A proximity-based labelling approach to analyse lipid binding in proteins

A Thesis

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by

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INDIAN INSTITUTE OF SCIENCE EDUCATION AND RESEARCH PUNE

Certificate

This is to certify that this dissertation entitled “A proximity-based labelling approach to analyse lipid binding in proteins” towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Swayam Prakash Singh at Indian Institute of Science Education and Research under the supervision of Thomas Pucadyil, Professor, Department of Biology, Indian Institute of Science Education and Research Pune, during the academic year 2023-2024.

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Declaration

I hereby declare that the matter embodied in the report entitled “A proximity-based labelling approach to analyse lipid binding in proteins” are the results of the work carried out by me at the Department of Biology, Indian Institute of Science Education and Research (IISER) Pune, under the supervision of Thomas Pucadyil and the same has not been submitted elsewhere for any other degree. Wherever others contribute, every effort is made to indicate this clearly, with due reference to the literature and acknowledgement of collaborative research and discussions.

Swayam Prakash Singh

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This thesis is dedicated to Dr Sucharita Mohanty and Dr Gyan Prakash Singh.

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Contributions

Contributor name	Contributor role
Thomas Pucadyil, Swayam Prakash Singh	Conceptualization Ideas
Thomas Pucadyil, Swayam Prakash Singh	Methodology
-	Software
Swayam Prakash Singh	Validation
Swayam Prakash Singh	Formal analysis
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Swayam Prakash Singh	Writing - original draft preparation
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Swayam Prakash Singh	Visualization
Thomas Pucadyil	Supervision
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Thomas Pucadyil	Funding acquisition

Contents

Abstract

Membranes act as sites of several biochemical processes occurring inside a cell. Soluble proteins that bind these membranes often do so by interacting with resident lipids. A preliminary step in understanding these proteins is thus studying their binding specificity and affinity to these lipids. Various methods exist to probe for these interactions, often relying on liposomes as the membrane substrate of choice. Yet, distinguishing protein-lipid binding from protein-membrane binding is impossible on these substrates. In addition, liposomes suffer from several limitations, such as the involved preparation methodologies, longer-term structural instability and the amount of lipids consumed in each prep. Here, we propose detergent micelles as a simpler yet useful substrate for detecting and quantifying protein-lipid interactions. Detergents are doped with small amounts of test lipids along with a bifunctional photoactivable fluorescent lipid to form the substrate for protein binding. Proteins that bind the test lipid are covalently labelled by the bifunctional lipid in a proximity-based manner upon exposure to UV, which can then be detected and quantified by in-gel fluorescence as a readout for binding. At low concentrations of test lipid, this assay functions as a lipid-protein binding assay. The ease of preparation, along with the relatively inexpensive reagents and equipment required, make this a more accessible assay.

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1. Introduction

1.1 Background

Eukaryotic cells are compartmentalised into various organelles, most of which are delimited by a lipid membrane. These compartments are uniquely defined by the presence of Rab-GTPases and specific lipids (Jean and Kiger, 2012) such as various phosphatidylinositol phosphates (PIPs) or phosphoinositides (Behnia and Munro, 2005; Posor *et al.*, 2022). These lipids are generated by phosphorylation of the inositol head group of phosphatidylinositol at one or more positions (C3, C4, C5) **(Fig 1)**. These lipids are recognised by peripheral membrane proteins (effectors), which are recruited to membranes and perform various downstream functions. The affinity and specificity for lipids influences their recruitment to these membranes in addition to other partner proteins on the membrane. Understanding these interactions provides the basis for further biochemical assays that can be performed with the protein in the presence of membranes containing its cognate lipid.

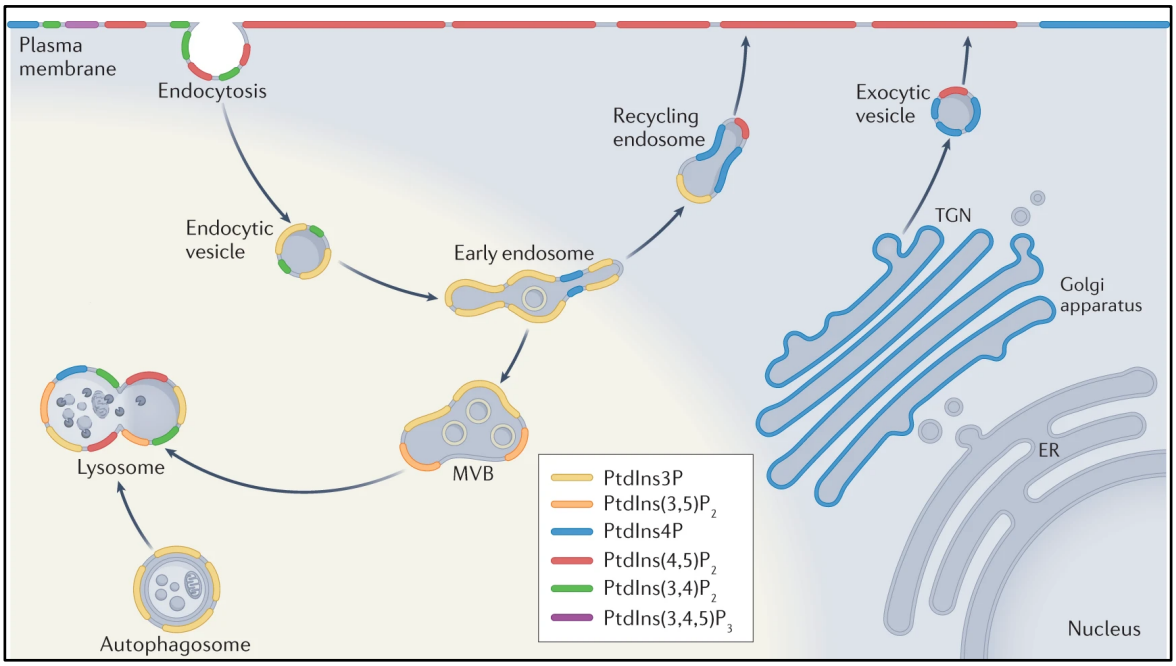

Figure 1. Schematic showing the distribution of various phosphoinositides across organelles. Reproduced from Posor *et al.*, 2022

1.2 Peripheral membrane proteins

Peripheral membrane proteins perform various functions when associated with membranes through specific lipid interactions. For instance, cytoskeletal proteins associate with the membrane to maintain cell structure (Sheetz et al. 2006). Proteins such as phosphoinositide 3-kinase (PI3K), upon recruitment to the plasma membrane, generate signalling lipids to recruit other proteins (Franke *et al.*, 1997). Membrane remodelling proteins such as the BAR (Bin-Amphyphysin-Rvs) domain-containing and dynamin family proteins perform membrane tubulation and fission by multivalent lipid and protein interactions that are key to vesicular trafficking.

