

**PROTEIN AND LIPID FINGERPRINTING OF NATIVE-LIKE MEMBRANE COMPLEXES BY COMBINING
THIN LAYER CHROMATOGRAPHY AND PROTEIN ELECTROPHORESIS: THE EXAMPLE OF LUNG
SURFACTANT**

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ABSTRACT

Traditionally, thin layer chromatography (TLC) has been used for the analysis of lipids isolated from membrane complexes. Here, we describe a method based on the combination of TLC and SDS-polyacrylamide gel electrophoresis (SDS-PAGE) for the qualitative analysis of the protein/lipid profile of membrane complexes such as those of lung surfactant. For this purpose, native lung surfactant was applied onto a silica TLC plate in the form of an aqueous suspension, preserving not only hydrophilic proteins associated to lipids, but also native protein-lipid interactions. Using native membrane complexes in a TLC allows the differential migration of lipids and their separation from the protein components. As a result, (partly) delipidated protein-enriched bands can be visualized and analysed by SDS-PAGE to identify proteins originally associated with lipids. Interestingly, the hydrophobic surfactant protein SP-C, which interact tightly with lipids in native membrane complexes, migrate through the TLC plate configuring specific bands that differ from those corresponding to lipids or proteins. This method therefore allows the detection and analysis of strong native-like protein-lipid interactions.

Supplementary Keywords (5 from the list)

LUNG SURFACTANT; PHOSPHOLIPIDS; PROTEOMICS; DIAGNOSTIC TOOLS; LIPOPROTEIN

INTRODUCTION

Thin layer chromatography (TLC) is a traditional technique typically used to separate and study lipid composition in many biological tissues (1-5) and membrane complexes. An example of this is lung surfactant (LS) (6-13), a protein/lipid complex lining the air-liquid interface in alveoli, which allows breathing and facilitates gas exchange. LS composition is conserved in a fair number of mammalian species (14-16) and comprises 90% by weight of lipids and 8-10% proteins. From the lipid fraction, phospholipids are the most abundant components. Among them, phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylethanolamine (PE), phosphatidylserine (PS) and small amounts of sphingolipids such as sphingomyelin (SM) have been identified by TLC. Neutral lipids, especially cholesterol, are also present.

Proteins co-isolated together with LS complexes are typically in close contact with lipids, such as in the case of surfactant protein A (SP-A) (17), whereas other soluble, non-lipid interacting, proteins present in the bronchoalveolar lavage (18) are lost during LS purification (19).

The so-called surfactant-associated proteins, SP-A, SP-B, SP-C and SP-D, have been traditionally studied after their separation from its native lipid context by means of electrophoresis and western blot (WB). However, due to the high lipid content in bronchoalveolar lavage or isolated surfactant, electrophoresis and blotting often results in deformed protein bands and poor detection. Moreover, small fractions of strongly interacting lipids are usually co-isolated with the hydrophobic proteins of surfactant, SP-B and SP-C, making their study by WB a difficult task.

Here, we present a methodology that overcomes those difficulties by combining TLC and electrophoresis to qualitatively study the lipid and protein fingerprint of whole native surfactants or other native membrane complexes. Lipid characterization by TLC typically includes the organic extraction of the lipid component from a given biological sample. Extracted samples solubilized in organic solvents are then directly applied on the TLC silica plates, avoiding water-soluble "contaminants" such as proteins. We propose an alternative method that preserves polar lipid-protein interactions and allows the simultaneous lipid/protein characterization of LS samples and other membrane complexes. An example of application to the study of protein/lipid interactions of small membrane proteins is also presented.

MATERIALS AND METHODS

All phospholipid standards were purchased from Avanti Polar Lipids (Alabama, USA) and organic solvents were HPLC grade from LabScan (Gliwice, Poland). The N-terminal peptide from endothelial Nitric Oxide Synthase (eNOS) was provided by Dr. Ignacio Rodriguez-Crespo, from Complutense University in Madrid, and bacteriorhodopsin was a kind gift of Prof. Esteve Padros, from Autonomous University of Barcelona. Surfactant protein SP-C was isolated from porcine lungs as described (20). SP-C and SP-B analogues (KL4: KLLLLLLLLLLLLLLLLL; KL2A2: KLAALKLAALKLAALKLAALK; KL4PQ: KLLLLLLLLPQLLLLLLLLLL) were synthesized by F-moc matrix-assisted chemistry at the laboratory of Prof. David Andreu, from Pompeu Fabra University in Barcelona. Protein stocks were prepared as follows: eNOS (1mg/ml) in methanol, SP-C and SP-B (1 mg/ml) in chloroform: methanol 2:1 v/v. Native SP-C and bacteriorhodopsin were preserved as isolated. Lipid stocks were prepared and stored at the desired concentrations in chloroform/methanol (2:1 v/v).

