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Stabilized phospholipid membranes in chromatography: towards membrane protein-functionalized stationary phases

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Abstract

Transmembrane protein (TMP)-functionalized analytical platforms represent a powerful new paradigm in chemical analysis. Of particular interest is the development of high throughput, TMP-functionalized stationary phases for affinity chromatography of complex analyte libraries. A number of natural and synthetic phospholipids and lipid-mimics have been utilized for TMP reconstitution, though the resulting membranes often lack the requisite chemical and temporal stability for long term utilization, a problem that is exacerbated in flowing separation systems. Over the past two decades, polymerizable lipids have been developed that exhibit markedly increased membrane stability, while still supporting TMP function. More recently, these lipids have been incorporated into a range of analytical platforms, including separation-based platforms, and are now poised to make significant impacts in TMP-based separations. Here, we describe the current methods for preparing TMP-containing stationary phases and examine the potential utility of polymerizable lipids in TMP affinity chromatography.

Keywords

Affinity chromatography; Immobilized artificial membrane; Immobilized liposome chromatography; Polymerizable phospholipid; Stationary phase; Transmembrane protein

Introduction

Transmembrane proteins (TMPs) represent important classes of biomolecules that aid in the transmission of signals from the extracellular to intracellular environment. Important TMPs include cellular receptors (G-protein coupled receptors, growth factor receptors, etc.), signaling enzymes, and ion channels. Based on the ubiquitous nature of TMPs in biological function, a large fraction of pharmaceuticals are selective for TMP targets. Thus, methods that allow rapid screening and detailed characterization of physiological and pharmacological TMP-modulators from chemically complex matrices are highly valued.

The most commonly used screening assays for TMP modulators in biological and synthetic matrices rely upon detection of down-stream cellular function in cell-based assays. These methods suffer from irreproducibility caused by variability among heterogeneous cell

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populations, exhibit false positives and false negatives due to non-specific interactions, and are difficult to interpret because of the complexity associated with monitoring a downstream effect of signal transduction [1]. Further, cell-based assays require pre-fractionation or separation of complex mixtures (e.g. combinatorial libraries) prior to cell-based analysis. In contrast, affinity chromatography presents a synthetic platform containing a single target capable of monitoring both competitive and noncompetitive analyte binding while minimizing non-specific interactions [2]. Affinity columns have been prepared with immobilized antibodies, enzymes, and other soluble proteins to provide the needed combination of throughput, specificity, reproducibility, and selectivity to allow qualitative and quantitative analysis of biological ligands and pharmaceuticals [2]. Incorporation of TMPs into affinity columns requires the presence of a phospholipid membrane into which the TMP is reconstituted. A number of innovative approaches have been employed to integrate the phospholipid membrane into the separation platform, including fractionated cell membranes, synthetic lipid vesicles, and immobilized artificial membranes (Figure 1). It is our belief that the next step in the genesis of TMP-functionalized affinity columns lies in the use of highly stable, polymerized phospholipid membranes that have shown strong promise in the preparation of TMP-functionalized sensor platforms and have more recently been incorporated into separation platforms.

Phospholipid-derived stationary phases for affinity chromatography

From a historical perspective, immobilized liposome chromatography (ILC) represents the first, and still most utilized approach, to TMP-functionalized separation matrices (Figure 1). In ILC, liposomes prepared from natural or synthetic lipids are integrated into stationary phase supports including agarose or acrylamide gels [3,4], and silica-based particles and monoliths [5,6] by steric, hydrophobic, covalent, or avidin-biotin interactions [3,6]. ILC is primarily used to study small molecule partitioning through lipid membranes [7], and interactions between peptides and phospholipids [8]. Two-dimensional chromatography with ILC phases in the first dimension provides a novel platform for analysis of complex natural and synthetic mixtures [9].

A particularly powerful application of ILC is the analysis of ligand binding to TMP-functionalized liposomes. TMP-ILC phases are prepared in one of two ways: a) fractionation of cell membranes into liposomes and b) preparation of synthetic liposomes followed by reconstitution of purified TMPs. In the former, natural lipid compositions are retained, a key functional requirement for many TMPs [10], though at the expense of increased nonspecific interactions. In the latter, highly homogenous backgrounds are obtained, provided sufficient protein can be purified and reconstituted.