Several protein domains are identified to bind specific lipids with a wide range of affinities. These proteins interact with membranes in two broad modes –(i) by recognising general physical properties of membranes, such as overall surface charge and topology, or (ii) by binding to certain membrane components, such as specific phospholipids and resident proteins. PLCδ1 was one of the first proteins shown to bind a phospholipid headgroup (Inositol-1,4,5-trisphosphate from phosphatidylinositol-4,5-bisphosphate ($PI(4,5)P_2$)) in a stereospecific manner via its Pleckstrin homology (PH) domain (Ferguson *et al.*, 1995; Lemmon *et al.*, 1995). Others, such as the Fab1-YOTB-Vac1-EEA1(FYVE) domain-containing proteins, bind strongly to phosphatidylinositol-3-phosphate (PI(3)P) containing membranes but do not bind the isolated headgroup with such affinity. Instead, they rely on membrane insertion for stronger binding (Kutateladze *et al.*, 1999). Protein Kinase B (PKB/Akt) binds specifically to the lipid second messenger phosphatidylinositol-3,4,5-trisphosphate ($PI(3,4,5)P_3$), which is generated only transiently by PI3K, allowing for the spatiotemporal control of recruitment and activation of this protein involved in the PI3K/Akt/mTOR signalling pathway (Brazil and Hemmings, 2001). There are many other domains, such as the PX (Phox homology), PHD (Plant Homeodomain), C1, Discoidin C2, Protein Kinase C (PKC) C2, F-BAR, N-BAR domains that show binding to selective lipids (DiNitto *et al.*, 2003; Lemmon, 2008). Identifying the lipid binding partner of these proteins allows us to perform further biochemical studies with appropriate substrates to understand them better.

1.3 Models used to study membranes

A few artificial membrane mimics have been used to study the binding and activity of the aforementioned proteins. Lipid vesicles, or liposomes, are the most widely used membrane mimics. These are spherically arranged bilayers with one or more lamellae which resemble cellular compartments **(Fig 2.A)**. Unilamellar vesicles are the most used kind of liposomes and are categorised further by size. Liposome preparation has largely remained unchanged as a process since it was pioneered in 1980 (Szoka and Papahadjopoulos, 1978; Mui *et al.*, 2003). Dried thin films of lipids, upon hydration, produce multilamellar vesicles of ~1μm diameters. These can then be extruded through filters to produce large unilamellar vesicles (LUVs) of diameters ranging from 100-500 nm or small unilamellar vesicles (SUVs) of diameters ranging from 30-100 nm. Giant unilamellar vesicles (GUVs) are unique as they range in size from 10-300 μm and can be micromanipulated to form a variety of structures. These are produced by electroformation or hydration for much longer durations than is required for LUVs or SUVs. These are not diffraction-limited objects and are used to study protein binding and activity under microscopy.

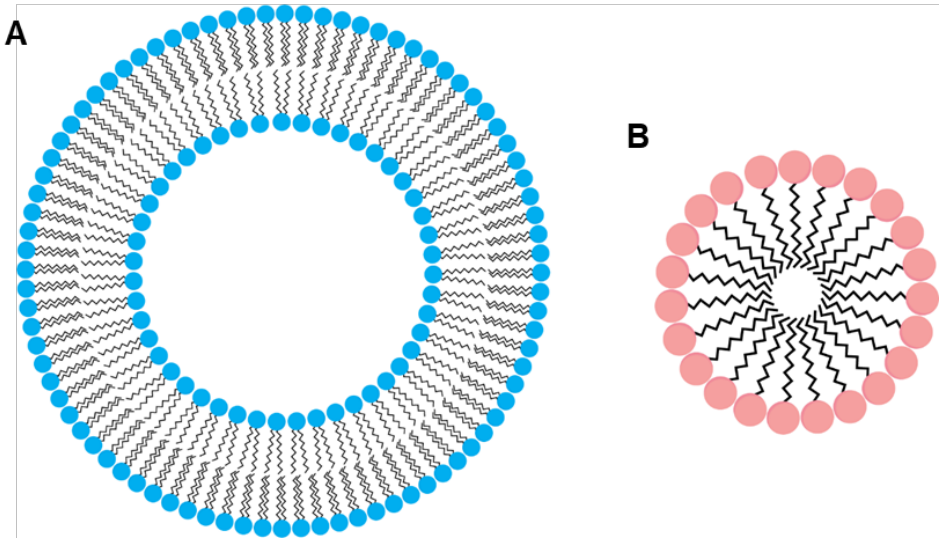

Figure 2. Schematic depicting **A** a unilamellar vesicle, **B** a micelle.

In contrast to lipids, detergents exclusively form self-assembled entities called micelles at appropriate concentrations in aqueous media. These consist of the detergent molecules arranged in spherical and ellipsoidal structures similar to liposomes, with the hydrophilic ends exposed to the medium and the hydrophobic

ends buried at the core of the micelle **(Fig 2.B)**. Lipids incorporated into micelles also orient themselves as such (Singh *et al.*, 2007). However, micelles only consist of a monolayer of molecules and are devoid of an aqueous lumen. While liposomes with diameters less than 30 nm are unstable, micelles are even smaller entities. Triton X-100 micelles have diameters of around 10 nm, which may provide an advantage for these systems over liposomes. These systems are, however, sparsely studied using biochemical methods and offer an interesting avenue for exploring protein-lipid interactions.

1.4 Methods used to detect soluble protein-lipid interactions

Various biochemical assays exist to study protein association and function with lipids and membranes. The most well-known assays are elucidated here.

1.4.1 Immobilised lipid-based assays

The well-known high throughput dot-blot assay is performed by spotting lipids on PVDF/nitrocellulose membranes to which the desired proteins are allowed to bind, and the signal is generated by immunoblotting against the desired protein (Dowler *et al.*, 1999). Dot blot assays use relatively small amounts of lipids and are quick and easy to perform. However, since phosphoinositides are highly charged lipids, they tend to get washed off in a charge-dependent manner before probing the lipids with the test protein when performing the dot blot assay. This is a major disadvantage of testing phosphoinositide specificity by dot-bot assays. There are other assays in which lipids are immobilised on resin beads where proteins are bound via specific lipid interactions, which can then be processed for mass-spectrometry to determine binders or densitometric assays to assay for specific affinity. However, both these kinds of assays are performed with lipids in non-native conditions with little to no control over localised concentrations and thus lead to artefacts, which require orthogonal assays to verify the results (Narayan and Lemmon, 2006)

1.4.2 Bulk liposome assays

The methods employ liposomes (LUVs and SUVs) made with different compositions of lipids to be tested. Liposome co-sedimentation is a classic, widely used membrane binding assay, where liposomes mixed and incubated with

desired protein/s are pelleted down by ultracentrifugation. Any protein bound to the liposomes is pulled down into the pellet along with them. The unbound protein remains in the supernatant. Some membrane-binding proteins tend to oligomerise; hence, they get sedimented upon ultracentrifugation even when they are not bound to liposomes, which reduces the detection range in this assay. In the case of flotation assays, ultracentrifugation is performed on a mixture of protein with liposomes suspended at the bottom of a sucrose density gradient. Any protein bound to the liposomes floats up the density gradient, while unbound proteins remain at the bottom in a pellet. Floatation assays, however, are a more involved and time-intensive process than sedimentation.