Isolation of porcine lung surfactant

Native porcine lung surfactant was purified from bronchoalveolar lavage (BAL) by NaBr density gradient as previously described (21). Briefly, BAL was obtained by introducing and recovering ice cold Tris 5mM in 0.9% NaCl into the trachea of porcine lungs obtained from the slaughterhouse. BALs were gauze-filtered and centrifuged to remove cells and debris. Supernatant containing cell-depleted BALs were collected and stored at -20°C until use. To obtain the full membrane fraction, samples were thawed and centrifuged at 100.000 g, 4°C for 1h (70 Ti fixed-angle rotor, Beckman Coulter). Pellets were resuspended in NaBr 16% NaCl 0.9% and pooled. This fraction was placed at the bottom of a centrifuge tube, onto which two less dense solutions, NaBr 13% NaCl 0.9% and NaCl 0.9%, were carefully deposited. Samples in this discontinuous density gradient were centrifuged at 120.000xg, 4°C for 2h (SW40 Ti swinging-bucket rotor, Beckman Coulter). Surfactant complexes, comprising phospholipids and associated proteins, formed a compact layer on top of the NaBr 13% NaCl 0.9% solution. Samples were carefully recovered avoiding the collection of NaBr 13% NaCl 0.9% solution, further diluted in NaCl 0.9% and flash frozen in liquid N₂. These samples are termed native surfactant (NS).

Organic extraction of lung surfactant

For LS organic extraction, the protocol of Bligh and Dyer was followed (22). Briefly, samples were mixed with methanol and chloroform in a 1:2:1 volumetric ratio and vigorously mixed for 30sec. Soluble proteins were flocculated by 30 min incubation at 37°C. Addition of one volume of chloroform and one volume of water resulted in the formation of two phases that were fully separated by centrifugation (2.000xg, 5min, 4°C). The organic (bottom) phase was collected whereas the aqueous (top) phase was subjected to two consecutive extraction steps yielding the maximum phospholipid extraction. Organic phases were then pooled and stored at -20°C in borosilicate tubes. These samples are termed organic extracts (OE).

Lipid and lipid/protein sample preparation

To prepare liposome suspensions, lipids alone or a combination of lipid and hydrophobic proteins were mixed in chloroform/methanol (2:1 v/v) in the proportions indicated for each experiment. Samples were dried out under a nitrogen stream and left in vacuum for 2h in a SpeedVac Concentrator System (Thermo Scientific). Multilamellar suspensions were prepared by rehydrating samples in Tris buffer (5 mM Tris, 150 mM NaCl pH 7) at 50 °C for 1 h with intermittent shaking.

For samples containing bacteriorhodopsin, the protein was added during the rehydration step. Per sample, 100 µg of phospholipid was used to a final concentration of 10 mg/ml.

Thin layer chromatography: plates and application of samples

Chromatoplates, 20x20 cm, pre-coated with Silicagel 60 (ref. 1.05748.0001, Merck, Germany) were used in this study. Both samples and standards were applied at a phospholipid concentration of 10mg/ml as aqueous suspensions (10µl) or organic extracts as indicated.

Samples and standards were applied subsequently in small volumes (3-5 µl steps) using a 10 µl Hamilton syringe or pipette forming a line at the bottom of the plate, here and thereafter called the application point (AP). Samples were air-dried at room temperature before the next sample application. Once the solvent was evaporated, dried samples appeared indistinguishable from the background. Plates with the applied samples were placed in a fume-hood and further dried out at room temperature for 15-20 min. The developing solvent system used in these TLC experiments was chloroform/methanol/water 65:25:4 (v/v/v). The chromatographic chamber (Shandon Scientific Co. Ltd, UK) was lined on three sides with Whatman No. 1 filter paper wetted with the developing solvent system. The chamber was saturated with fresh solvent for 30 min before running the chromatography. Thereafter, the developed plate was removed and air-dried completely before staining.

For the detection of lipids, the plate was placed vertically in a second chamber saturated with iodine vapour (Sigma Aldrich, Missouri, USA). Iodine binds to unsaturated carbon bonds of lipid molecules resulting in dark bands along the TLC plate.