Lundahl et. al. were the first to utilize TMP-containing stationary phases for affinity chromatography [11]. The type 1 glucose transporter (GLUT1) from red blood cells was reconstituted to yield proteoliposomes that were immobilized through steric interactions inside support matrix gel-bead pores [11]. The resulting affinity stationary phases were used to study D-glucose transport across lipid bilayers. In the presence of L-glucose or other channel modifiers (i.e. cytochalasin B and forskolin), GLUT1-functionalized proteoliposome phases demonstrated stereo-selective glucose binding. The primary limitation of this important method was the limited temporal stability of the immobilized proteoliposomes, with the phospholipid and protein loss dependent on the phospholipid mixture used to prepare the liposomes. In the least stable column, 75 % of the protein was lost within 10 days [11]. This early work in TMP-functionalized stationary phases illustrated that the mobile phase (pH, ionic strength, presence of organic solvents or detergents), separation parameters (temperature, flow rate, etc.), and interactions between small molecules and phospholipids affect the liposome, and thus stationary phase, stability and reproducibility [11,3]. Since this pioneering work, ILCs have been prepared with a range of

important receptors including those for endothelins, acetylcholine, cannabinoids, and many others [4,12,13]. Thus, while ILC presents a powerful approach, the limited stability precludes the use of this approach in affinity separations where high flow rates are desired.

Immobilized artificial membranes (IAMs) were developed as a more stable and more reproducible alternative to ILC stationary phases (Figure 1). IAMs are prepared via covalent attachment of a lipid monolayer to an amine-modified silica particle through an amide bond at the terminus of the lipid tail [14]. Due to the covalent bonds, IAM stationary phases are highly stable with little phospholipid loss during separations or storage [3]. IAMs have lipid densities similar to biomembranes [3], allowing analytes to interact with the hydrophilic head groups via ion-pairing and hydrogen bonding, or through hydrophobic interactions with the lipid tails. Based on these properties, IAMs are routinely used in drug analysis and characterization [14].

Importantly, multiple TMPs, including nicotinic acetylcholine receptors, opioid receptors, P-glycoprotein transporters, and others have been immobilized in IAM stationary phases, most commonly, via fusion of solubilized membranes obtained from transfected cellular models where the protein of interest was overexpressed [2,15,16,17]. The result is either direct incorporation of the TMP into the IAM monolayer or deposition of a bilayer onto the IAM. Studies with IAM-TMP columns have shown that relative drug binding affinities can be determined for overexpressed TMPs [15,16]. However, direct inclusion into the lipid monolayer may limit incorporation of TMPs that require both leaflets of the bilayer to support the native protein conformation [18] and TMPs that do incorporate into the monolayer may exhibit altered binding affinity compared to native phospholipid bilayers [16,18]. Furthermore, the non-covalent interactions between cellular membrane fractions and the underlying IAM monolayer lead to decreased stability [3].

The success of ILC and IAM stationary phases clearly demonstrate the utility and importance of TMP-functionalized affinity stationary phases and provide the foundations upon which next generation TMP-functionalized affinity phases will be designed. Specifically, further advancements in this area require development of chromatographically-compatible membranes with greater stability that more closely resemble natural phospholipid bilayers to support the structure and function of an extended range of TMPs [3,16]. Several key advancements in membrane engineering are likely to play roles in achieving these goals, including the development of polymerizable phospholipid membranes.

Polymerized lipid membranes

Fluid phospholipid membranes composed of natural lipids are by nature dynamic and inherently unstable structures, posing a significant obstacle to their utilization in bioanalytical and biotechnological applications. Specifically, fluid phospholipid membranes lack the desired chemical, thermal, and mechanical stability to serve as long-term biomimetic coatings on stationary phase materials. This problem is further exacerbated in high pressure systems, such as chromatography, where shear forces are sufficient to remove the lipid coatings. Moreover, fluid phospholipid membranes are readily damaged by brief exposures to common chemical and physical insults that may be encountered in chemical separations, including air bubbles, exposure to organic solvents, and surfactants.

