▼), or 0% (◆) POPS. Titrations were performed at 25 °C in 5 mM TES buffer, 145 mM NaCl, pH 7.4.

Table 2 $K_{association}$ ($\times 10^5$ M^{-1}) for receptor–membrane binding[a]

	Composition of POPC vesicles				
Receptor	POPC only	50% POPS	20% POPS	10% POPS	5% POPS
1	—	55.5 ± 14.0	94.6 ± 31.8	10.2 ± 9.6	3.0 ± 1.8
2	—	1.4 ± 0.1	0.4 ± 0.1	—	—
3	—	0.6 ± 0.1	—	—	—
4	—	—	—	—	—

[a] Each value represents the average of at least three separate experiments. All binding constants were determined at 25 °C in aqueous solutions of 5 mM TES, 145 mM NaCl, pH 7.4.

Experimental

General

Indicators **5** and **7** were purchased from Aldrich and used without further purification. Coumarin methylsulfonate **7** was prepared as previously described.[27] All fluorescence spectroscopy was performed on either a Perkin-Elmer LS 50 A or Jobin Yvon LambdaMax-3 fluorescence spectrometer with jacketed water-cooled cell holders and internal magnetic stirrers. UV-Vis spectroscopy was performed on a Perkin-Elmer Lambda 2 with a jacketed water-cooled cell holder. All experiments were performed in aqueous solution at 25 °C in 1 × 1 × 5 cm quartz cuvettes.

Curve fitting and determination of association constants

All nonlinear curve fitting for determination of receptor-indicator association constants was performed using Origin 5.0 software and the 1 : 1 binding model previously described.[26] Previous work has shown that closely related dinuclear coordination compounds form 1 : 1 bridging complexes with catechol-type ligands.[33] In the case of the Cu^{2+}–calcein system, the tight-binding titration curves clearly indicated a 1 : 1

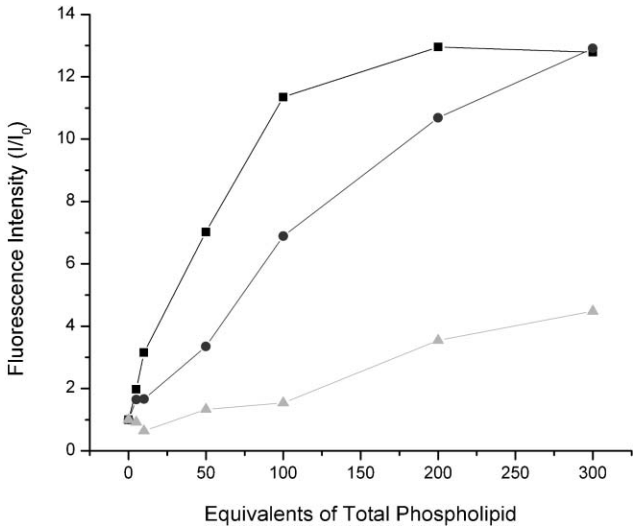

Fig. 10 Fluorescence intensity of **7** as a function of total phospholipid titrated into a 1.4 : 1 mixture of Cu^{2+}–**7**. Vesicles consisted of POPC containing 0% (▲), 5% (●), or 20% (■) POPS. Titrations were performed at 25 °C in 5 mM TES buffer, 100 mM NaCl, pH 7.4.

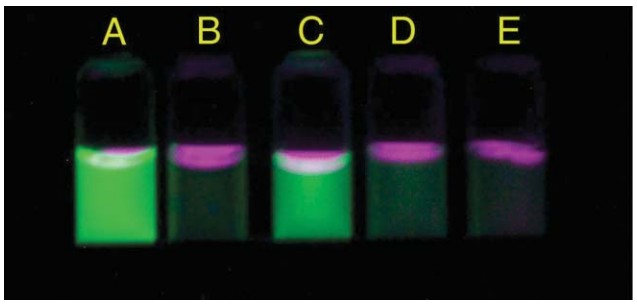

Fig. 11 Fluorescence emission of **7** (10 μM) upon treatment with POPC vesicles containing various amounts of POPS. (A) Aqueous solution of **7** in the absence of Cu^{2+}. (B) Aqueous solution of **7** and 1.4 equivalents of $CuBr_2$. (C)–(E) Aqueous solutions of **7** and 1.4 equivalents of $CuBr_2$ in the presence of POPC vesicles containing 50, 20, and 5% POPS, respectively.

stoichiometry for complete calcein quenching. Determination of association constants for receptor binding to the phospholipid vesicles employed the standard mathematical method for indicator displacement assays.[28] We have previously shown that these Zn^{2+}–DPA coordination complexes do not diffuse across the membrane, and do not induce vesicle leakage.[34] The calculations assumed a phospholipid concentration of 60% of total lipid due to unavailability of the phospholipid in the inner monolayer of the unilamellar membranes.