Isothermal titration calorimetry employs proteins binding free liposomes in solution to directly measure the heat absorbed or released upon binding. Still, it requires large quantities of reagents, making it prohibitively expensive.

Surface plasmon resonance is a sophisticated approach to detect ligand binding by the changes in refractive index caused by binding events. This employs liposomes displayed on a dextran substrate to probe for protein binding and can detect affinity, specificity, and even kinetics of the interaction, but it requires dedicated equipment.

1.4.3 Single liposome assays

A recently developed liposome microarray (LiMA) assay in which a large number of GUVs are generated on a micropatterned agarose substrate to allow for studying the binding of lipids in a high throughput methodology under a microscope (Saliba *et al.*, 2014). Binding is scored by measuring the fluorescence intensity of a labelled protein on the GUVs. However, fluorescent labelling of the protein is not feasible in certain cases or can directly affect the binding of the protein when performed prior to testing, thus affecting the validity of the data.

1.4.4 Proximity-based Labelling of Membrane-Associated Proteins

Proximity-based Labelling of Membrane-Associated Proteins (PLiMAP) is an assay that was developed to increase the versatility of bulk liposome binding assays in terms of throughput and dynamic range. This assay takes advantage of a bifunctional synthetic lipid probe – BODIPY-Diazirine Phosphatidylethanolamine

(BDPE) – which has a BODIPY fluorophore on its tail and a diazirine moiety conjugated to the phosphatidylethanolamine headgroup. Upon exposure to UV, it generates a short-lived, highly reactive carbene at the diazirine moiety, which can covalently bond to proteins in a fairly residue-indifferent manner. Liposomes of desired sizes (LUVs and SUVs) are made of compositions to be tested and doped with a small amount of BDPE (1 mol%) probe, which labels proteins that are associated with membranes when exposed to UV. The mix can then be resolved in SDS-PAGE, and the labelled protein can be imaged for in-gel fluorescence. The fluorescence readout is sensitive and offers a higher dynamic range of detection than high throughput methods such as dot-blots and liposome co-sedimentation assays. Being a simple biochemical assay, it also maintains an edge over more exotic methods that involve large amounts of reagents or sophisticated equipment (Jose *et al.*, 2020).

2. Rationale for the study

Assays that probe for direct lipid binding often do so with lipids immobilised on solid substrates. The highly charged phosphoinositides have a tendency to get washed off of the solid substrate if they are not covalently attached to the substrate, which leads to additional artefacts. These assays offer little control over the local concentration of lipids on the substrate at various locations, which may introduce artefacts..

Liposomes address these drawbacks with the inherent property of lipids to laterally diffuse across membranes, preventing large variations in local concentrations. However, their curvature and general physical properties can confound recording the ability of a protein to bind specific lipids in isolation. Additionally, in contrast to bimolecular protein-ligand interactions in solution, protein-lipid interactions often involve sequestration of cognate lipids on the membranes, which may mask true binding affinity. Extrusion produces liposomes in a range of sizes, which makes probing for single lipid-protein binding difficult. Diacyl-lipids have a cylindrical geometry, which predisposes them to form planar bilayers as opposed to the highly curved nature of liposomes, thus making liposomes unstable over longer periods of time.

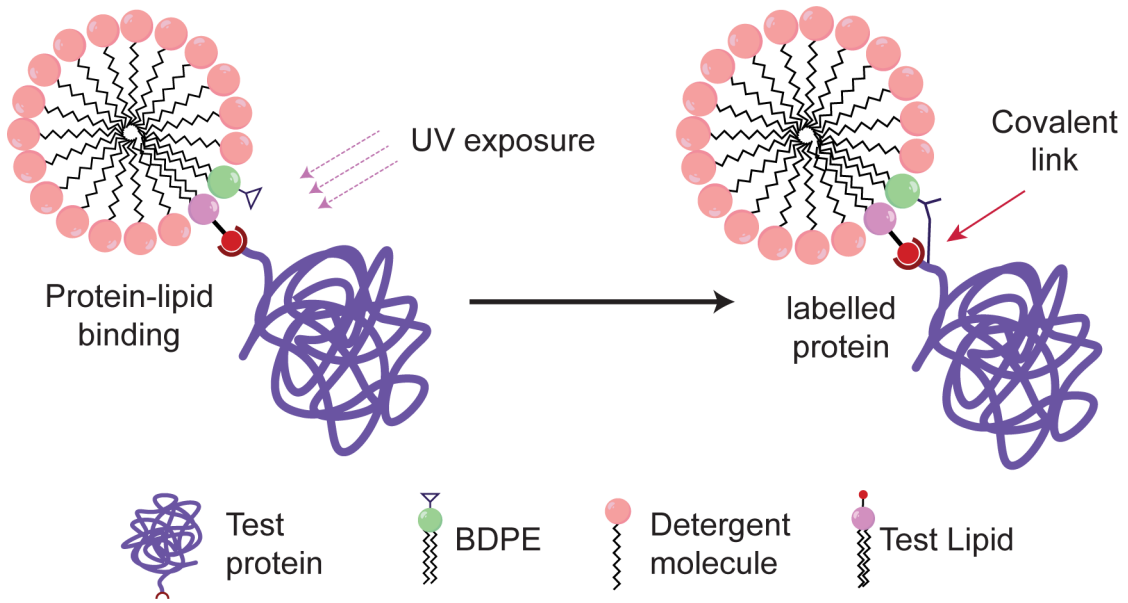

Figure 3. Schematic depicting micelle-based binding assay.