To overcome these limitations, stabilized phospholipid membranes have been developed. Stabilized membranes provide an analog to ILC membranes that, once fully developed, will play a key role in TMP-functionalized affinity matrices. A variety of strategies have been employed to increase the stability of phospholipid membranes (Figure 2), including: a) doping of biological components to the membrane, e.g. cholesterol; b) adsorption of a

protective overlayer; c) polymer templating; d) direct polymerization of phospholipid monomers; and e) membrane tethering. Of these approaches, direct polymerization yields the most stable membranes, though often at the expense of membrane fluidity, whereas polymer templating facilitates direct utilization of cell membrane fractions, analogous to cell-derived ILC phases.

Polymerization of lipid membranes significantly stabilizes the membrane architecture via direct covalent coupling between adjacent lipid molecules. Native phospholipids lack the requisite reactive moieties to yield polymeric membranes, thus synthetic alternatives are required. A relatively simple approach is the partitioning of reactive monomers into the hydrophobic region within a liposome bilayer [19,20]. Upon suitable initiation, a polymer scaffold forms within the bilayer, markedly stabilizing the liposome. Importantly, this approach is relatively universal in that it can be applied to any bilayer membrane of any lipid composition.

A more specialized approach is the utilization of synthetic lipids produced with reactive monomer functionalities. The stability of the lipid polymer is dependent on the identity of the polymerizable moieties, polymerization efficiency, density of polymerization sites, and initiation approach. Polymerizable moieties can be incorporated in the hydrophilic or hydrophobic region of the lipid [21]. Lipids with polymerizable headgroups have been used to tether phospholipids to solid supports; however, polymerizable headgroups may alter biological binding events. Phospholipids with naturally occurring headgroups can be functionalized in the hydrocarbon tails at the chain terminus, mid-chain, or near the glycerol backbone [21]. Examples of common reactive moieties integrated into phospholipid tails are shown in Figure 3 [21].

The number of polymerizable groups in the phospholipid dictates the elastomeric properties and net stability of the resulting polymer. Functionalization of one reactive moiety per lipid molecule yields linear polymers, often of lower chain length. Conversely, functionalization of two or more reactive moieties, typically one in each fatty acid tail, yields high chain-length, cross-linked polymers that are characterized by their insolubility in surfactants or organic solvents, illustrating the enhanced stability of these polymerized structures [21]. The resulting enhancement in chemical, mechanical, and environmental stability suggests that cross-linked phospholipid membranes might provide a key advancement for fabrication of highly stable ILC phases.

Both polymer templating and reactive lipids generate polymerized liposomes, whereas reactive lipids are most commonly used to prepare polymerized black lipid membranes, and planar supported lipid bilayers (PSLBs) that demonstrate enhanced stability while retaining TMP function [22,23].

Of the reactive moieties, sorbyl and diacetylene lipids are most commonly used to prepare polymerized PSLBs and liposomes. For sorbyl lipids, high polymerization efficiency results when polymerization occurs in the liquid phase where the hydrocarbon chains are tightly packed, maintaining the lipids in close proximity, yet allowing the lipids to diffuse through the bilayer to react with the growing polymer chain. In comparison, diacetylene moieties require polymerization in the crystalline state where the lipid tails form rigid, ordered structures with low packing efficiencies [21].

When polymerized under the appropriate conditions, both diacetylene and sorbyl moieties result in highly stabilized lipid polymers. For example, polymerized diacetylene lipids were used in non-aqueous capillary electrophoresis to prepare highly stabilized-surface coatings by fusing small unilamellar vesicles (SUVs) of DiynePC (1,2-bis(10,12-tricosadiynoyl)-*sn*-

glycero-3-phosphocholine) (Figure 3) with bare silica capillaries, resulting in vesicle fusion, and formation of PSLBs. After flushing capillaries with 80 or 250 column volumes of methanol or acetonitrile, respectively, the unpolymerized lipid coatings were removed from the capillary surface. However, once polymerized, the lipids showed significantly greater stability, with the bilayer coatings remaining stable for over 1500 or 600 capillary volumes for methanol or acetonitrile, respectively. Additionally, the polymerized DiynePC coating was stable after 4 months of dried storage [24].

