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Hydrophobic and electrostatic forces control the retention of membrane peptides and proteins with an immobilised phosphatidic acid column

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Abstract

The retention behaviour of four membrane-associated peptides and proteins with an immobilized phosphatidic acid (PA) stationary phase was evaluated. The solutes included the cytolytic peptides gramicidin A and melittin, the integral membrane protein bacteriorhodpsin and cytochrome c, a peripheral membrane protein. Gramicidin has no nett charge and exhibited normal reversed phase-like behaviour which was largely independent of mobile phase pH. In contrast, melittin, which has a positively charged C-terminal tail, exhibited reversed phase like retention at pH 5.4 and 7.4, and was not retained at pH 3 reflecting the influence of electrostatic interactions with the negatively charged phosphatidic acid ligand. Bacteriorhodpsin was eluted at high acetonitrile concentrations at pH 3 and 5.4 and cytochrome c was only eluted at pH 3. Moreover, cytochrome c eluted in the breakthrough peak between 0 and 100% acetonitrile, demonstrating the role of electrostatic interactions with the PA surface. Overall, the results demonstrate that pH can be used to optimize the fractionation and separation of membrane proteins with immobilized lipid stationary phases.

Keywords: Membrane proteins; Chromatography; Immobilised phospholipid chromatography; Phosphatidic acid; Membrane protein purification

1. Introduction

Membrane proteins are involved in many essential functions as a result of their position between the cell and the surrounding environment. They are involved in signal transduction, energy generation, molecule transport, cell adhesion, intracellular communication, immune recognition and cell structure. As a result of these important biological functions, many membrane proteins are also involved in a number of diseases while also being important for pharmacological action, representing 70% of the known protein drugs targets [1]. Despite their importance, membrane proteins are notoriously difficult to isolate and analyse [2–4] and although the human genome predicts that 30% of all proteins encoded by genes are membrane proteins [5,6], they account for only a small proportion of proteins of known structure [1].

There are many methods available for the isolation of membrane proteins for functional studies through the use of mild detergents that provide solubility without denaturation followed by for example, ion-exchange or size exclusion chromatography [7]. However, the purification and separation of membrane proteins for structural and proteomic analysis remains a major challenge [2–4]. Most of the techniques currently utilized were originally developed for the isolation of soluble proteins and are associated with a number of limitations when applied to membrane proteins [2]. For example, two-dimensional gel electrophoresis is widely used for the analysis of membrane proteins, but is limited by solubility, low resolution and poor reproducibility and may have reached its limits when used specifically in the separation of membrane proteins. Chromatographic separations on the other hand have significant potential due to the ability to develop a wide range of stationary phases designed specifically for membrane proteins. Furthermore, when coupled to mass spectrometry, chromatographic separations can provide the high-throughput method required for the separation and identification of membrane proteins from complex samples for example in proteomic applications. [8–10].

An alternative approach to membrane protein purification has also been developed, namely high performance liquid chromatographic stationary phases containing phospholipids [11]. These immobilised phospholipid monolayers have the potential to act as model biomembrane surfaces to separate membrane proteins due to their resemblance to the naturally occurring phospholipids

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with which membrane proteins interact. To date, immobilized artificial membrane chromatography has been widely used to study partition coefficients between small drugs/molecules and membranes [12–19]. In contrast, only a limited number of individual membrane associated or interacting peptides/proteins such as PLA_2 [20], cytochrome P450 [21], and membrane-active peptides [22–24] have been purified and/or analysed using the immobilized lipid stationary phases. Overall, the potential application of these stationary phases to membrane protein purification has not been widely exploited.

In order to explore the application of different phospholipid materials, we previously synthesised and characterized a series of immobilised phospholipid monolayers including a phosphatidylcholine and phosphatidylglycerol stationary phase which were shown to be a stable and sensitive system for the analysis of peptide-membrane interactions [22]. In the present study, the potential of the immobilized phosphatidic acid (PA) material for the purification of membrane proteins was evaluated by analysis of the retention behavior of peptides and proteins of varying molecular size and membrane interacting properties. The majority of the published studies on membrane protein isolation were performed with an immobilized phosphatidylcholine ligand. The results of this study demonstrate that glycerophospholipid columns have the potential to be used in the separation of membrane proteins. Moreover, the optimisation of different phospholipid surfaces and mobile phases could provide the basis of a specific and selective membrane protein separation tool that can be incorporated into current technologies applied to membrane protein profiling, crystallography, drug design and biomarker discovery.