Detergent micelles offer an interesting middle ground between isolated lipids, their soluble headgroups and their incorporation into liposomes. Above the critical micelle

concentration, the detergent molecules self-assemble to form micelles. Structurally, they consist of a monolayer of molecules devoid of a lumen and instead have a hydrophobic core akin to a bilayer. Any lipid dispersed in a micelle would thus be exposed to the solvent (Singh *et al.*, 2007). Previous studies have also shown the activity of phospholipases on lipids dispersed in detergent micelles, giving precedent that proteins can indeed interact with lipids in detergent micelles and even catalyse reactions (Deems *et al.*, 1975; Singh *et al.*, 2008). The tight distribution of micellar diameter and, thus, approximate aggregation number for various detergents can enable modulation of the number of lipids present per micelle based on concentration. At a low enough concentration of lipid relative to the detergent, one can obtain a distribution of no more than 1 lipid per micelle. This provides a substrate for single lipid-protein binding, which could truly represent the affinity of a protein towards a specific lipid in a bimolecular binding paradigm. Adapting the bifunctional probe BDPE to this micellar system serves to augment the PLiMAP assay, as will be discussed in this thesis **(Fig 3)**.

3. Materials and Methods

3.1 Protein constructs

All constructs used were already cloned and available in the laboratory. Table 1 lists all the constructs used.

Vector	Expressed protein construct	Description
pET17B	Strep II-Mff(ΔTMD)-6xHis	Transmembrane domain deleted human Mitochondrial fission factor with N-terminal Strep II tag and C-terminal 6xHis tag
pET15B	6xHis-(TEV)-Pfu-Strep II	Pfu polymerase with N-terminal 6xHis tag and C-terminal Strep II tag
pGEX4P-1	GST-2xP4M	P4M domain from *L. pneumophila* SidM with N-terminal GST tag
pET15B	6xHis-(TEV)-C2-Strep II	C2 domain from bovine Lactadherin with N-terminal 6xHis tag and C-terminal Strep II tag

Table 1. List of all constructs used in the study with brief descriptions for each.

3.2 Expression and purification

Constructs were transformed in NiCo21 (DE3) cells and grown in 1L of autoinduction medium (0.5g/L Glucose + 2g/L α-lactose monohydrate) at 18° C for 36-40 hrs. For constructs with hexa-histidine (6xHis) and Strep II tags, the purification is performed as follows. Pelleted cells were resuspended in 500-HBS (500 mM NaCl, 20 mM HEPES-NaOH (pH 7.6)) and sonicated for 10 mins at 60% amplitude with 1s pulse and 3s off time. Following sonication, the lysate was given a 30,000g spin for 20 minutes to pellet down unlysed cells and debris. Meanwhile, TALON metal affinity beads were equilibrated with 500-HBS and the supernatant was further incubated with these beads for 30min. Excess supernatant is flowed through, followed by 50 mL of washes using 500-HBS. Elution is performed using 15 mL of 500-HBS with 250 mM imidazole. This elution is then bound to a StrepTactin XT Strep II affinity column pre-equilibrated with 500-HBS. The column is then washed with 50 mL of 500-HBS. In the case of Pfu polymerase, this is followed by a 10 mL wash with 1M NaCl, 20 mM HEPES (pH 7.6) buffer to remove DNA bound to the protein, followed by buffer exchange to 150-HBS (150 mM NaCl, 20 mM HEPES (pH 7.6)). For all other proteins, the buffer is directly exchanged with 150-HBS. Final elution is

performed using 150-HBS with 2.5 mM biotin. The elutions were kept on ice until further usage.

For constructs with an N-terminal GST tag, the purification is as follows. Pelleted cells were resuspended in 500-HBS-DTT (500 mM NaCl, 20 mM HEPES-NaOH (pH 7.6), 1 mM DTT), sonicated and pelleted as described previously. The supernatant is incubated for 30 mins with 1mL of Glutathione Sepharose 4B beads pre-equilibrated with 500-HBS-DTT. Excess supernatant is flowed through, followed by 50 mL of washes using 500-HBS-DTT and subsequent buffer exchange to 150-HBS-DTT (150 mM NaCl, 20 mM HEPES-NaOH (pH 7.6), 1 mM DTT). Elution is performed using GST elution buffer (150 mM NaCl, 75 mM HEPES-NaOH (pH 7.6), 5 mM DTT, 15 mM reduced Glutathione). The elution is then dialysed against 150-HBS and stored on ice for later use.

3.3 Lipids and detergents

All lipids were purchased from Avanti polar lipids (Table 2). All inositol phosphate phospholipids were stored as methanol stocks. All other phospholipids were stored as chloroform stocks. BODIPY-diazirine phosphatidylethanolamine (BDPE) was synthesised as described in (Jose and Pucadyil, 2020) and stored as a chloroform stock. The Triton X-100 used in the study was purchased from Sigma-Aldrich (Catalog no. T8787).

Lipids	Abbreviation	Catalog. No.
1-palmitoyl-2-(dipyrromethene boron difluoride)undecanoyl-sn-glycero-3-phosphoethanolamine	TopFluor® PE	810282
1,2-dioleoyl-*sn*-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl] (nickel salt)	DGS-NTA(Ni^{2+})	790404
1-oleoyl-2-hydroxy-sn-glycero-3-phosphocholine	Lyso-PC	845875C
1,2-dioleoyl-sn-glycero-3-phosphocholine	PC	850375C
1,2-dioleoyl-sn-glycero-3-phosphoethanolamine	PE	850725C
1,2-dioleoyl-sn-glycero-3-phospho-L-serine	PS	840035C
1,2-dioleoyl-sn-glycero-3-phosphate	PA	840875C

1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol)	PG	840475P
1,2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol)	PI	840042
1,2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol-3'-phosphate)	PI(3)P	850150
1,2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol-4'-phosphate)	PI(4)P	840045
1,2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol-5'-phosphate)	PI(5)P	850152
1,2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol-3',4'-bisphosphate)	$PI(3,4)P_2$	850153
1,2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol-3',5'-bisphosphate)	$PI(3,5)P_2$	850154
1,2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol-4',5'-bisphosphate)	$PI(4,5)P_2$	850155

Table 2. List of all lipids used in the study.

3.4 Preparation of micelles

Required amounts of each test lipid, except BDPE, were aliquoted into separate glass tubes. Since all micelles used in the assay contain BDPE, the total amount of BDPE required for all micelles is aliquoted in a separate glass tube. Lipids are then dried under high vacuum for >3 hours to ensure complete evaporation of solvents. To make the micelles, 150-HBS containing the required molar concentration of Triton X-100 is used to hydrate the lipids. First, BDPE is hydrated with the Triton X-100 solution followed by vortexing at low speed for 30 sec to make a main stock of BDPE containing micelles in proportion. Required volumes of this stock of BDPE-containing micelles are further used to hydrate each test lipid to make the stocks of micelles containing BDPE and probe lipid. These stocks are then stored in microcentrifuge tubes at 4°C for up to two weeks.

