A change in metal cation switches selectivity of a phospholipid sensor from phosphatidic acid to phosphatidylserine†

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Phosphatidic acid and phosphatidylserine are anionic phospholipids with emerging signalling roles in cells. Determination of how phosphatidic acid and phosphatidylserine change location and quantity in cells over time requires selective fluorescent sensors that can distinguish these two anionic phospholipids. However, the design of such synthetic sensors that can selectively bind and respond to a single phospholipid within the complex membrane milieu remains challenging. In this work, we present a simple and robust strategy to control the selectivity of synthetic sensors for phosphatidic acid and phosphatidylserine. By changing the coordination metal of a dipicolylamine (DPA) ligand from Zn(II) to Ni(II) on the same synthetic sensor with a peptide backbone, we achieve a complete switch in selectivity from phosphatidic acid to phosphatidylserine in model lipid membranes. Furthermore, this strategy was largely unaffected by the choice and the position of the fluorophores. We envision that this strategy will provide a platform for the rational design of targeted synthetic phospholipid sensors to probe plasma and intracellular membranes.

Introduction

The outer boundaries of mammalian cells, as well as subcellular organelles, are defined by membranes comprised of a bilayer of phospholipids.1 Phospholipid structures vary in both the length and saturation of the lipid tail and by substitution of the phosphate headgroup, leading to a huge variety of possible structures.2 Compositional characterisation of cellular membranes has revealed that each membrane is formed from a complex and dynamic mixture of different phospholipids. Membrane compositions vary between the inner and outer leaflets, across different regions of the membrane surface, and over time. The most abundant phospholipids are the zwitterionic species phosphatidylcholine (PC) and phosphatidylethanolamine (PE); however, anionic phospholipids—characterised by negatively charged headgroups—play important structural and functional roles in all cellular membranes.1,2

The most common anionic phospholipids in mammalian membranes, which each account for approximately 1–10% of the total phospholipid mass, are phosphatidylserine (PS), phosphatidic acid (PA), and phosphatidylinositol (PI).3 Phosphatidylglycerol (PG) is a mitochondrial phospholipid, which amounts to ~1–2% of total cellular phospholipids in mammalian cells.4 A small proportion of phosphorylated PI accounts for less than 1% of total cellular phospholipids.5

There is growing evidence that this repertoire of anionic phospholipids plays vital roles as dynamic signalling molecules, since their appearance in the membranes and variation between the membrane leaflets can induce downstream processes in cells.1,6,7 For example, healthy mammalian cells maintain higher concentrations of PS in the inner leaflet of the plasma membrane than the outer leaflet; however, in cells undergoing apoptosis PS flips to the outer leaflet and becomes exposed to the extracellular environment.8 This translocation acts as an important “eat me” signal for dead cells. Similarly, PA’s emerging roles implicate it in cellular growth, death, and metabolic regulation.9 Elucidation of further functional roles of anionic lipids necessitates the development of selective sensors that can recognize and signal the presence of one phospholipid within complex mixtures, in real time, to track changes as cells respond to stimuli.
A small number of synthetic sensors have been developed for several anionic phospholipids, including PA, PG, and PS. For example, Umeda and co-workers developed a large tetravalent peptide (MW > 6 kDa) which displayed selective binding to PA over PS, PI, PG, and PC, at as little as 1% PA liposome composition. Analysis of PA binding was conducted through changes in tryptophan fluorescence in the UV region. However, the mechanism of this selectivity is unknown and further rational design of PA sensors based on this system has not eventuated. In a system applicable to bacterial membranes, which contain high concentrations of PG, small molecule receptors developed by Busschaert and co-workers were found to selectively bind to 100% PG liposomes but not to 100% PC liposomes. Smith and co-workers have reported several small molecule sensors for anionic biomembranes and their applications in imaging.

In previous work, some of us have demonstrated that a small molecule fluorescent sensor for PS is effective for analysis of cell-surface PS in real-time cell imaging and cytometry applications, with several advantages (including a fluorogenic response, faster binding and no need for addition of Ca(II) to the binding buffer) over the PS-binding protein, Annexin V. However, more work is needed to understand and improve upon the selectivity of synthetic sensors, and the rational design of selective sensors capable of recognizing phospholipid headgroups within mixtures and concentrations that are biologically relevant remains a challenge.

