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Immobilized lipodisks as model membranes in high throughput HPLC-MS analysis

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Abstract

Lipodisks, also referred to as polyethylene glycol (PEG)-stabilized bilayer disks, have previously been demonstrated to hold great potential as model membranes in drug partition studies. In this study, an HPLC-MS system with stably immobilized lipodisks is presented. Functionalized lipodisks were immobilized on two different HPLC support materials either covalently by reductive amination or by streptavidin-biotin binding. An analytical HPLC column with immobilized lipodisks was evaluated by analysis of mixtures containing 15 different drug compounds. The efficiency, reproducibility and stability of the system were found to be excellent. *In situ* incorporation of cyclooxygenase-1 (COX-1) in immobilized lipodisks on a column was also achieved. Specific binding of COX-1 to the immobilized lipodisks was validated by interaction studies with QCM-D. These results taken together open up for the potential to study ligand interactions with membrane proteins by weak affinity chromatography (WAC).

Keywords

lipodisks, COX-1, HPLC-MS, model membrane, drug partition studies, membrane protein, WAC, weak affinity chromatography

1. Introduction

Lipid-based model membranes, that accurately reflect the structure and properties of biological membranes, constitute essential tools for studies in various biological, analytical and pharmaceutical areas. Model membranes, such as liposomes and immobilized lipid mono- and bilayers, are for instance frequently employed in studies designed to investigate the interaction between drugs or biomolecules and biological membranes (1–3). Bio-similar model membranes are furthermore needed for structure/function studies of membrane proteins. This class of proteins is of great interest not least since membrane proteins represent 60% of current drug targets (4). However, not many methods are available to study membrane proteins due to the significant difficulties associated with their poor aqueous solubility. In addition, many membrane proteins require their natural lipid environment in order to maintain full activity (5).

Among the wide variety of different techniques used for investigations of membrane- and membrane protein interactions, chromatographic methods hold a unique position for fast and reproducible screening of large numbers of analytes. Stable immobilization of suitable model membranes to inert and robust chromatographic media has thus the potential to open up for efficient high-throughput analysis of drug- or biomolecular interactions with membranes. Providing that membrane proteins can be reconstituted into, or adsorbed onto, the immobilized model membrane, systems enabling protein interaction studies by means of techniques such as weak affinity chromatography (WAC) (6) can furthermore be envisioned. WAC, which is emerging as a promising method for the study of weak interactions ($K_D = \text{mM} - \mu\text{M}$), has previously been successfully used for the study of protein interactions with carbohydrates (7,8), fragment screening (9–11) and for chiral separation (12). The possibility to perform WAC-based investigations involving membrane proteins embedded in their natural lipid milieu would considerably increase the potential of the technique.

Liposomes constitute one of the most frequently used model membranes, and have been used in combination with a number of chromatographic techniques for the purpose of membrane interaction studies. Several studies on drug partition behavior have for example employed liposomes passively immobilized to chromatographic gel filtration media (2,3). More recently, chromatographic systems based on covalent coupling of liposomes to silica gels has also been reported (13–15). Although liposomes certainly have proven useful as model membranes in numerous studies, their use in interaction studies is associated with some potential complications. First, since liposomes are closed bilayer structures comprising an inner aqueous core, only the lipids in the outer bilayer leaflet stand in direct contact with the surrounding bulk media. This, together with the fact that liposome preparations normally contain a fraction of bi- and multilamellar structures, means that a substantial, and typically unknown, fraction of the lipids initially are shielded from interaction with analytes dissolved in the bulk media. The presence of an effectively hidden lipid fraction may slow down or hamper analyte equilibration and thereby prevent reliable and reproducible collection of interaction data. Another drawback is that when reconstituting membrane proteins into liposomes, a fraction of the protein as a rule incorporates with the active site facing towards the liposome interior, thus being inaccessible for interaction with potential ligands in the bulk solution. Finally, conventional liposomes have a rather limited shelf life and, over time, tend to aggregate and fuse into larger, less well- defined structures.

PEG-stabilized bilayer disks, henceforth referred to as lipodisks, have emerged as an interesting alternative to liposomes for the use as model membranes in interaction studies (16–20). The lipodisks are flat circular lipid aggregates consisting of a lipid bilayer surrounded by a highly curved rim (see schematic picture in Figure 1). Lipodisks are obtained by mixing

lipids that spontaneously form bilayers with lipids that have a large polyethylene glycol (PEG) chain covalently attached to their head group. The latter, so called PEG-lipids, are micelle-forming compounds and therefore prefer aggregates of high positive curvature. The components in the lipodisks partially segregate and the PEGylated lipids are predominantly found at the disk rim (21). The size of the disks can be varied by alteration of the PEG-lipid content (17,22). Furthermore, the lipid composition can be adjusted to closely mimic that of biological cell membranes (16,18). Similar to the case with liposomes, membrane spanning, as well as peripheral, membrane proteins can be incorporated into the lipodisks (16,23). However, in contrast to the case with liposomes, the open structure of the disks ensures that both lipid bilayer leaflets are readily available for interaction with analytes present in the bulk aqueous phase. Further, the heavy PEGylation of the disks protects them against fusion and ensures excellent long term stability of the lipodisk preparations (17). Taken together the lipodisks possess properties that make them highly interesting for use as model membranes in interaction studies.

Lipodisks have in previous studies (16,17) been successfully used to collect drug partition data by means of chromatographic techniques. The disks were in these cases passively immobilized to chromatographic gel filtration media in a way similar to that suggested and used by Lundahl et al. for immobilization of liposomes (24,25). As indicated before, stable immobilization of the disks to a more robust chromatographic media would considerably increase the applicability of lipodisk-based systems for interaction studies. Previous studies have shown that lipodisks can be stably linked to different surfaces by incorporation of PEGylated lipids carrying functional groups at the end of the polymer chain. Lipodisks functionalized with biotin have thus been successfully immobilized to streptavidin-covered sensor surfaces and employed in studies based on the surface plasmon resonance (23) and quartz crystal micro balance (QCM) (20) techniques. The possibility of providing the lipodisks with functionalized PEG-lipids could potentially be used to link these promising model membranes to a suitable HPLC support medium.