We have extended the utilization of polymerized lipid membranes to the preparation of open tubular capillary coatings using bis-SorbPC (1,2-bis[10-(2',4'-hexadienoxy)decanoyl]-*sn*-glycero-3-phosphocholine) (Figure 3). Polymerized bis-SorbPC coatings were stable to surfactant solutions, shear forces associated with flushing the capillary, pH extremes, and applied electric fields [25,26]. The resulting membranes were stable in excess of one year and retained a number of key phospholipid membrane properties, including resistance to non-specific protein adsorption and surface fouling. More recently, we prepared polymerized supported lipid bilayers on silica nanoparticles and microparticles with structures and activities similar to planar analogs [27]. Further, the polymeric membrane-microparticles were used to prepare packed columns that retained the lipid membrane in excess of 2000 PSI back pressure (unpublished data), suggesting the utility of bilayer coated particles for chromatographic matrices, particularly as polymerized lipids become more integrated into the chromatographic community.

Outlook

The long-term development of polymer-stabilized, TMP-functionalized affinity phases requires that membranes of sufficient fluidity and stability be generated that support native TMP conformation and function. While no TMPs have been incorporated into polymerized lipid stationary phases to date, a number of studies in the analogous polymerized black lipid membrane and PSLB geometries suggest the feasibility of this approach.

Though polymerization increases bilayer stability, it decreases membrane fluidity. Bilayer fluidity is a critical parameter for TMP structure and function [28,22]; thus, formation of TMP-functionalized stationary phases requires that some degree of fluidity be retained, a key challenge with highly stable polymeric membranes. Diffusion coefficients in polymerized membranes are decreased by 10 – 20 % after UV-initiated polymerization of bilayers composed of 100 % bis-SorbPC or by a factor of 6 in UV-polymerized bilayers formed from a 1:1 mixture of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine and a cross-linking diacetylene lipid [29]. Preparation of binary membranes provides an effective alternative that facilitates tuning of membrane fluidity, though at the expense of membrane stability. Binary membranes comprised of mixtures of either bis- and mono-functionalized lipids or polymerizable and unpolymerizable lipids yield varying degrees of fluidity [29]. These results support the feasibility of engineered polymer membranes that support functional TMP reconstitution for affinity separation matrices.

Both diacetylene and sorbyl lipids have been used to prepare stabilized platforms that retain TMP activity. Though functional receptors have not been reconstituted into pure diacetylene lipid membranes, patterned membranes consisting of regions of polymerized DiynePC and fluid lipid membranes have been demonstrated [30]. Similar results were obtained with bis-SorbPC after photoinitiated polymerization in fused silica capillaries [26,31].

To better demonstrate the impact of fluidity, gramicidin was reconstituted into polymerized bilayers formed from PTPE (1-palmitoyl-2-(10,12-tricosadiynoyl)-*sn*-glycero-3-phosphoethanolamine) in combination with an unknown mole fraction of unpolymerizable lipids (1,2-diphytanoyl-*sn*-glycero-3-phosphocholine) [23]. Though the stability of the

bilayer increased after polymerization, the conductance of the channels showed that the protein monomers were able to diffuse through the bilayer to form functional pores; however, the fluidity is likely associated with the presence of unpolymerized lipids and the formation of linear polymers via the mono-functionalized lipid.

Rhodopsin, a GPCR, was incorporated into PSLBs of polymerized bis-SorbPC (Figure 4) [22]. Activation of rhodopsin was studied using planar waveguide resonance to confirm activity of the protein following membrane polymerization. Yellow light activation of rhodopsin resulted in an orthogonal stretch of the TMP, which the polymerized bis-SorbPC lipids were able to accommodate. These examples illustrate that polymerized membranes can be generated that support TMP function yet retain enhanced stability, paving the way for TMP-functionalized affinity phases.