Many of the synthetic receptors and sensors for carboxylate and phosphate species reported in the literature rely on metal complexes containing dipicolylamine (DPA) ligands as the central anion recognition unit. The DPA group forms high affinity complexes with a range of divalent metal cations, even in a highly competitive aqueous environment. In turn, such-formed metal DPA (MDPA) complexes display high affinity binding to a range of anions, and in particular to phosphate species. Early examples by the groups of Hamachi, Yoon, Ahn, and Smith have demonstrated the utility of MDPA sensors incorporating a wide range of fluorophores. The strength and versatility of the MDPA group for binding to numerous anionic guests has inspired the continued and prolific use of this recognition motif. However, with the exception of a few select examples, MDPA-based sensors have exclusively featured divalent copper or zinc as the complexed cation. Moreover, the property of copper(II) to quench the fluorescence of nearby fluorophores means that DPA-based sensors featuring this metal typically operate by an indicator displacement mechanism, rendering such sensors ineffective for imaging in cellulo. Zn(II) displays a number of favourable properties, including: strong binding to both the DPA ligand and to anionic analytes; a diamagnetic metal centre that is compatible with NMR analysis; and minimal quenching of nearby fluorophores. This has led to the widespread use of the Zn(II)DPA motif in fluorescent sensing and discrimination of biologically relevant phosphate derivatives.

Several Zn(II)DPA-based sensors (mostly comprising two Zn(II)DPA units) have been previously reported to bind to the anionic PS headgroup. However, the selectivity profiles of Zn(II)DPA-based sensors in regard to anionic phospholipid sensing remain underexplored. Given the diverse and dynamic phospholipid composition of cell membranes, it is of particular interest to investigate whether selective MDPA-based sensors can be identified for other anionic phospholipid headgroups.

In previous work, we investigated sensor 1 (Fig. 1A)—featuring a single Zn(II)DPA binding unit and the environment-responsive NBD fluorophore—which displays an increase in fluorescence intensity when bound to liposomes. The sensor was evaluated against liposomes containing 100% PC, or 50%
PC and 50% of either PA, PS, or PG. Sensor 1 was found to bind more strongly to PA-containing liposomes (log $K_a = 3.9 \pm 0.5$) compared to PS- and PG-containing liposomes (log $K_a = 2.4 \pm 1.5$; 1.8 ± 1.2 respectively), and did not bind appreciably to liposomes only of PC. However, this apparent selectivity was not further explored.

Therefore, in this work we set out to investigate the selectivity profile of DPA-based phospholipid headgroup sensors complexed with divalent zinc towards a range of anionic phospholipids (Fig. 1B). Furthermore, we investigated sensors complexed with nickel, cobalt or manganese to determine the suitability of alternative MDPA complexes for use in fluorescent sensors, and for comparison of their selectivity profiles.

**Results and discussion**

**Sensor design and synthesis**

In order to systematically explore the influence of different divalent cations on selectivity towards phospholipids, we prepared three sets of phospholipid sensors, each containing a single DPA ligand (Fig. 1A). These sensors were each complexed with a small range of divalent cations: Zn(n), Ni(n), Co(n), and Mn(n). To account for any potential effects of physical or electronic interactions between the metal complex and fluorophore on the response, two different environment-sensitive fluorophores that have been previously used in phospholipid sensors—NBD and PRODAN—were employed.36,41 Furthermore, to see whether any selectivity differences towards anionic phospholipids arise from the position of the fluorophore relative to the MDPA group, we varied the point of attachment of the NBD fluorophore to the peptide backbone.42 The peptide backbone and DPA group of each sensor were prepared using solid phase peptide synthesis (SPPS) on Rink amide resin (details in ES1†). Consequently, the C-terminal methyl ester of sensor 1 was replaced by a carboxamide in this work, to produce sensor NBD-1 (Fig. 1A).