A fluorescamine solution (Sigma Aldrich, MO) 0.5% in acetone was sprayed on the plate in order to detect primary amine-free containing molecules (23), such as amine-containing phospholipids or proteins in our case. The detection of fluorescence is possible under UV light. Images were recorded using a VersaDoc imaging system (Biorad, Hercules, CA) in the epifluorescence mode. For the visualization of both lipids and proteins in the sample plate, fluorescamine was applied and the images were recorded before iodine staining to avoid fluorescence quenching by iodine vapor.

SDS-PAGE

TLC bands were scratched out and boiled at 99°C for 10 min in a Thermomixer (Eppendorf, Hamburg, DE) after the addition of electrophoresis Laemmli buffer (2% SDS, 62.5 mM Tris pH 6.8, 10% glycerol, 0.03% bromophenol blue) containing 4% β-mercaptoethanol. Before application into the electrophoresis gels, samples were spin down pelleting most of the silicagel. Samples were applied either into 13% or Mini-protean precast gradient polyacrylamide gels (4-16%, BioRad, Hercules, CA) and electrophoresis were performed in a Mini-protean BioRad system (BioRad, Hercules, CA). Visualization of the bands was achieved by silver staining.

Identification of proteins: Western Blot and mass spectrometry

The analysis of the protein content was assessed by Western Blot. Proteins were transferred onto PVDF membranes with a semi-dry system (BioRad) for 1h at 2.3mA/cm² membrane and blocked in PBS-T (100mM Na₂HPO₄/KH₂PO₄- 1% Tween) 5% skimmed milk at room temperature for 2h. Membranes were then incubated overnight with the primary antibody in PBS-T 5% milk at 4°C, washed thoroughly in PBS-T and incubated with the secondary antibody for 2h at room temperature. Before development, membranes were further washed, and then developed using a commercial ECL system (Millipore, MA). Primary antibodies used were: anti-SP-A (1:2000) kindly provided by Prof. Joe Wright, anti-SP-B (1:2000) from Seven Hills (WRAB-48604), anti-SP-C

(1:2500) from Seven Hills (WRAB-76694). Secondary antibody (1:5000) used in all cases was anti-rabbit (P0217) from Dako.

Bands from silver stained gels were analysed at the proteomic facility in CNB-CSIC (Madrid, Spain) using Peptide Mass Fingerprinting MS - MS/MS by MALDI TOF/TOF.

RESULTS

The lipid bands resolved in a TLC of a native surfactant sample applied as aqueous suspension (NS) or as organic extracted surfactant in organic solvent (OE) did not substantially differ, and comprised mainly phosphatidylcholine (PC), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), sphingomyelin (SM) and neutral lipids (NL) (Fig. 1). Interestingly, the main difference in the band pattern when comparing surfactant in aqueous and organic solvent solution lied in the application point (AP). Phospholipids were not detected at the AP after organic phosphorous detection (data not shown). However, hydrophilic components of surfactant, such as soluble proteins, were expected to stay at the AP. To analyse the protein content, AP bands were scratched out and subjected to SDS-PAGE and silver staining (Fig. 2).

A number of protein bands were visible in the AP of native surfactant samples (Fig. 2A). As it could be anticipated, those proteins were also detectable in BAL fluid in larger amounts, whereas many other proteins observed in BAL were absent in NS samples (Fig. 2B). Therefore, only proteins with a significant interaction with lung surfactant are co-isolated with it and hence, the protein bands identified in the AP NS represented specific surfactant-associated proteins. Among them, SP-A, SP-B and SP-C could be identified by western blot (Fig. 2C). Mass spectrometry analysis revealed that proteins appearing at the AP in NS were not classical surfactant-associated proteins but involved in the immune response (IgM, IgA), characteristic of blood (albumin, hemoglobin, serotransferrin) or tissue (beta-actin) (Supp. Fig. 1).

SP-A, the main hydrophilic surfactant protein, was only found in NS and not in OE as expected, whereas SP-B was present in both AP of NS and OE. SP-C, however, did not appear at the AP when samples were applied as aqueous solution but rather run together with PC. To confirm this special behaviour, PC liposomes containing increasing amounts of SP-C were also applied as aqueous solutions and analysed by this method. As observed in Fig. 2D, SP-C configured a different band below that of PC. Higher SP-C content resulted in an increase in the fluorescamine-stain intensity, confirming the presence of the protein in those particular bands. These findings suggest that some native SP-C-lipid interactions might still be preserved even under the presence of organic solvents in the developing phase of the TLC. Upon organic extraction this interaction no longer remained, as deduced from the presence of lipid-free SP-C at the AP when organic extracted surfactant was analysed.