In summary, TMP-functionalized affinity phases will continue to play a key role in drug discovery and screening applications, and may eventually move into diagnostic applications as the technique further matures and more stable TMP-functionalized materials become available. Recent advances in lipid membrane engineering, particularly the utilization of highly stable, polymeric lipid membranes present significant opportunities for the continued development of TMP-functionalized affinity phases. The capability to modulate the stability and fluidity of the polymeric membrane, coupled with capacity to retain TMP function within the polymeric membrane, will lay the foundation for the next generation of reusable, homogenous and highly tunable ILC- and IAM-like materials that present a wide range of enhanced properties. These novel stationary phases will facilitate screening of combinatorial libraries, verification of pharmaceutical targets, biophysical analyses of novel ligands, including the identification of important structural features and binding parameters, and elucidation of biological pathways and novel binding interactions.

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Abbreviations

bis-SorbPC	1,2-bis[10-(2',4'-hexadienoyloxy)decanoyl]- <i>sn</i> -glycero-3-phosphocholine
DiynePC	1,2-bis(10,12-tricosadiynoyl)- <i>sn</i> -glycero-3-phosphocholine
GLUT1	Type 1 glucose transporter
GPCR	G-protein coupled receptor
IAM	Immobilized artificial membrane
ILC	Immobilized liposome chromatography
PSLB	Planar supported lipid bilayer
PTPE	1-palmitoyl-2-(10,12-tricosadiynoyl)- <i>sn</i> -glycero-3-phosphoethanolamine
SUV	Small unilamellar vesicle
TMP	Transmembrane protein
UV	Ultraviolet

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Fig. 1.

Schematic of some common lipid-functionalized separation media. a) Immobilized liposome chromatography often involves the steric encapsulation of liposomes in a biocompatible polymer matrix. b) Immobilized artificial membranes are prepared via covalent linkage of a lipid monolayer onto an underlying silica or polymer support. c) Polymeric planar supported lipid bilayers are prepared via vesicle fusion of small unilamellar vesicles onto silica supports followed by polymerization. Note: schematics not to scale

Fig. 2.

Schematic representation of common bilayer stabilization strategies: a) Incorporation of cholesterol (purple) or other biological scaffold materials; b) Formation of protective overlayers to sandwich the bilayer between hydrophilic polymers or polyelectrolyte layers; c) Polymer templating via the formation of cross-linked polymer networks from monomers partitioned into the lamellar region; d) Cross-linking polymerization of synthetic reactive lipids; and e) Bilayer tethering via inclusion of reactive lipids.

Fig. 3. Polymerizable lipid structures. a) Common polymerizable functional groups that are incorporated into phospholipid tails to increase lipid bilayer stability. b) Structures of sorbyl and diacetylenyl lipids used for preparing stabilized structures that maintain TMP activity.

Fig. 4.

Polymerized PSLBs of bis-SorbPC have the fluidity necessary to maintain the activity of TMPs. bis-SorbPC contains a polymerizable moiety in each lipid tail, which results in chemical crosslinking of the lipid network and results in a highly stabilized phospholipid bilayer. The activity of rhodopsin, a TMP, was monitored by plasmon waveguide resonance after incorporating the TMP into PSLBs of bis-SorbPC. s-Polarized plasmon waveguide resonance spectra collected for 10 mM phosphate buffer, pH 5.5 (1), after formation of a bis-SorbPC PSLB (2), after incorporation of rhodopsin (3), after photoinitiated polymerization with UV light (4), and after activation of rhodopsin by exposure to yellow light (5). Reprinted with permission from: Subramaniam V, Alves ID, Salgado GFJ, Lau P-W, Wysocki RJ, Jr., Salamon Z, Tollin G, Hruby VJ, Brown MF, Saavedra SS (2004) *J Am Chem Soc* 127:5320-5321 [22]. Copyright 2005 American Chemical Society.