The main objective of the present work was to develop a robust HPLC-MS system with stably immobilized lipodisks that enables high throughput analysis of drug partition. Another objective was to immobilize lipodisks carrying the membrane protein cyclooxygenase-1 (COX-1) to the HPLC support in order to take the first steps towards future applications of the lipodisk-HPLC-MS system for studies of protein-ligand interactions by means of the WAC technique.

2. Materials and Methods

2.1 Chemicals

Sephadex G-50 was purchased from GE Healthcare Lifescience (Uppsala, Sweden). Dry powder of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocoline (POPC), soy L- α -phosphatidylethanolamine (Soy PE), N-palmitoyl-sphingosine-1-{succinyl[methoxy(polyethylene glycol)2000]} (Ceramide-PEG₂₀₀₀), 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[biotinyl(polyethylene glycol)-2000] (DSPE-PEG₂₀₀₀biotin) and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG₂₀₀₀amine) were purchased from Avanti Polar Lipids (Alabaster, USA). Ovine cyclooxygenase I (COX-1) was purchased from Cayman Europe (Tallinn, Estonia). Cholesterol, octyl β -D-glucopyranoside (OG), alprenolol, pindolol, lidocaine, promethazine, propranolol, theophylline, diclofenac, ibuprofen, indomethacin, naproxen, warfarin, cortisone, hydrocortisone, prednisolone, corticosterone, periodic acid, sodium cyanoborohydride, N-hydroxysuccinimide (NHS), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC), sodium sulfite, sodium metabisulfite, ammonium molybdate, 4-amino-3-hydroxy-1-naphthalenesulfonic acid, dimethyl sulfoxide (DMSO), sodium dodecyl sulphate (SDS), diethyldithiocarbamate (DDC) and streptavidin were purchased from Sigma Aldrich Chemical (Steinheim, Germany). The analytes for HPLC were dissolved in ethanol (Kemetyl, Haninge, Sweden) at 5 mM and were further diluted in water to working concentrations.

2.2 Preparation and Characterization of Lipodisks

2.2.1 Preparation of lipodisks

The lipodisks used in this study were composed of POPC, Soy PE, cholesterol, Ceramide-PEG₂₀₀₀ and DSPE-PEG₂₀₀₀biotin or DSPE-PEG₂₀₀₀amin in the molar ratio 30:28:17:21:4. The lipids were dissolved in chloroform and thereafter dried under a gentle stream of nitrogen gas. Remaining chloroform was then removed under vacuum overnight. The lipid film was dissolved in an OG-solution and the solution was allowed to equilibrate for at least 4 h with intermittent vortex mixing. The lipid to detergent molar ratio was 1:10 (21.5 mM extra OG, i.e. corresponding to the OG critical micelle concentration (CMC) (26) was added to the sample). The solution was run on a Sephadex G-50 column (35 x 1.9 cm). A Gilson Pump Minipuls 2 (Gilson International, Den Haag, Netherlands), was used to control the flow rate to approximately 0.7 mL/min. As mobile phase, 0.1 M sodium phosphate, 0.15 M sodium chloride buffer, pH 7.0 (coupling buffer), was used. The lipodisks and the detergent were eluted from the column as two well separated fractions. A Dual Path Monitor UV-2 (Pharmacia Fine Chemicals, Uppsala, Sweden) was used to detect the fractions. The lipodisk fraction was collected manually and concentrated on a Miniprep concentrator (Millipore, Billerica, MA, USA) and stored at 4 °C until further use.

2.2.2 Cryo-Transmission Electron Microscopy

The lipodisks were characterized using cryogenic transmission electron microscopy (cryo-TEM) using a Zeiss EM 902A Transmission Electron Microscope (Carl Zeiss NTS, Oberkochen, Germany). Observations were made in zero loss bright-field mode at an accelerating voltage of 80 kV. Digital images were recorded under the low dose conditions with a BioVision Pro-SM Slow scan CCD camera (Proscan, Münster, Germany). An underfocus of 1–2 μ m was used to enhance the image contrast. The sample preparations were performed in a custom-built climate chamber at 25 °C and >99% relative humidity. First a small drop (~ 1 μ L) of the lipodisk solution was deposited on

a copper grid covered with a carbon reinforced holey polymer film. Thin sample films were prepared by blotting the grid with a filter paper. The liquid film was vitrified by immediately plunging it into liquid ethane kept just above its freezing point. Samples were kept below -165 °C and protected against atmospheric pressure throughout the analysis. The technique has been described in more detail by Almgren et al. (27).

2.2.3 Dynamic Light Scattering

The aggregate size in the lipodisk preparations was assessed using dynamic light scattering (DLS). The experimental setup consisted of a Uniphase He-Ne laser (Milpitas, CA) emitting vertically polarized light with a wavelength of 632.8 nm operating at 25 mW. The scattered light was detected at 90° scattering angle using a Perkin Elmer (Quebec Canada) diode detector connected to an ALV-5000 multiple digital autocorrelator (ALV-laser; Vertriebsgesellschaft, Germany).

2.3 Immobilization of lipodisks on HPLC support materials

Two different HPLC support materials (Nucleosil silica; 10 µm in diameter, 1000 Å pore size; Macherey-Nagel, Düren, Germany) which had been silanized into diol-substituted silica according to standard procedures (7) (25 mg samples) and POROS® AL Self Pack® media with aldehyde functionality (20 µm in diameter, 500 – 10 000 Å pore size; Applied Biosystems, Carlsbad, USA; 14 mg samples) were suspended in MilliQ water and ultrasonicated for a few minutes. The diol silica was oxidized into aldehyde silica by 0.5 mL 0.1 g/mL periodic acid at ambient temperature for 2 h. Both materials were washed with coupling buffer (5 mL) and immobilization was carried out identically on the two different materials.