3.5 Micelle based binding assay

Proteins and micelles were mixed to a final molar ratio of 1:400 protein to detergent in a final volume of 30 µL of 150 HBS on transparent 96-well plates. The reaction mixes are incubated for 30 minutes at room temperature in the dark. The plate is

placed at a distance of ~3 cm from the lamp of a UVP crosslinker and exposed to 200 mJ/cm^2 of 365 nm UV light (UVP crosslinker CL-1000L) for 1 minute, as described in (Jose and Pucadyil, 2020). The entire volume of each well is aliquoted into respective MCTs. Each well is then washed with 30 µL of 2x Laemmli sample buffer, which is then transferred into the corresponding MCT.

3.6 SDS-PAGE and gel imaging

Samples were mixed with Laemmlli buffer and boiled at 99°C for 15 mins, cooled to ~70°C and then flash spun. 20 µL of each sample is loaded and resolved with SDS-PAGE. After removing the dye-front, the unstained gel is imaged for BODIPY fluorescence from the labelled protein. The gel is later fixed and imaged for Coomassie Brilliant Blue staining. Fluorescence imaging is performed on Typhoon biomolecular imager (Amersham) and iBright 1500 (Invitrogen). Stained gels are imaged on iBright 1500 (Invitrogen).

3.7 Analysis of images

All images were saved as TIFF files and analysed using FIJI ImageJ software. Processing and analysis is performed as described in Jose and Pucadyil, 2020. The required bands are straightened, cropped out from the fluorescence images, and rotated 90°. Line profiles across the band on the perpendicular axis are obtained using a region of interest (ROI) that accommodates the length of every band to be analysed. To account for variation in the background, line profiles are obtained from ROIs in the gaps following each band. The signal is calculated by subtracting the background line profile from the band line profile. The corrected line profile for each band is plotted and fitted to a Gaussian distribution. The amplitude of the fitted curve is taken as the signal value. Binding data was first corrected for background binding. The corrected values were then fitted to a one-site binding isotherm to estimate K_d and B_{max}. Binding data was then normalised to the corresponding estimated B_{max} for comparison. Curve fitting is performed on GraphPad Prism.

4. Results and Discussion

4.1 Proof of concept

The formation of detergent micelles is dictated by the critical micelle concentration (CMC). This is the concentration above which additional molecules of detergent self-assemble to form micelles. Different detergents have varied CMC values depending on the structure and chemistry of the molecular unit. At any concentration above CMC, the concentration of molecules present as solvent-dispersed monomers remains constant at a value equal to the CMC of the detergent while the rest of the molecules self-assemble to form micelles. A lower CMC value thus allows one to have a larger fraction of molecules in the form of micelles, which form the substrate for the assay.

Two polyhistidine-tagged constructs were used for preliminary validation of the assay – StrepII-Mff(ΔTMD)-6xHis, the C-terminal cytosolic portion of mitochondrial fission factor and 6xHis-(TEV)-Pfu-StrepII, the DNA polymerase – both of which are known to not bind membranes on their own. We initially tested the validity of this assay using detergents with particularly low CMCs as substrates. Lyso-lipids have reported CMCs as low as 0.4 μM for 18:0 lyso-PC (Marsh, 2013). Thus, 18:1 lyso-PC (1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphocholine) was chosen to form the bulk of the micelle. The lyso-PC micelles were doped with 1 mol% of BDPE and 5 mol% of DGS-NTA(Ni^{2+}). When incubated with polyhistidine-tagged proteins at a protein:lipid ratio of 1:100 for 30 mins and exposed to UV, bound proteins were successfully labelled with BDPE. However, when the proteins were tested with only 1 mol% BDPE and no DGS-NTA(Ni^{2+}), a smaller fraction of the protein was still getting labelled **(Fig 4.A)**, indicating a basal binding of these proteins independent of the polyhistidine-DGS-NTA(Ni^{2+}) interaction, which would interfere with this assay.

To address this issue, we replaced lyso-PC with Triton X-100, a non-ionic detergent with a reported low CMC of 0.019-0.02% w/v, which is ~320 μM assuming the average weight of Triton X-100 as 625g/mol. The molecule has a large polyethylene oxide headgroup that can cause steric crowding at the surface of the micelle, which may allow the polyhistidine-tagged proteins to stably interact with the micelle only via specific binding to the DGS-NTA(Ni^{2+}) lipid. Triton X-100 micelles with 1 mol% BDPE and 5 mol% DGS-NTA(Ni^{2+}) show significant binding, while those with only 1 mol%

BDPE and no DGS-NTA(Ni^{2+}) show no detectable binding **(Fig 4.B)**. This establishes the ability of Triton X-100 to exclude non-specific binding from membrane non-interacting proteins. In addition, Triton X-100 is a significantly inexpensive reagent as compared to a lyso-lipid such as lyso-PC.