To further analyse whether SP-C behaviour was shared by any other small membrane proteins, different proteins and peptides were reconstituted into PC membranes and tested (Fig. 3). The palmitoylated N-terminal fragment of the enzyme eNOS behaved similarly to SP-C (Fig. 3A), revealing that protein-lipid interactions non-specific to SP-C might be responsible for this migration pattern. However, a membrane protein of a larger size, such as bacteriorhodopsin, does not migrate out of the application point of the TLC under these conditions, in spite that its sequence is little more than 7 transmembrane helices. Small synthetic peptides used as potential surrogates of surfactant proteins in the design of synthetic surfactant clinical preparations, such as KL4 or KL2A2, were also able to run along the TLC plate (Fig. 3B) not only associated to PC species but also to PG (such as KL4PQ). Again, this behaviour was only detectable when samples were applied as aqueous suspensions, as none of the proteins analysed migrated through the plate when they were applied in organic solvent (Fig 3).

DISCUSSION

In this work we have shown a new application for a rather classical technique, TLC. The main novelty relies on the application of native-like lipid/protein samples in aqueous suspension instead of in organic solvents. Thus, sample composition is fully retained as well as many of the molecular polar and hydrophobic interactions especially relevant in the context of membranes

The added value provided by sample analysis using this methodology is illustrated by the behaviour of SP-C, which showed a differential TLC migration pattern compared with other proteins and lipids. SP-C is dually palmitoylated (24), and because of its extremely hydrophobic character, its migration pattern through the TLC plate could be interpreted as SP-C adopting a lipid-like configuration. However, given that SP-C only migrated when the protein was applied as part of lipid/protein complexes in aqueous solutions, SP-C hydrophobicity is not sufficient to explain this behaviour. Strong SP-C/phospholipid interactions might then be likely preserved, possibly involving some solvation water molecules. The confirmation of a similar behaviour for other small membrane-associated proteins such as the eNOS N-terminal sequence or the surfactant peptide analogues KL4, KL4PQ and KL2A2, further supports that this migration pattern is due to the preservation of native protein-lipid interactions. Interaction with different (phospho)lipids and protein/lipid ratio are relevant factors to consider when analysing specific protein/lipid interactions. In the case of SP-C, its migrating behaviour was observed from small to large amounts until 10% by weight, suggesting that up to 10 wt%, SP-C/lipid interactions are not saturated. However, this cannot be extrapolated to other membrane proteins, for which the role of different protein/lipid ratios and particular lipid species remain to be investigated.

Depending on their polarity, samples components partition between the hydrophobic (solvent) mobile and polar (silica) phases. For this reason, lipids are carried by the developing phase whereas most proteins remain delipidized at the AP. Upon application as aqueous solutions, tight protein/lipid/water complexes withstanding organic solvent solubilization could allow their migration as a unit along the TLC plate, thus opening the possibility for studying functional lipid/lipid and lipid/protein interactions in native cell membranes.

The behaviour of the protein examples analysed here suggests that for a protein to migrate associated with lipids in a "native" TLC it needs to have a dominant hydrophobic character, so that an important fraction of its structure could be mobilized while exposed to the organic mobile phase. In the case of SP-C, its α -helix as well as its palmitoylated cysteines are likely fully exposed to the organic solvent, whereas the charged N-terminal segment of the protein could preserve the interaction with some phospholipid headgroups thanks to remaining solvation water molecules. Other small amphipathic proteins and peptides could adopt a similar configuration. However, for a protein such as bacteriorhodopsin, with polar groups at the two sides of the membranes, such an orientation towards the organic solvent would be incompatible hindering its development through the plate. Whether a few specific lipid molecules are retained by these AP-immobilized proteins warrants further investigation.

The methodology presented here also allows to study the hydrophilic component of a sample such as surfactant considering those soluble proteins strongly associated to membrane/surfactant complexes. This protein fingerprinting could be useful not only for assessing the purity and quality of clinical surfactant preparations, but also to understand and diagnose lung diseases. For instance, the identification of albumin in surfactant samples might be indicative of edema and fluid leaking into the alveolar spaces, indicating ARDS risk or different stages of the

disease (25, 26). Other potential molecular markers identified in this work are haemoglobin, which is over-expressed in the lung under hypoxic conditions (19, 27), and IgM and IgA, whose increase is linked to different interstitial lung diseases such as sarcoidosis (28), idiopathic pulmonary fibrosis or chronic hypersensitivity pneumonitis (29). Beta actin might also be considered as a marker of cell damage related to lung disease whereas serotransferrin levels could be a good indicator of redox activity as detected under the exposure to cigarette smoke (30, 31), NO₂ (32), O₃ (33, 34), hyperoxia (35, 36) or hypoxia (19, 37).