For immobilization of lipodisks of biotin functionality (DSPE-PEG₂₀₀₀biotin), 1.25 mg streptavidin dissolved in 1.25 mL coupling buffer was added to the material samples and sodium cyanoborohydride was added to a final concentration of 9 mg/mL. The samples were incubated at ambient temperature for 20 h and washed with coupling buffer. The eluates from washing of the samples were collected and the amount of immobilized streptavidin was determined indirectly from the protein concentration of the eluates and of the applied sample, as measured by absorbance readings at 280 nm. Lipodisks with biotin functionality (345 µL, 60 mM lipid) were mixed with the HPLC support materials with immobilized streptavidin.

Lipodisks with amine functionality (DSPE-PEG₂₀₀₀amin; 106 mM lipid) were mixed with samples of Nucleosil aldehyde silica (215 µL lipodisk solution in each sample) and POROS material (170 µL lipodisk solution in each sample). Sodium cyanoborohydride was added to the samples to a final concentration of 9 mg/mL. Samples of HPLC media and lipodisks were incubated for 67 h at ambient temperature and washed with MilliQ water (5 mL) to remove all phosphate from the coupling buffer. Immobilized lipodisks were dissolved from the materials by incubation in 1 mL 121.5 mM octylglycosid for 18 h. The dissolved lipids were quantified by phosphorous analysis as described by Bartlett et al. (28).

2.4 Analysis by HPLC-MS

2.4.1 Preparation of Nucleosil silica with immobilized lipodisks

Two different batches of Nucleosil silica were oxidized into aldehyde silica as described above. The first batch, which contained a total of 990 mg silica, was used to quantify passive and active immobilization of lipodisks to the silica material. To check for passive immobilization, i.e., possible immobilization caused by non-specific interactions between the lipodisks and the silica surface, 30.5 mg silica was mixed with lipodisks (72.5 µL, 63 mM

lipid) and left to incubate without addition of cyanoborohydride. The remaining silica from the first batch was used for active immobilization. This silica was mixed with amine functionalized lipodisks (1800 μ L, 63 mM lipid) and sodium cyanoborohydride was thereafter added to a final concentration of 9 mg/mL. Both samples were incubated for 68 h at ambient temperature. The silica was washed with coupling buffer and 15 mg samples were taken out from both passive and active immobilization for phosphorous analysis. The second silica batch contained 664 mg Nucleosil silica and was used for active immobilization alone. The silica was mixed with functionalized lipodisks (1500 μ L, 50 mM) and sodium cyanoborohydride (final concentration of 9 mg/mL) and incubated for 70 h at ambient temperature. The silica was washed with coupling buffer and a sample of 20 mg silica was taken out for phosphorous analysis.

The samples for phosphorous analysis were washed with MilliQ water and incubated in 1 mL 173 mM SDS for 16 h at ambient temperature. Phosphorous analysis by means of the method described by Bartlett et al (28) was used to quantify the dissolved lipids.

2.4.2 Preparation of aldehyde Nucleosil silica

Aldehyde silica was prepared to pack a reference column. Nucleosil diol silica was suspended in MilliQ water and ultrasonicated for 8 min. The diol silica was oxidized into aldehyde silica by incubation in 1.25 ml 0.1 g/mL periodic acid at ambient temperature for 2 h and washed with MilliQ water.

2.4.3 Packing of columns

The silica material obtained by active immobilization of functionalized lipodisks to Nucleosil silica from the first batch (see section 2.4.1) was used to pack two 35 x 2.1 mm stainless steel columns (column 1 and 2) and the aldehyde silica was used to pack one 35 x 2.1 mm stainless steel column (column 3). In addition, a 50 x 3.2 mm stainless steel column (column 4) was packed with the silica material obtained by active immobilization of functionalized lipodisks to the second Nucleosil silica batch (see section 2.4.1). Packing was performed using an air-driven liquid pump (Haskel, Burbank, USA) at 300 bar for 15 min. PBS pH 7.4 (0.01 M sodium phosphate, 0.15 M sodium chloride) (lipodisk columns) and MilliQ water (aldehyde silica column) were used as mobile phase during packing. The columns were stored in PBS pH 7.4 or ammonium acetate buffer (20 mM) pH 6.8 -7.0 at 4°C between analyses.

2.4.4 Analysis of drug compounds on a lipodisk HPLC column

Screening was performed on an Agilent 1200 series capillary HPLC system equipped with a diode-array detector (DAD) and a single quadrupole mass spectrometry detector (MSD; Agilent Technologies, Santa Clara, USA). On MS detection, analytes were ionized by electrospray at atmospheric pressure (API-ES) in positive mode. Drying nitrogen gas flow was 12 L/min at 350°C. The nebulizer pressure was 50 psig and the capillary voltage was 3000 V. MS signal acquisition was set at selected ion monitoring (SIM) mode on sample target masses. The $[M+1]^+$ ion was monitored for each analyte. The fragmentor was set to 100 V. On UV detection, analytes were detected at a wavelength of 214 $\pm$ 4 or 254 $\pm$ 4 nm with a reference wavelength of 360 $\pm$ 50 nm. Retention times were based on peak apexes of the extracted ion chromatogram (EIC). Chromatograms were analyzed with the Agilent ChemStation version B.04.01 chromatography data system.

15 analytes of various charges were analyzed in triplicates on one of the lipodisk columns (column 1) and the reference column packed with aldehyde silica (column 3). Screening was performed with an injection volume of 1 μ L and a flow rate of 0.2 mL/min. The column temperature was 22°C. Analysis was performed isocratically on both columns using two different mobile phases - PBS pH 7.4 or ammonium acetate buffer (20 mM) pH 6.8 – 7.0.