**Response of sensors to anionic liposomes**

In previous work, sensor 1 bound to PA-containing liposomes more strongly than PS-containing liposomes when liposomes contained 50% of the target phospholipid. To validate these results for liposomes containing the phospholipids of interest at levels closer to those found in cells, we prepared liposomes containing 4% of the target phospholipid (PS as DOPS, PA as Egg PA, PG as Egg PG, and PI as Soy PI) with PC (as DOPC) fulfilling the remaining liposomal composition. NBD-1-Zn showed a prominent response to PA as judged by an increase in fluorescence intensity and a slight hypsochromic shift (up to 10 nm) in the emission peak (Fig. S1A†), and little to no response to other anionic phospholipids (Fig. S1B†). This closely matches results previously obtained for the sensor 1.36,41

Remarkably, changing the cation complexed within the DPA ligand to Ni(n) was found to have a significant impact on the phospholipid selectivity. In direct contrast to the analogous Zn(n) complex, which responded to PA-containing liposomes only, NBD-1-Ni displayed a large increase in fluorescence intensity in the presence of PS-containing liposomes (Fig. 2A) and minimal response to PA-containing liposomes (Fig. 2B).

NBD-1-Co exhibited similar responses to both PA- and PS-containing liposomes, however this response was small in both cases (Fig. S1C and S1E†). NBD-1-Mn showed small, non-selective increases in intensity in response to increasing concentrations of all phospholipids tested (Fig. S1D and S1E†). Changing the position of the fluorophore, as in NBD-2, resulted in a small drop in the extent of response and degree of selectivity; however the effect of changing the complexed metal was the same as for NBD-1-M (Fig. 2C). NBD-2-Zn showed the most prominent response to PA-containing liposomes (Fig. 2C and Fig. S1F†), whereas NBD-2-Ni gave a response to PS-containing liposomes only (Fig. 2C and Fig. S1G†). Hence, the fluorophore position does not profoundly impact the interaction between the MDPA group and the phospholipid headgroups. Hereafter, we used NBD-1-M complexes for subsequent experiments and comparison with PRODAN-1-M.

Results for the metal complexes of PRODAN-1 roughly matched those observed for NBD-1 and NBD-2, with PRODAN-1-Ni maintaining high selectivity for PS-containing liposomes over the other phospholipids (Fig. 2C). A selective response to PA-containing liposomes was observed for PRODAN-1-Zn; however, this was much less prominent than the result obtained with NBD-1-Zn or NBD-2-Zn. To determine whether this slight difference in response is due to the inherent binding differences of fluorophores, we tested the binding of PRODAN and NBD derivatives that lack the MDPA binding site to liposomes containing 4% anionic lipids (PS, PA, PG or PI). NBD hexanoic acid showed no selective binding to liposomes (Fig. S2A, S2B and S2D†). On the other hand, acrylodan showed a slight preference for liposomes containing 4% PS (Fig. S2A, S2C and S2D†). These data may explain the superior selectivity of NBD-1-Zn towards PA-containing liposomes when compared to PRODAN-1-Zn. Despite their lower selectivity, one useful feature of the metal complexes of PRODAN-1 is a significant hypsochromic shift (up to 40 nm) in the emission peak upon binding to target phospholipids (Fig. S3A and S3B†), which makes the PRODAN-1 sensors suitable for ratiometric analysis by examination of the response at two separate wavelengths (Fig. S3C and S3D†).41

**Comparison of sensor affinities**

In order to determine if the differences observed for the Zn(n) and Ni(n)-complexed sensors were due to differences in binding affinities or differing fluorescence responses, we conducted titrations of these sensors with PC-, PA-, and PS-containing liposomes and evaluated apparent binding affinities (Fig. 3).43,44 Apparent binding affinities could not be determined for PC-containing liposomes for any of the probes evaluated, due to minimal changes in fluorescence intensity (Fig. 3A and Fig. S4A, S4D, S4C†). Similarly, NBD-1-Ni displayed very little change in fluorescence intensity in response
to PA-containing liposomes, and so a binding affinity could not be determined (Fig. S4F†).

Titrations of NBD-1-Ni and PRODAN-1-Ni with PS-containing liposomes did not fit satisfactorily to a binding model; however, the presence of an inflection point in the binding isotherm is suggestive of multiple binding events (Fig. S4L and S4M†).

Overall, apparent binding affinities for the phospholipid sensors complexed with Zn(ii) were reflective of the differences in fluorescence response observed for PA- and for PS-contain-
ing liposomes. Binding affinities were found to be higher towards PA-containing liposomes than for PS-containing liposomes for both NBD-1-Zn and PRODAN-1-Zn (Table 1, Fig. S4J and S4K†), indicating that the selectivity observed is due to selective binding, rather than a selective response.