2. Materials and methods

2.1. Chemicals

Glacial acetic acid and sodium phosphate dibasic (Na_2HPO_4) were purchased from BDH AnalR® (Kilsyth, Vic, Australia). Sodium phosphate monobasic (NaH_2PO_4) was purchased from Amresco (Solon, OH, USA). HPLC grade methanol (MeOH), and acetonitrile (ACN) were purchased from Merck Pty. Ltd. (Kilsyth, Vic, Australia).

2.2. Protein and peptide preparation

Lyophilised melittin from honey bee venom (Auspep, Parkville, Australia) and Gramicidin A from *Bacillus Brevis* (≥90% HPLC) (Sigma–Aldrich, St Louis, MO, USA) was dissolved first in a small volume of methanol, and then milliQ ddH_2O to a final concentration of 1 mg/ml (15% methanol). Lyophilised Bacteriorhodopsin (BR) S9 from the Halobacterium Salerium strain purple membrane (Sigma, St. Louis, MO, USA) and cytochrome *c* from chicken heart (≥95% HPLC) (Sigma–Aldrich, St Louis, MO, USA) was dissolved in milliQ ddH_2O to a final concentration of 1 mg/ml. Samples were aliquoted into 100 μl eppendorf tubes and stored at either −20 °C or 4 °C according to manufactures guidelines until use.

2.3. Chromatographic procedures

All chromatographic measurements were performed on an Agilent Series 1100 Analytical HPLC (Agilent Technologies, Palo Alto, CA, USA) equipped with a quaternary pump to maintain solvent conditions and flow rate, a degasser, an auto-sampler for automatic injections, a thermostated column compartment allowing temperature control and a variable wavelength detector. The column temperature was maintained at 25 °C and the chromatographic separation was monitored at 214 nm. Retention times were measured using ChemStation software (Revision A.10.02) (Agilent Technologies, Palo Alto, CA, USA). Water was quartz-distilled and deionised in a Milli-Q system (Millipore, Bedford, MA, USA). Bulk solvents and mobile phases were filtered through a 0.22 μm nylon membrane filter (Alltech associates, Pty. Ltd., Baulkham Hills, NSW, Australia) by vacuum suction using a Millipore solvent filtration apparatus (Millipore, Bedford, MA, USA).

The immobilisable phosphatidic acid was covalently attached to 3-isothiocyanatopropyltriethoxy (ITCPS) modified ZORBAX Rx-SIL silica particles (5 μm diameter and 300 Å average pore size) as previously described [25]. The silica was dried under reduced pressure for 24 h and stored under anhydrous conditions until use. The phosphatidic acid-modified silica was packed into a stainless steel cartridge with dimensions of 4 cm × 4.6 mm ID and the structure of the monolayer is shown in Fig. 1.

Isocratic elution of melittin, gramicidin, bacteriorhodopsin and cytochrome *c* was performed using three different mobile phases, namely: 0–100% (v/v) acetonitrile in milliQ water, 0–100% (v/v) acetonitrile in milliQ water (0.1% acetic acid) and 0–60% (v/v) acetonitrile with 10 mM Na_2PO_4 buffer at a flow rate of 1 ml/min. The column was equilibrated in each solvent for at least 30 min before sample injection. All injections were carried out in duplicate with retention times typically varying by less than 1 s. The injection sample size was 10 μg for cytochrome *c* and bacteriorhodopsin, 2 μg for melittin and 3 μg for gramicidin.

The retention of solutes on the phosphatidic acid chromatographic surface under isocratic conditions with organic solvent can in the first instance be considered analogous to reversed phase HPLC where hydrophobic interactions are dominant and can be expressed using the isocratic retention factor *k* according to:

$$k = \frac{(t_r - t_0)}{t_0} \quad (1)$$

where t_r is the retention time of the peptide or protein and t_0 corresponds to the dead time of the column [26]. The column dead time (t_0) was calculated as the breakthrough peak of the water blank (a bulk refractive index change) monitored as an absorbance increase at 214 nm. Under regular reversed phase conditions [24,26,27], $\log k$ is inversely linearly dependant on the mole fraction of organic solvent (φ) that is required to elute the solute from the hydrophobic surface according to Eq. (2) as