During analysis using PBS pH 7.4 as mobile phase, detection was only performed using UV detection and the MSD was bypassed to avoid contamination of the MSD by non-volatile salts. The analyte concentration was 10 μ M (0.2% ethanol). The analytes were analysed as single injections in both mobile phases. During analysis in ammonium acetate buffer, the analytes were also studied in mixtures in sets of 6 or 7 analytes in each mixture (1.2 or 1.4% ethanol). The retention time of ibuprofen was determined from a single injection at a sample concentration of 0.1 mM (2% ethanol) in order to facilitate detection during analysis on the lipodisk column with ammonium acetate as mobile phase and during analysis on the aldehyde column using PBS as mobile phase. The columns were stored at 4°C in either of the mobile phases between analyses. All organic solvents were avoided at all times to prevent dissolution of the lipodisks.

The void time of the column with immobilized lipodisks was determined from the retention time of an injection of water, as detected by a negative peak by the DAD at 200 $\pm$ 2 nm. Since the MSD is located after the DAD, the void time to the MSD is slightly longer. The difference in void times between the detectors was determined by single injections of theophylline. The void time of the DAD and the difference in retention times of theophylline between the two detectors were used to determine the void time of the MSD. For the reference column, the void time was determined from the retention time of 0.05% DMSO (214 nm).

2.4.5 Data Analysis

The drug partitioning was evaluated from the retention time on the lipodisk column. The normalized capacity factor K_s (M^{-1}) was calculated for each analyte according to (29):

$$K_s = \frac{(t'_{R, \text{lipodisk}} - t'_{R, \text{reference}}) \cdot F}{A} \quad (1)$$

where $t'_{R, \text{lipodisk}}$ is the adjusted retention time on the lipodisk column, $t'_{R, \text{reference}}$ the adjusted retention time on the reference column, F the flow rate during analysis and A the amount of lipids (mol) on the column. The adjusted retention times were calculated by subtraction of the void time from the retention times of the analytes.

2.4.6. Production of a COX-1 column by *in situ* incorporation

COX-1 was incorporated *in situ* into immobilized lipodisks of one of the lipodisk columns (column 2). Similar conditions were used as during incorporation of COX-1 into liposomes according to MirAfzali et al. (30). The column was equilibrated with mobile phase (80 mM Tris-HCl buffer pH 8.0). COX-1 (94 μ g/ml) dissolved in 80 mM Tris-HCl buffer pH 8.0, 0.019% Tween 20, 300 μ M DDC was incorporated into the immobilized lipodisks on the column by 14 x 100 μ L injections with a flow rate of 0.1 mL/min. DDC acts as a reductive agent and a conservative for the protein. The flow rate was stopped for 2.25 min between each injection. The column temperature was 37°C and 1 h was required to incorporate all protein. The column was rinsed with mobile phase.

The material of the lipodisk column and the COX-1 column was taken out and washed with MilliQ water. The amount of immobilized lipids on the columns was determined by phosphorous analysis. The amount of incorporated protein on the COX-1 column was determined by amino acid analysis.

2.4.7. Stability and reproducibility studies on column 4

HPLC analysis of the 15 drug compounds were performed on column 4 with immobilized lipodisks using PBS pH 7.4 as mobile phase and detection was carried out by UV detection. Analytes were analysed in triplicates. The injection volume was 2 μ L. In all other respects, HPLC analysis on column was performed as described above. Column 4 was then stored in PBS pH 7.4 at 4°C for 82 days. Three analytes – diclofenac, propranolol and indomethacin – were analysed again on the column for evaluation of the columns' long-term stability.

2.5 QCM-D analysis

The binding behavior of COX-1 to the lipodisks was followed using a Quartz Crystal Microbalance with Dissipation monitoring (QCM-D). A QCM-D D300 (Q-Sense, Gothenburg, Sweden) instrument thermostated at 21°C was employed for all measurements.

A QCM-D gold sensor was cleaned with hot piranha solution (3:1 sulfuric acid:hydrogen peroxide), rinsed with MilliQ water and absolute ethanol, and then incubated overnight in 1 mM 11-mercaptoundecanoic acid (MUA) dissolved in ethanol. Before use, the sensor was rinsed with absolute ethanol and dried under a gentle nitrogen flow. After mounting of the sensor, the system was equilibrated with MilliQ water until a stable baseline was obtained. The surface was then activated for 10 minutes with a freshly prepared 0.1 M NHS : 0.4 M EDC 1:1 mixture. A suspension of amine functionalized lipodisks (50 μ M total lipid concentration) in 80 mM acetate buffer (pH 4.5) was then loaded. The amine groups bind to the active surface, resulting in a layer of immobilized lipodisks. After rinsing with the acetate buffer to remove any non-bound lipodisks, the remaining active surface was inactivated by addition of 1 M ethanolamine. After a 10 minutes inactivation period, the system was finally equilibrated with the working buffer (80 mM Tris-HCl pH 8.0).

In order to determine the COX-1 binding isotherms, solutions with increasing concentrations of the protein were sequentially loaded into the system. Before each protein addition the system was rinsed with the working buffer in order to remove any non-specifically bound material. The binding of the protein to the lipodisks is recorded as negative shifts in the oscillation frequency of the quartz crystal. Quantitative results were obtained by fitting the obtained frequency and dissipation shifts with the viscoelastic model described by Voinova et al. (31).

As Tween 20 is present in the COX-1 solution, analysis of the results assume that the recorded mass changes arise from the binding and partition of the protein and the detergent in the same weight proportions in which they are found in the original solution (32:68 COX-1:Tween 20). Therefore, the results provided represent the lower limit for the binding of COX-1 to the lipodisks.