Receptor–pyrocatechol violet (5) association constant determination

A 1.0 mL aqueous solution of **5** (50 μM final concentration) was prepared in a buffer of 5 mM TES (*N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid) containing 145 mM NaCl, pH 7.4. Aliquots of an aqueous 10 mM Zn^{2+}–DPA receptor solution in the same buffer containing 50 μM **5** were titrated into the indicator solution and dispersed thoroughly by repeated pipette mixing. Aliquots were added until the Zn^{2+}–DPA receptor concentration reached 400 μM. After each addition, the UV-Vis absorbance spectrum of the solution was acquired. Plots of absorbance values (600 nm) as a function of Zn^{2+}–DPA receptor concentration were generated for the addition of **1–4** and fit to a 1 : 1 binding model[26] to determine the receptor–indicator association constant for receptors **3** and **4**. Binding of **1** and **2** to **5** was found to be too tight for accurate determination of an association constant.

Receptor–coumarin methylsulfonate (6) association constant determination

A 3.0 mL aqueous solution of **6** (10 μM final concentration) was prepared in a buffer of 5 mM TES, 145 mM NaCl, pH 7.4. While stirring, aliquots of an aqueous 10 mM Zn^{2+}–DPA receptor solution in the same buffer were titrated into the indicator solution until the final receptor concentration reached 100 μM. After approximately 30 s for solution equilibration, the fluorescence emission intensity of **6** was measured (λ_{ex} = 347 nm, λ_{em} = 480 nm) after each addition. Plots of fluorescence intensity as a function of Zn^{2+}–DPA receptor concentration were generated for the addition of **1–4** and fit to a 1 : 1 binding model[26] to determine the receptor–indicator association constant for each receptor.

Cu^{2+}–calcein (7) association constant determination

A 2.0 mL aqueous solution of **7** (5 μM final concentration) was prepared in a buffer of 5 mM TES, 100 mM NaCl, pH 7.4. Aliquots of a 100 μM solution of $CuBr_2$ in the same buffer were titrated into the indicator solution while stirring. After approximately 30 s for solution equilibration, the fluorescence intensity of **7** was measured after each addition (λ_{ex} = 450 nm, λ_{em} = 530 nm). Plots of fluorescence intensity as a function of Cu^{2+} concentration were generated and fit to a 1 : 1 binding model[26] to determine the Cu^{2+}–indicator association constant.

Preparation of vesicles

All phospholipids were purchased as 10 mg mL^{-1} stock solutions in $CHCl_3$ from Avanti Polar Lipids (Alabaster, AL) and stored at −20 °C until used. Appropriately sized aliquots of POPC, POPS, POPG, and POPA were added to a 10 mL round bottom flask and the $CHCl_3$ removed by rotary evaporation. Residual solvent was removed under vacuum for at least 1 h. Lipids were then rehydrated with the appropriate buffer and a glass ring was added to the flask to ensure complete removal of all lipid from the flask wall. The flask was then vigorously vortexed for approximately 30 s and the suspension extruded 29 times through a 19 mm polycarbonate membrane with 200 nm pore diameter (100 nm vesicles were used for the Cu^{2+}–calcein experiments). Vesicles were used on the day of preparation. For the short-chain phospholipids DHPC and DHPS, analogous procedures were followed except lipid suspensions were not extruded.

Displacement assays for detecting PS-rich membranes

Assay 1. To a 3.0 mL volume of a 1 : 1 mixture of Zn^{2+}–DPA receptor (**1–4**) and indicator **5** (50 μM each) in an aqueous buffer of 5 mm TES, 145 mM NaCl, pH 7.4, were

added aliquots of a 10 mM suspension of 200 nm POPC vesicles containing between 0 and 50% POPS until the total phospholipid concentration reached 400 μM. The vesicle suspension contained 50 μM **5** in order to offset the dilution effect realized during titration. After repeated pipette mixing, the absorbance spectrum of the solution was acquired. Procedures were repeated for POPC vesicles enriched in POPA and POPG, as well as for suspensions of the monodispersed phospholipids DHPC and DHPC–DHPS.

Assay 2. To a 3.0 mL volume of a 1 : 1 mixture of Zn^{2+}–DPA receptor (**1**–**4**) and indicator **6** (10 μM each) in an aqueous buffer of 5 mM TES, 145 mM NaCl, pH 7.4, were added aliquots of a 10 mM suspension of 200 nm POPC vesicles containing between 0 and 50% POPS until the total phospholipid concentration reached 100 μM. After approximately 30 s for solution equilibration, the fluorescence emission intensity of **6** was measured (λ_{ex} = 347 nm, λ_{em} = 480 nm) after each addition. The intensity values as a function of total phospholipid concentration were then used to determine a receptor–phospholipid membrane association constant by methods previously described. Procedures were repeated for POPC vesicles enriched in POPA and POPG, as well as for suspensions of the monodispersed phospholipids DHPC and DHPS.

Assay 3. To a 3.0 mL volume of a 1.4 : 1 mixture of $CuBr_2$ and indicator **7** in an aqueous buffer of 5 mM TES, 100 mM NaCl, pH 7.4, were added aliquots of a 10 mM suspension of 100 nm POPC vesicles containing between 0 and 50% POPS until the total phospholipid concentration reached 420 μM. After approximately 30 s for solution equilibration, the fluorescence intensity of **7** was measured after each addition (λ_{ex} = 450 nm, λ_{em} = 530 nm). Procedures were repeated for POPC vesicles enriched in POPA and POPG, as well as for suspensions of the monodispersed phospholipids DHPC and DHPC–DHPS.

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