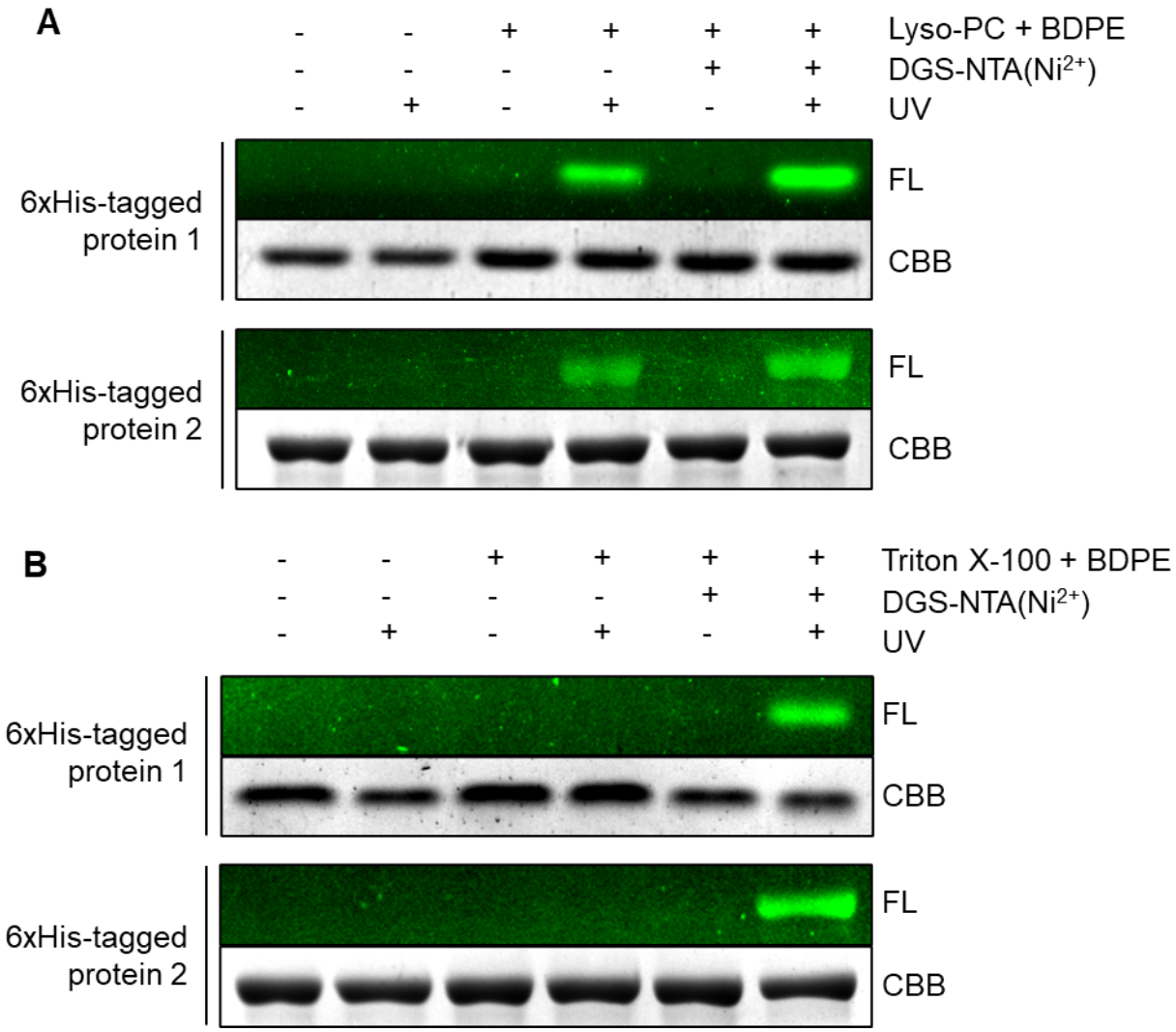

Figure 4. Validating protein-lipid interaction on detergent micelles. Representative images showing BDPE-crosslinked in-gel fluorescence (top) and CBB-stained (bottom) of protein 1 (MffΔTMD) and protein 2 (Pfu) **A** Micelle composition is 100 μM Lyso-PC with 1 μM BDPE, with (+) or without (-) 5 μM DGS-NTA(Ni^{+}). **B** Micelle composition is 100 μM Triton X-100 with 1 μM BDPE, with (+) or without (-) 5 μM DGS-NTA(Ni^{+})

4.2 Protein-Lipid binding: Phosphoinositides

Once the detergent was established, we decided to put the assay to the test using a well-known high-affinity binder of phosphoinositides, the P4M domain of SidM/DrrA protein from *Legionella pneuophila*. This P4M domain has been established as a

phosphatidylinositol-4-phosphate (PI(4)P) binding protein and thus used as a biosensor for PI(4)P in living cells.

Considering that the aggregation number of Triton X-100 micelles is 100-155 (Johnson, 2013), we settled on a micelle composition of 1 mol% BDPE and 0.1 mol% PIPs, to ensure that (i) all micelles contain at least one molecule of the labelling lipid BDPE, (ii) no more than one molecule of PIPs is present on any micelle, and (iii) the concentration of the PIPs is in the range of their physiological concentration on membranes.

We tested whether this assay can be used to determine the lipid binding specificity of the phosphoinositide binding P4M domain. Across a range of phosphoinositides, the P4M domain showed binding in the order – PI(4)P > $PI(4,5)P_2$ > PI(3)P – as has been reported previously **(Fig 5)** (Del Campo *et al.*, 2014; Jose *et al.*, 2020).

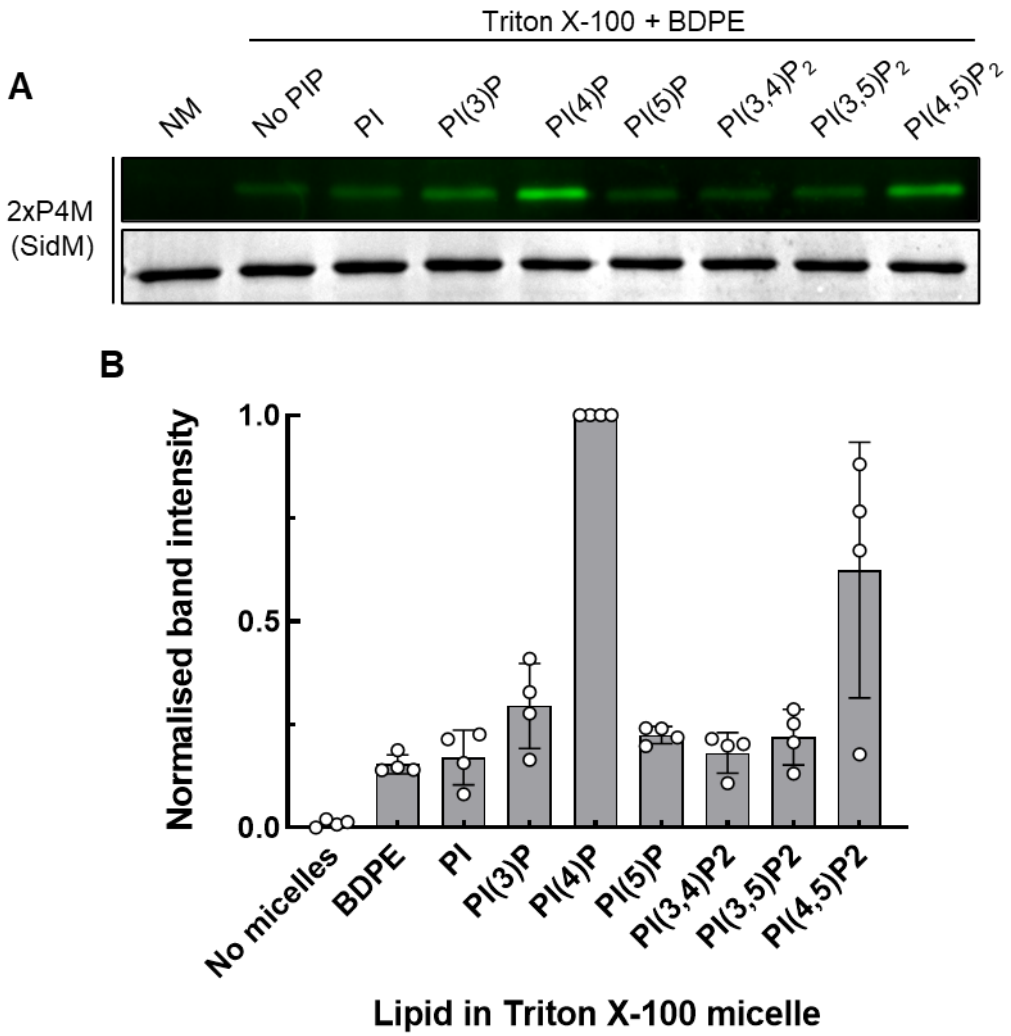

Figure 5. Specific protein-phosphoinositide binding on detergent micelles. **A** Representative images showing in-gel fluorescence of BDPE-labelled (top) and CBB-stained (bottom) GST-2xP4M domain of SidM. Micelles consist of 400 μM Triton, 4 μM BDPE and 0.4 μM phosphoinositide as indicated. **B** Quantitation of BDPE fluorescence on GST-2xP4M domain of SidM. Data represents mean ± SD of 4 experiments.