3. Results and Discussion

3.1 Lipodisk characterization

Lipodisks were produced by the detergent depletion method, which enables preparation of lipodisks with a small diameter suitable for immobilization into the pores of the HPLC support materials. Cryo-TEM was used in combination with DLS to determine the size and shape of the lipid structures in the preparations. The cryo-TEM investigation showed that the lipodisk samples contained mainly disk shaped aggregates. No structural difference was observed between the samples prepared with biotinylated PEG and those prepared with amine functionalized PEG. A representative micrograph is shown in figure 2. Note that due to the poor contrast of the polymer, the PEG-chains are invisible in the micrograph. By studying a large amount of micrographs and measuring more than 500 structures the apparent radius of the amine functionalized lipodisks was determined to 6.4 ± 2.2 nm. By adding 3.5 nm for the

length of the PEG chains (26) the full disk radius was calculated to 9.9 nm. According to DLS analysis the hydrodynamic radius (R_h) of the same disks was 9.3 ± 1.3 nm. For the biotinylated disks the corresponding values were 8.6 ± 2.3 nm (with PEG) from cryo-TEM and 8.9 ± 1.2 nm from DLS.¹

3.2 Immobilization of lipodisks on HPLC support materials

As a first step, immobilization of lipodisks by various coupling methods, and on different HPLC materials, was evaluated. Lipodisks with amine and biotin functionalities were immobilized onto Nucleosil silica and POROS media. For immobilization of lipodisks with biotin functionality, streptavidin was immobilized by reductive amination coupling of mainly the lysine side chains of the protein to the aldehyde groups of the materials. The coupling yield was close to 100% ($0.45 \mu\text{mol protein/mL}$) on the Nucleosil silica and 55% ($0.31 \mu\text{mol protein/mL media}$) on the POROS media. The amount of immobilized lipids on each material was determined by phosphorous analysis. Coupling by reductive amination of lipodisks resulted in $12 \mu\text{mol lipids/mL}$ Nucleosil silica and $19 \mu\text{mol lipids/mL}$ POROS media. Coupling by streptavidin-biotin binding of lipodisks with biotin functionality resulted in $8.2 \mu\text{mol lipids/mL}$ Nucleosil silica and $9.3 \mu\text{mol lipids/mL}$ POROS media. No lipodisks were found in the reference samples, representing passive coupling.

Although the amount of immobilized lipodisks on the POROS media was slightly higher compared to that on the Nucleosil silica, Nucleosil silica was chosen for further studies, as this material is more commonly used for HPLC. Since coupling via reductive amination is more straightforward and also more cost effective than streptavidin-biotin binding, we opted to use this method in our further studies based on HPLC analysis. Furthermore, non-specific interactions between analytes and immobilized streptavidin may interfere when studying membrane protein-ligand interactions.

During a second immobilization of lipodisks with amine functionality on Nucleosil silica, $8.2 \mu\text{mol lipids/mL}$ silica was immobilized. Similar to in the previous immobilization experiment, lipodisks were added to the silica material in great excess (coupling yields corresponding to 2 and 13%, respectively). Since the amount of immobilized lipodisks was about the same in both experiments, it is likely that $\sim 10 \mu\text{mol lipids/mL}$ silica is the maximum amount of lipid that can be immobilized in the form of lipodisks on the limited surface area of the HPLC support materials. Choosing the optimal HPLC support material for immobilization of lipodisks is a tradeoff between pore size and surface area, since the pores must be big enough to harbor the disk, while the available surface area decreases dramatically with increasing pore size.

3.3 HPLC analysis of drug-disk interactions

The performance of one of the lipodisk columns (column 1) packed with Nucleosil silica with immobilized lipodisks was tested by analysis of 15 drug compounds and the reproducibility and stability of the column was evaluated. Figure 3 demonstrates typical chromatograms from analysis of a mixture of 7 compounds on the lipodisk column using ammonium acetate buffer

¹ The hydrodynamic radius obtained with DLS is determined based on the assumption that all particles are spherical. The hydrodynamic radius can be recalculated into the radius of a lipodisk via a model described by Mazer et al. (32). In order to do this accurately the thickness of the disks must be known. Since the thickness of the lipodisks used in this study is unknown and not easily estimated no recalculation of the hydrodynamic radius into the disk radius was done here.

as mobile phase, showing both the chromatogram from UV detection ($\lambda=214$ nm), the total ion chromatograms (TICs) of SIM positive mode and the extracted ion chromatograms (EICs) of individual analytes in the mixture. The retention time of the analytes on the lipodisk column, corrected for the retention time on the reference column, indicates the extent of interaction with immobilized lipodisks.

Analysis on the lipodisk column resulted in reproducible retention times, as evidenced by coefficients of variation (CV values) of 0.5% in average for the retention times of all 15 drug compounds. Analysis of the 15 drug compounds on the lipodisk columns column 1 and column 4 showed excellent correlation ($R^2 = 0.9902$). These results demonstrate that lipodisk columns can be produced in a reproducible manner and with correlating screening results.

No differences in retention times were observed for the analytes throughout the study (a few weeks), which indicates that the immobilized lipodisks were stable and no leakage of the column occurred. The long term stability of column 4 in PBS pH 7.4 at 4°C was found to be excellent. After 82 days of storage, the retention times of the three compounds diclofenac, propranolol and indomethacin were measured to be on average 99% of the retention times observed on day 0. The long term stability of lipodisks has been reported elsewhere (16).

The analytes were analysed both as single injections and in mixtures on the lipodisk column using ammonium acetate as mobile phase and UV detection in combination with MS detection. The differences in retention times during analysis in mixtures compared to single injections were very small showing that the partition behavior of individual drugs was not affected by the presence of other drugs in the mixture. Figure 4 shows retention times of the 15 drug compounds on the lipodisk column (analysis as singles and in mixtures) and the reference column (analysis in mixtures). Retention times on the reference column were short for all analytes. Hence, interactions with the silica matrix or remaining aldehyde groups did not significantly contribute to the overall retention on the lipodisk column.