Taken together, our findings support that this novel application of TLC represents a promising tool to analyse and identify native protein/lipid interactions not only restricted to surfactant samples. Additionally, protein/lipid fingerprinting of surfactant samples from patients, animal models or surfactant clinical preparations, may contribute to the understanding and diagnosis of lung diseases.

ACKNOWLEDGEMENTS

This work has been supported by grants from the Spanish Ministry of Economy (BIO2015-67930-R) and the Regional Government of Madrid (S2013/MIT-2807). N.R. acknowledges a FPU fellowship from the Spanish Ministry of Education.

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FIGURES

Figure 1

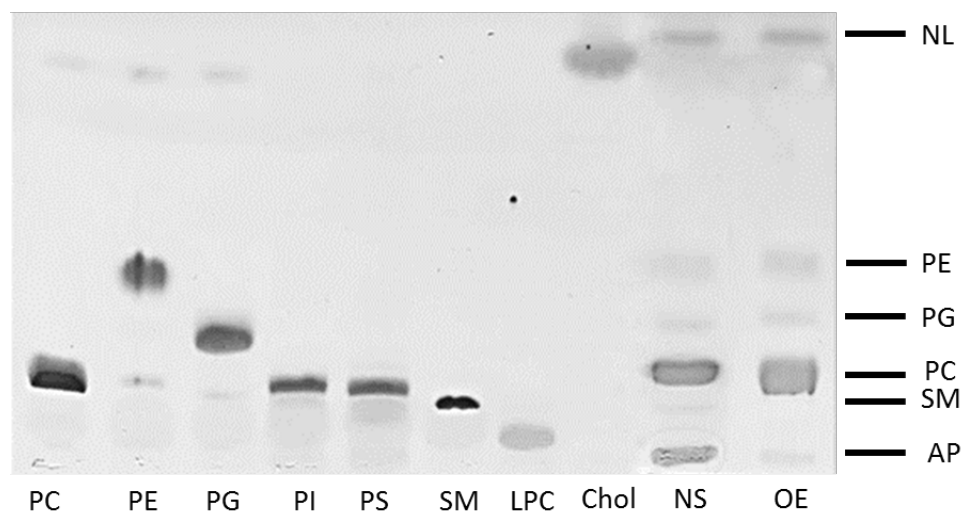


Figure 1

Thin layer chromatography of surfactant in aqueous solution (NS) and in organic solvent (OE)

Samples applied include aqueous suspensions of native surfactant purified from porcine bronchoalveolar lavage (NS) and its organic extract applied as a chloroform/methanol solution (OE). Applied lipid standards include: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylinositol (PI), phosphatidylserine (PS), sphingomyelin (SM), lysophosphatidylcholine (LPC), and cholesterol (Chol). Bands stained with iodine revealed SM, PC, PG, PE, NL (neutral lipids, including cholesterol) and the AP (application point) band where water-soluble proteins remain.

Figure 2

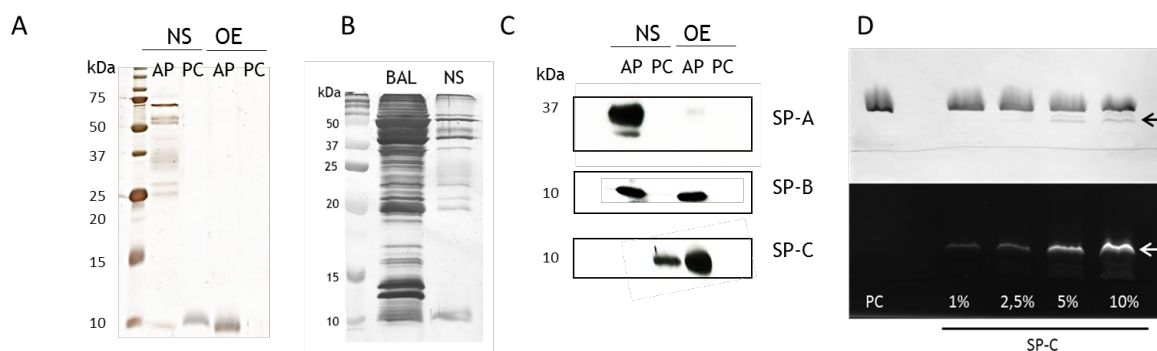


Figure 2

Electrophoretic analysis of proteins associated with surfactant samples as extracted from TLC plates