The binding affinity of the P4M domain was also probed with detergent micelles with increasing concentrations of PI(4)P. The constant of dissociation was observed to be 0.93 μM, which is within the range of previously reported values. This shows that this assay is useful not only in determining lipid specificity but also in determining the binding affinity of proteins to lipids.

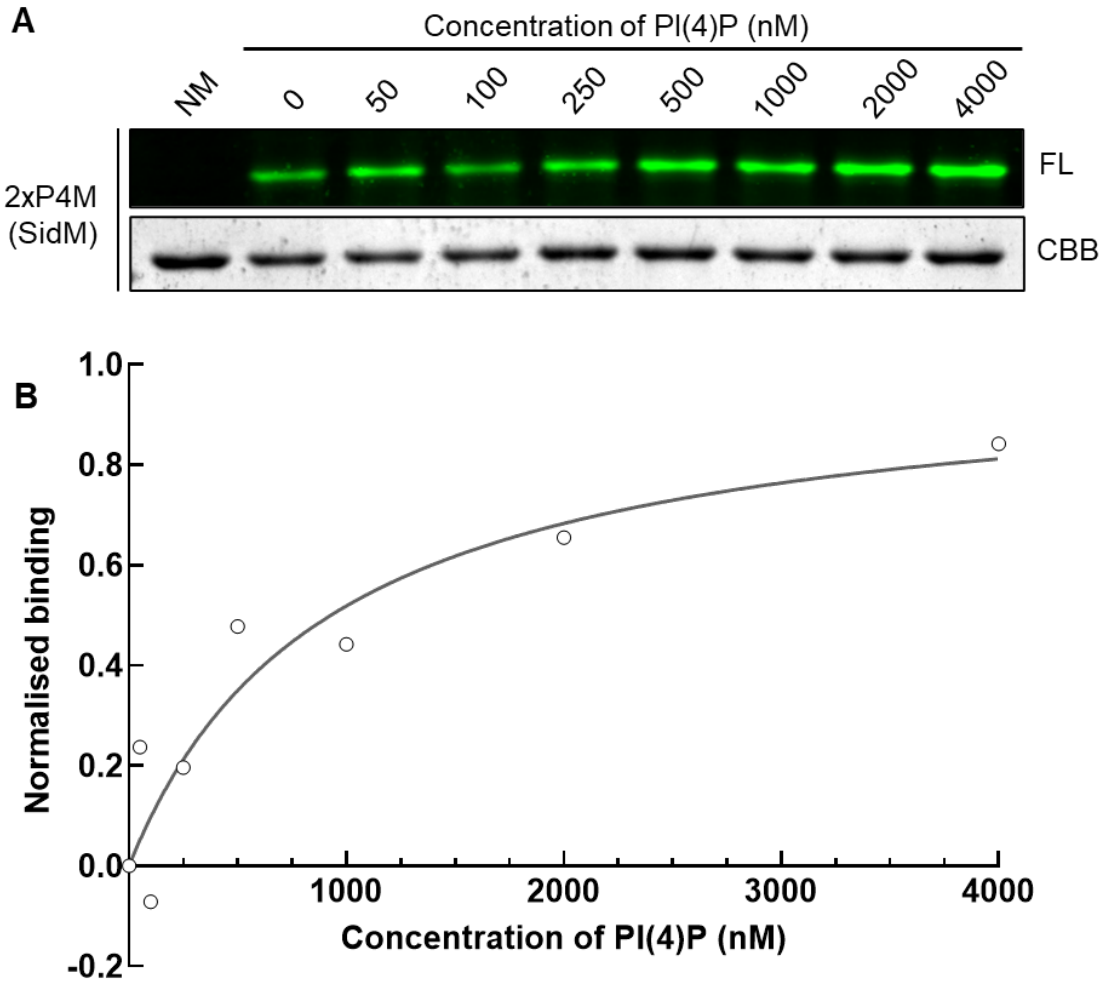

Figure 6. Binding affinity for phosphoinositides on detergent micelles. **A** Representative image showing in-gel fluorescence of BDPE-labelled (top) and CBB-stained (bottom) GST-2xP4M domain of SidM. Micelles consist of Triton X-100 with 1 mol% BDPE and increasing concentrations of PI(4)P. **B** Quantitation of BDPE fluorescence from GST-2xP4M domain of SidM. Data represents 1 experiment.

4.3 Expanding the usage to other phospholipids

The C2 domain from Lactadherin is known to bind phosphatidylserine (PS) with a high affinity (Andersen *et al.*, 2000; Otzen *et al.*, 2012). In contrast to the P4M domain, which interacts only with the inositol headgroup of PI(4)P, the interaction of Lactadherin with PS involves the insertion of a portion of the peptide into the hydrophobic core of the membrane. Whether this kind of binding can be probed with micelle substrates is worth addressing, as it broadens the scope of this assay. When tested against a range of different phospholipids, the binding to PS was the highest **(Fig 5)**, thus indicating that this assay works for non-phosphoinositide lipids as well.