The amount of lipids immobilized on the lipodisk column was determined to 1.18 μmol (9.8 $\mu\text{mol/mL}$ silica) by phosphorous analysis. Analysis of the 15 drug compounds on the lipodisk column using PBS instead of ammonium acetate buffer as mobile phase resulted in small differences in obtained $\log K_S$ values for charged compounds (Table 1, Figure 5). As expected, electrostatic effects between the negatively charged DSPE-PEG-lipids and the analytes becomes more apparent in the ammonium acetate buffer due to the considerably weaker ionic strength as compared to the PBS. The influence of electrostatic effects on the interaction between charged analytes and lipodisks has previously been reported and discussed by Johansson et al. (16).

Taking the above-mentioned electrostatic effects into account, it can be concluded that the $\log K_S$ values obtained using the two different mobile phases correlated well. Moreover, there was no difference in the drug retention times obtained from single injections and mixtures. These results suggest that individual drug compounds present in mixtures can be analyzed with high throughput using ammonium acetate buffer as mobile phase in combination with MS detection. It should be noted that the maximum number of analytes in each mixture likely is considerable higher than seven, as was used in the present study to avoid overlapping mass-to-charge ratios. Duong-Thi and coauthors have recently verified high throughput analysis in a similar, but lipodisk-free, system using up to 65 compounds in each mixture (11). The robust chromatographic medium, together with the possibility to analyze multiple compounds simultaneously, makes the current lipodisk-HPLC-MS system far more efficient for drug partition studies than previous systems based on immobilization of lipodisks to gel filtration media (16, 17).

3.4 COX-1 incorporation into immobilized lipodisks

In a previous study by Lundquist et al. (23) the peripheral membrane protein COX-1 was incorporated into biotin-functionalized lipodisks, and the resulting proteodisks were subsequently successfully immobilized on streptavidin-covered sensor surfaces [20]. A similar approach can likely be used to immobilize proteodisks by streptavidin-biotin binding also to HPLC support material. However, in the present study a COX-1 column was produced by *in situ* incorporation of COX-1 into lipodisks immobilized by reductive amination to the silica material (see section 2.4.6 for details). This strategy has the potential to work well for peripheral proteins, such as COX-1, that can be added to the column after lipodisk immobilization and deactivation of remaining aldehyde groups. However, for transmembrane proteins that need to be incorporated into the lipodisks prior to immobilization, streptavidin-biotin coupling might be preferable in order to avoid amine coupling of lysine side chains of the protein to the support material.

The COX-1 column (column 2) was emptied and the content was analyzed by amino acid and phosphorous analysis. The amount of incorporated COX-1 on the column was determined to 0.6 nmol (5.0 nmol COX-1/mL Nucleosil silica) and the amount of lipids to 1.32 μ mol (10.9 μ mol lipid/mL silica). Hence, the number of lipids on the column for each COX-1 dimer was about 4400.

Specific binding of COX-1 to immobilized lipodisks was validated by interaction studies by QCM-D. Lipodisks from the same batch as was used for HPLC-MS experiments were successfully immobilized onto the QCM-D sensor (data not shown). Addition of COX-1 resulted in negative frequency shifts. Blank measurements performed on a modified gold sensor surface that had been inactivated with ethanolamine prior to protein addition showed that the non-specific binding of the protein to the surface is negligible. Therefore, it is safe to assume that the observed shifts result from binding of the protein (and associated Tween 20) to the lipodisks. The binding isotherm shown in figure 6 was obtained from the experiments.

Given that the associated protein to lipid ratio is rather low, it is unlikely that more than one COX-1 dimer is located on each lipodisk at saturation conditions. Furthermore, the amount of immobilized lipodisks was kept comparably low (calculated to ~ 300 ng/cm², in contrast to the maximum obtained coverage at long immobilization times of ~ 900 ng/cm²). The system can then be treated as an array of separated binding sites. Each lipodisk constitutes an independent binding site to which only the binding of a single protein is possible. Also, given the low degree of coverage, the lipodisks can be assumed to be immobilized with some distance to each other, implying that the binding of COX-1 to one lipodisk will not affect the properties of other lipodisks. Hence, the Langmuir association isotherm can be employed as an approximation to describe the binding behavior of COX-1 to immobilized lipodisks. The effective associated protein to lipid ratio at saturation ($R_{\text{eff, max}}$) can therefore be estimated from the experiments.

According to the QCM-D results, at saturation, one COX-1 dimer is found for every 2200-2900 lipid molecules ($R_{\text{eff, max}} = 3.9 \times 10^{-4} \pm 5.5 \times 10^{-5}$). This finding correlates reasonably well with the results obtained from the amino acid analysis of the COX-1 column. The difference in protein to lipid ratio obtained from the QCM-D and amino acid analysis may partly be due to the fact that the former analysis was carried out at 21°C, whereas incorporation of COX-1 *in situ* on the lipodisk column was performed at 37°C. Further, it is possible that the accessibility of the disks for protein binding is somewhat compromised in the narrow pores of the Nucleosil silica..

These QCM-D results also support that COX-1 was bound specifically to immobilized lipodisks on the column rather than non-specifically to other parts of the column. An indication of the affinity of the protein for the lipodisks would be given by the association

equilibrium constant K (M^{-1}), which can be obtained by generation of a binding hyperbola on basis of the experimental data. However, given the limited number of measurement points, the experimentally determined value of K can only be determined with rather large error margins ($0.78 \pm 0.38 \text{ mL}/\mu\text{g} = (1.1 \pm 0.5) \times 10^8 \text{ M}^{-1}$). Nonetheless, it is safe to state that equilibrium concentrations slightly above $10 \mu\text{g/mL}$ (7.1 nM) are enough to achieve over 90 % saturation of the lipodisks with the COX-1 protein. The QCM-D experiments thus indicate that the lipodisks are saturated with bound COX-1 at significantly lower protein concentrations than what was used during the *in situ* incorporation of COX-1 on the lipodisk column.