To further assess the selectivity of the sensors—and their ability to provide a response in the presence of more complex phospholipid compositions—NBD-1-Zn and NBD-1-Ni were evaluated against a series of mixed liposomes containing combinations of PA, PS, PC, PI, and PG. Phospholipid compositions were chosen to ensure that either the overall charge of the liposomes or the total amount of target phospholipid was comparable (Fig. 4). Pleasingly, a prominent increase in the intensity of NBD-1-Ni was only observed in liposomes containing PS (Fig. 4A). Comparison of liposomes containing 2% PS together with 2% of either PA, PG or PI indicated that the presence of other anionic phospholipids did not impair the response of NBD-1-Ni to PS. Interestingly, while there is no response to PI in the absence of PS, there appears to be a cooperative effect on the response when both PS and PI are present in the liposome. However, this is not expected to affect the ability of NBD-1-Ni to target PS selectively on the surface of mammalian cells as PS is generally more abundant than PI, and the levels of PS are ~3-fold higher than PI at the plasma membrane of mammalian cells.40 These data indicate that the Ni (ii) complexes display selective binding to PS-containing liposomes. In mixed liposomes comprising PC and one other phospholipid, the Zn(ii)-complexed sensor NBD-1-Zn showed a concentration-dependent increase only to PA-containing liposomes, whereas there was no such dose-dependent response to liposomes containing only PS and PC (Fig. S1A and S1B†). However, in mixed liposomes comprising three phospholipids, fluorescence enhancement at 530 nm was also observed in the additional presence of either PS or PI. Nevertheless, 4% PA-containing liposomes afforded the largest responses, and lower response to the liposomes bearing the same charge (2% PS and 2% PI) indicated that NBD-1-Zn is selective for PA (Fig. 4B). These data suggest that the sensors NBD-1 and PRODAN-1 may be applicable to in cellulo studies in which a complex mixture of phospholipids is present. In particular, we envision Ni(ii)DPA complexes as selective PS reporters in cellular studies.

**Rationale for cation-specific selectivity**

Analysis of the affinities of divalent metal cations with various anions and ligands has been widely investigated in founda-
tional work by Irving and Williams, and others.45–49 These data provide a possible rationale for the dramatic shift in selectivity observed for the Zn(u) and Ni(u) sensors. Although both Zn(u) and Ni(u) often form octahedral or distorted octahedral complexes—leaving three possible binding sites that are not inhabited by the DPA ligand—Ni(u) shows a higher tendency to form tridentate complexes with amino acids and related zwitterions than does Zn(u).46–50 In particular, it appears that Ni(u) forms stronger interactions with the α-amino group of amino acids, than does Zn(u), and at lower pH values.47 It is therefore likely that the head group of PS facilitates binding.12–14,34–36,39 Evidence from Zundel and coworkers suggests that the PS head group is involved in complex hydrogen-bonding networks within liposomes, which the sensors head group mimics (phosphoserine: log $K_a = 5.8$;50 methyl phosphate: log $K_a = 2.16$).35 However, previous MDPA-based PS sensors all contain two Zn(u)DPA moieties, suggesting that either the carboxylate and phosphate bind to different metal ions or that local clustering of PS groups within the membrane facilitates binding.12–14,34–36,39 The observed selectivity of NBD-1-Zn and PRODAN-1-Zn.

Conclusions

The choice of divalent metal cation in DPA-based phospholipid sensors has a strong impact on selectivity, with the choice of Ni(u) providing a simple method to target PS-containing model membranes, while the Zn(u) complexes target PA-containing membranes. This work paves the way towards more rational design of improved phospholipid headgroup sensors, through the informed choice of metal binding groups in combination with appropriate fluorophores.

Author contributions

S. M. B. contributed to investigation, formal analysis, and writing and review of the manuscript; B. E. contributed to investigation, methodology, data collection and curation, formal analysis, and writing and review of the manuscript; J. Y. contributed to investigation, data collection and curation; L. P. S. contributed to investigation; K. A. J. contributed to methodology, project conceptualisation, funding acquisition, supervision, and review of the manuscript.

Conflicts of interest

There are no conflicts to declare.

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