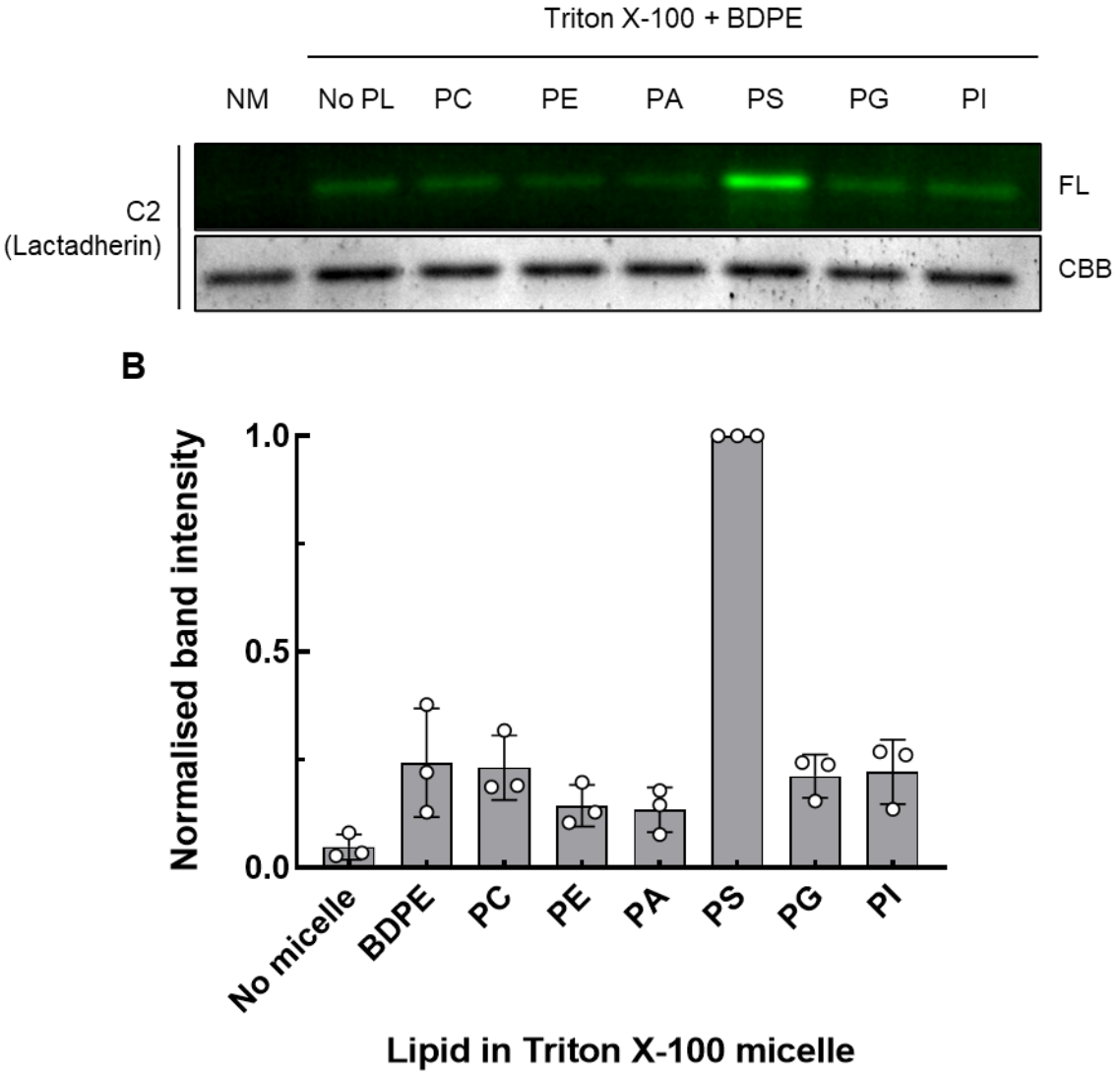

Figure 7. Specific protein-phospholipid binding on detergent micelles. **A** Representative image showing in-gel fluorescence of BDPE-labelled (top) and CBB-stained (bottom) 6xHis-Lact C2-Strep II, the C2 domain from Lactadherin. Micelles consist of 400 µM Triton, 0.4 µM BDPE and 1.2 µM of

phospholipid as indicated. **B** Quantitation of BDPE fluorescence on 6xHis-Lact C2-Strep II. Data represents mean ± SD of 3 experiments

4.3 Advantages of this assay

Preparation of liposomes is a time-intensive process and involves technicalities that may introduce random errors. The most commonly used method – reverse phase evaporation – relies on making a uniform and homogenous mix of lipids dried to a thin film, which is subsequently hydrated and incubated at temperatures above the highest phase transition temperature among the lipids in use. These generate multilamellar vesicles that require further processing, such as freeze-thaw cycling, extrusion, or sonication, to produce unilamellar vesicles of the desired sizes.

The usage of detergents in this assay system dramatically reduces the complexity of lipid binding assays while maintaining the sensitivity and range of detection offered by a fluorescence-based assay. Preparation of micelles doped with lipids requires no additional processing post-hydration, as described previously. The ability of micelles to incorporate lipids upon hydration, along with their dynamic nature, allows one to maintain the homogeneity of the micelles by the sheer diffusion of detergent molecules between micelles. All the lipids dispersed in a micelle are exposed to the solvent and are accessible to the protein. Since detergent micelles have a small range of aggregation numbers dictating the number of molecules in the micelles, it is possible to generate a distribution of micelles with one test lipid per micelle. This fine control is absent from liposome-based systems. This assay functions well even with significantly lower amounts of lipids and uses detergents instead of lipids for the bulk of the structure, which reduces the cost per reaction of this assay as well.

	Liposome-based assay lipid amount (nmol)	**Micelle-based assay Lipid amount (nmol)**
Bulk lipid	28.2	0
Test lipid	0.15	0.012
BDPE	0.03	0.12

Table 3. Comparing resource utilisation between liposome-based assay and micelle-based assay, per reaction.

5. Conclusions

Using established proteins with high-affinity binding to specific lipids, this study has established the proof of concept and validity of using detergent micelles as an alternative to liposomes for probing protein-lipid interactions. The usage of the promiscuously reactive photoactivable bifunctional lipid probe, BDPE, has been crucial in making this assay as facile as feasible while maintaining consistency with previously well-established methods to study lipid binding. The practicality of this assay is most apparent in the rapidity and ease of reagent preparation. Being a simple biochemical assay, it is far more accessible than other methods of similar sensitivity, while bridging the gap between high-throughput assays and high-sensitivity assays.

Since bound proteins are covalently labelled with a small molecule in this assay, it opens avenues for further investigation of the labelled proteins by mass-spectrometric methods. The BDPE probe is highly reactive and crosslinks with the nearest available residue. Since micelles are of comparable size to proteins, the footprint of the protein on the micelle itself is also smaller in comparison to that on a liposome, allowing precise labelling of the binding domain. While this may not be able to label the binding site itself, as that would be occupied by its cognate lipid, residues that are in close proximity will get labelled. The labelled protein can then be fragmented by peptidases and processed for mass-spectrometry to identify the fragments that bear the label, providing insights into the domains involved in lipid-interaction. Owing to the low concentration of lipids involved in this assay, it can be used to identify proteins that bind the test lipid with a high affinity from a mix of proteins, expanding the use of this assay as a screen for lipid binding proteins.

6. References

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