4. Concluding Remarks

In this work, lipodisks were successfully immobilized onto two different HPLC support materials by either reductive amination (lipodisks of amine functionality) or streptavidin-biotin binding (lipodisks of biotin functionality). Production of a HPLC column with covalently immobilized lipodisks resulted in an efficient HPLC system that showed high stability, and generated data with excellent reproducibility. MS detection enabled high throughput analysis of analytes in mixtures. The HPLC-MS system presented in this paper thus represents a new and improved technique for the determination of drug substance partition behavior.

Results of the present study show moreover that COX-1 can be stably bound to the immobilized disks via a straightforward protocol for *in situ* incorporation of the protein. Ultimately, the chromatographic lipodisk-protein system described in this work could be used to study protein-ligand interactions. Provided full activity of the protein, the COX-1 column produced in the present study could in fact theoretically be used to detect COX-1 binders with sub- μM affinities. However, in order to achieve this goal in practice immobilization of larger amounts of protein is required. This could potentially be achieved by utilization of a column of larger dimensions and hence with a higher protein load. Further optimization of the system in terms of mobile phase conditions such as pH and temperature is also an option. Studies along these lines are underway in our laboratories.

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6. References

1. Barenholz Y, Lasic DD. Handbook of non-medical applications of liposomes. Barenholz Y, Lasic DD, editors. Boca Raton, Florida, USA: CRC Press; 1996.
2. Cserhádi T, Szögyi M. Liposomes in chromatography. *Biomed Chromatogr*. 2010;24:1265–1272.
3. Wiedmer SK, Riekkola M-L, Jussila MS. Phospholipids and liposomes in liquid chromatographic and capillary electromigration techniques. *Trends Anal Chem* 2004; 23(8):562–582.
4. Arinaminpathy Y, Khurana E, Engelman DM, Gerstein MB. Computational analysis of membrane proteins: the largest class of drug targets. *Drug Disc Today* 2009;14(23-24):1130–1135
5. Gridley S, Shrout AL, Esposito EA. Challenges and Approaches for Assay Development of Membrane and Membrane-Associated Proteins in Drug Discovery. *Progress in Molecular Biology and Translational Science: Membrane Proteins as Drug Targets*. 91st ed. Worcester, Massachusetts, USA; 2010.
6. Zopf D, Ohlson S. Weak-affinity chromatography. *Nat* 1990;346(6279):87–88
7. Bergström M, Liu S, Kiick KL, Ohlson S. Cholera toxin inhibitors studied with high-performance liquid affinity chromatography: a robust method to evaluate receptor-ligand interactions. *Chem Biol Drug Des* 2009;73(1):132–141
8. Engström HA, Johansson R, Koch-Schmidt P, Gregorius K, Ohlson S, Bergström M. Evaluation of a glucose sensing antibody using weak affinity chromatography. *Biomed Chromatogr* 2007;22(3):272–277
9. Duong-Thi M-D, Meiby E, Bergström M, Fex T, Isaksson R, Ohlson S. Weak affinity chromatography as a new approach for fragment screening in drug discovery. *Anal Biochem* 2011;414(1):138–146.
10. Meiby E, Knapp S, Elkins J, Ohlson S. Fragment Screening of Cyclin G-associated Kinase by Weak Affinity Chromatography *Anal Bioanal Chem*. 2012;404:2417–2425.
11. Duong-Thi M-D, Bergström M, Fex T, Isaksson R, Ohlson S. High-Throughput Fragment Screening by Affinity LC-MS. *J Biomol Screen* 2012;18(2):160–171

12. Haginaka J. Protein-based chiral stationary phases for high-performance liquid chromatography enantioseparations. *J Chromatogr A* 2001;906(1-2):253–273
13. Xiqin M, Kong L, Li X, Guo B, Zou H. Unilamellar liposomes covalently coupled on silica gel for liquid chromatography. *Anal Bioanal Chem*. 2003;375:550–555.
14. Zhang C, Li J, Xu L, Shi Z-G. Fast Immobilized liposome chromatography based on penetrable silica microspheres for screening and analysis of permeable compounds. *J Chromatogr A*. 2012;1233:78–84.
15. Baños C-E, Wiedmer SK, Smått J-H, Sakeye M, Lokajová J, Riekkola M-L. Phospholipids covalently attached to silica particles as stationary phase in nano-liquid chromatography. *Journal of pharmaceutical and biomedical analysis* 2012;71:1–10
16. Johansson E, Lundquist A, Zuo S, Edwards K. Nanosized bilayer disks: attractive model membranes for drug partition studies. *Biochim Biophys Acta* 2007;1768(6):1518–1525
17. Johansson E, Engwall C, Arfvidsson MC, Lundahl P, Edwards K. Development and initial evaluation of PEG-stabilized bilayer disks as novel model membranes. *Biophys Chem*. 2005;113:183–192.
18. Boija E, Lundquist A, Edwards K, Johansson G. Evaluation of bilayer disks as plant cell membrane models in partition studies. *Anal Biochem*. 2007;364:145–152.
19. Boija E, Lundquist A, Nilsson M, Edwards K, Isaksson R, Johansson G. Bilayer disk capillary electrophoresis (BDCE): a novel method to study drug partitioning into membranes. *Electrophor*. 2008;29:3377–3383.
20. Vainikka K, Reijmar K, Yohannes G, Samuelsson J, Edwards K, Jussila M, et al. PEG-stabilized lipid disks as model membranes in interaction studies based on EKC and QCM. *Anal Biochem*. 2011;414:117–124.
21. Lundquist A, Wessman P, Rennie A, Edwards K. Melittin-lipid interactions: A comparative study using micelles, liposomes and bilayer disks. *BBA Biomembr*. 2008;1778:2210–2216.
22. Johnsson M, Edwards K. Liposomes, discs and spherical micelles: aggregate structure in mixtures of gel phase phosphatidylcholines and poly(ethylene glycol)-phospholipids. *Biophys*. 2003;85:3839–3847.
23. Lundquist A, Hansen SB, Nordström H, Danielson UH, Edwards K. Biotinylated lipid bilayer disks as model membranes for biosensor analyses. *Anal Biochem* 2010;405(2):153–159