Panel A, silver staining of a PAGE-SDS performed with the material scrapped from the AP (application point) and the PC (phosphatidylcholine) TLC-bands from NS or OE. *Panel B*, silver stained gel of the AP of BAL and NS after TLC. Equivalent amounts of phospholipid were applied (100 μ g). *Panel C*, immunodetection of surfactant associated proteins in the AP and PC lanes by western blot. *Panel D*, iodine-stained (upper panel) and fluorescamine-stained (lower panel) TLC of lipid/protein samples incorporating different amounts of SP-C expressed in % by weight and applied as aqueous suspensions onto the TLC plate. Arrows indicate the position of the band corresponding to SP-C.

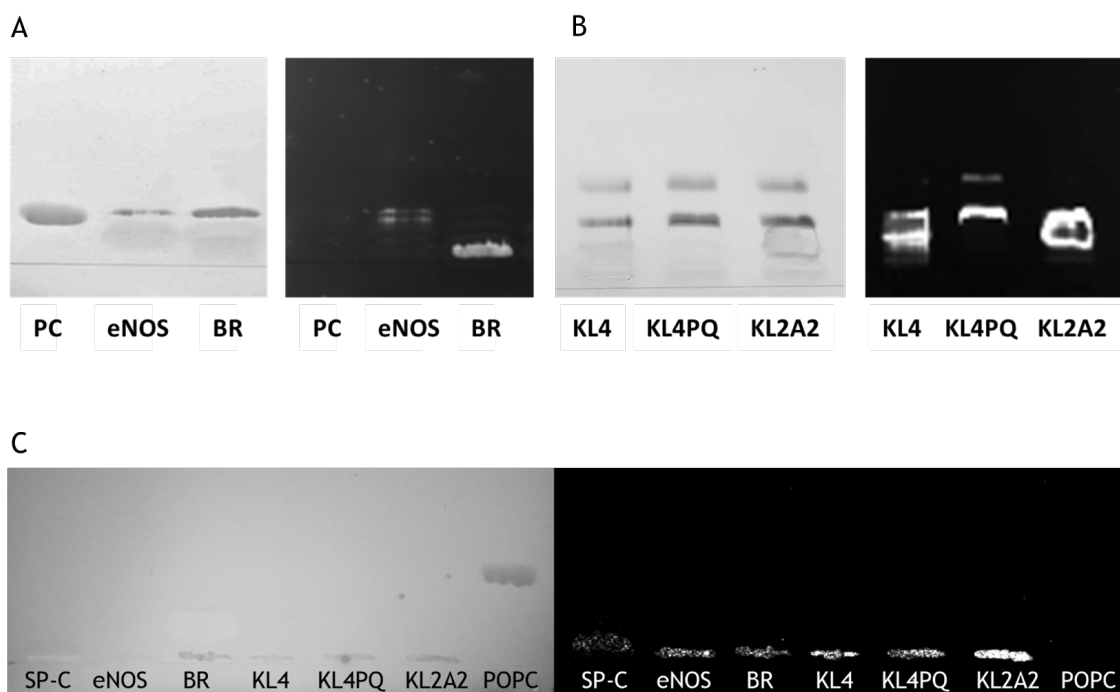


Figure 3

TLC analysis of different lipid/protein complexes applied as aqueous suspensions.

A: left panel, iodine-developed TLC plate of lipid or lipid/protein membrane complexes applied as aqueous solution of PC, PC/eNOS N-terminal segment, and PC/BR (bacteriorhodopsin); right panel, fluorescamine-developed TLC plate revealing the corresponding protein bands. B: left panel, iodine-developed TLC of lipid/protein membrane complexes applied as aqueous suspensions of DPPC:POPC:POPG membranes bearing peptides KL4, KL4PQ or KL2A2. Right panel, fluorescamine developed TLC plate revealing the corresponding peptide bands. C: left panel, iodine-developed TLC plate of different protein or peptide (SP-C, eNOS, BR, KL4, KL4PQ, KL2A2) or lipid (POPC) applied as in organic solvent; right panel, fluorescamine-developed TLC plate revealing the corresponding protein bands at the application point.