24. Beigi F, Yang Q, Lundahl P. Immobilized-liposome chromatographic analysis of drug partitioning into lipid bilayers. *J Chromatogr A*. 1995;704:315–321.
25. Lundahl P, Beigi F. Immobilized liposome chromatography of drugs for model analysis of drug-membrane interactions. *Adv Drug Deliv Rev*. 1997;23:221–227.
26. Johnsson M, Hansson P, Edwards K. Spherical micelles and other self-assembled structures in dilute aqueous mixtures of poly(ethylene glycol) lipids. *J Phys Chem B*. 2001;105(35):8420–8430.
27. Almgren M, Edwards K, Karlsson G. Cryo transmission electron microscopy of liposomes and related structures. *Coll Surf A - Physicochem Engineer Asp*. 2000;174(1-2):3–21.
28. Bartlett GR. Phosphorus assay in column chromatography. *J Biol Chem*. 1959;234(3):466–468
29. Beigi F, Gottschalk I, Hagglund CL, Haneskog L, Brekkan E, Zhang YX, et al. Immobilized liposome and biomembrane partitioning chromatography of drugs for prediction of drug transport. *Int J Pharm*. 1998;164(1-2):129–137.
30. MirAfzali Z, Leipprandt JR, McCracken JL, DeWitt DL. Fast, efficient reconstitution of the cyclooxygenases into proteoliposomes. *Arch Biochem Biophys*. 2005;443(1-2):60–65
31. Voinova M V., Rodahl M, Jonson M, Kasemo B. Viscoelastic acoustic response of layered polymer films at fluid-solid interfaces: Continuum mechanics approach. *Phys Scr*. 1999;59(5):391–396.
32. Mazer NA, Benedek GB, Carey MC. Quasi-elastic light-scattering-studies of aqueous biliary lipid systems - mixed micelle formation in bile-sat lecithin solution. *Biochem*. 1980;19(4):601–615.

Fig. 1 Schematic illustration of the cross-section of a lipodisk

Fig. 2 Cryo-TEM image of lipodisks composed of POPC/Soy PE/cholesterol/Ceramide-PEG₂₀₀₀/DSPE-PEG₂₀₀₀amine (30:28:17:21:4 mol%). The arrow and arrow head indicate lipodisks observed edge-on and face-on, respectively. Scale bar = 100 nm.

Fig. 3 Examples of analysis of a mixture of 7 compounds on the lipodisk column using ammonium acetate buffer as mobile phase. (A) UV detection 214 nm, (B) TIC of SIM positive mode, (C) EICs of individual analytes in SIM positive mode (1. theophylline, 2. naproxen, 3. prednisolone, 4. pindolol, 5. diclofenac, 6. indomethacin 7. propranolol). The void time was 0.63 min

Fig. 4 Retention times of 15 drug compounds during analysis on the lipodisk column and the reference column with ammonium acetate as mobile phase. Error bars represent the standard deviation (n=3). Ibuprofen was detected at a sample concentration of 0.1 mM, whereas the other analytes at 10 μ M.

Fig. 5 Comparison of $\log K_s$ values for uncharged (circles), positively (squares), and negatively (triangles) charged drugs obtained using covalently immobilized lipodisks in PBS compared to ammonium acetate buffer

Fig. 6 COX-1/lipodisks association isotherm. R_{eff} represents the effective associated COX-1 dimer/lipid mol ratio. $[\text{COX}]_{\text{eq}}$ is the equilibrium bulk concentration of the protein. Error bars represent the standard error from three repetitions of the experiment. The data at $[\text{COX}]_{\text{eq}} = 3$ and $4 \mu\text{g mL}^{-1}$ represent single experiments. The solid line represents the fitting of the data according to the Langmuir isotherm ($R_{\text{eff}} = K \cdot R_{\text{eff,max}} \cdot [\text{COX}]_{\text{eq}} \cdot (1 + K[\text{COX}]_{\text{eq}})^{-1}$)

Table 1. Log K_s values obtained using covalently immobilized lipodisks composed of POPC/Soy PE/cholesterol/Ceramide-PEG₂₀₀₀/DSPE-PEG₂₀₀₀amine (30:28:17:21:4 mol%).

Drug	Charge at pH 7-7.4	log K_s		
		Ammonium acetate buffer		PBS
		single injections	mixtures	
Alprenolol	+	3.10	3.09	2.74
Lidocaine	+	1.78	1.76	1.96
Pindolol	+	2.40	2.35	1.50
Promethazine	+	4.15	4.14	3.79
Propranolol	+	3.59	3.55	3.20
Theophylline	+	0.70	0.59	0.51
Diclofenac	-	2.79	2.81	3.05
Ibuprofen	-	1.65	1.65	1.81
Indomethacin	-	2.81	2.81	3.06
Naproxen	-	1.42	1.49	1.60
Warfarin	-	1.86	1.88	1.93
Corticosterone	0	2.25	2.25	2.28
Cortisone	0	2.30	2.31	2.34
Hydrocortisone	0	2.53	2.53	2.56
Prednisolone	0	2.06	2.05	2.11